

Cancer Genetics

Xin Wei Wang
Joe W. Grisham
Snorri S. Thorgeirsson
Editors

Molecular Genetics of Liver Neoplasia

 Springer

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Editors

Molecular Genetics of Liver Neoplasia

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Preface

Primary liver cancer is the third most deadly and fifth most common cancer worldwide, with an estimated 877,000 new cases and almost as many deaths in 2007. Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) are the major types of primary liver cancer. The 5 year survival rate of these cancers is less than 10% and during the last 50 years only minimal survival improvement has been realized. Although liver cancer is most frequent in sub-Saharan Africa and Asia, the incidence has increased sharply in the developed countries in recent years. The key etiological factors (i.e., Hepatitis B and C viruses, obesity, and type 2 diabetes) are known for HCC, but the etiological causes for CC are less well-defined. Furthermore, our current understanding of the molecular pathogenesis of primary liver cancer is still far from complete. Therefore, there is an urgent need for more comprehensive genetic and mechanistic understanding of primary liver cancer if improvements in treatment and prevention are to be realized.

Recent progress in the genetic and genomic understanding of liver cancer has generated both excitement and hope that this knowledge may offer approaches to improve the current situation. It is in this context that, we have brought together an international team of leading scientists and clinicians to prepare this monograph. The articles in this book provide an exciting overview of the most recent advances in the genetics, genomics, and biology of liver cancer, and how this new knowledge can be leveraged for improving diagnosis, treatment, and prevention of liver cancer. Each chapter starts with a state-of-the-art topic, ranging from genetics and environmental risk factors of liver cancer, genetics of liver development and pathogenesis, genetics and epigenetic changes associated with liver cancer, the utilities of genetic animal models, cancer stem cells, and translational genomics, to the relevance of these aspects to liver cancer. We are currently experiencing the most exciting time in liver cancer research with extraordinary opportunities for improving the treatment and prevention of this dreadful disease.

Bethesda, Maryland

Xin Wei Wang
Joe W. Grisham
Snorri S. Thorgeirsson

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Part I

Introduction

Chapter 1

Biology of Hepatocellular Carcinoma: Past, Present and Beyond

Xin Wei Wang, Joe W. Grisham, and Snorri S. Thorgeirsson

Abstract Primary liver cancer (PLC) is the third most deadly and fifth most common cancer in the world (Parkin et al. 1999), with an estimated 626,000 or 5.7% of new cancer cases and almost as many deaths in 2002 (Parkin et al. 2005). Liver cancer is an ancient disease and its description can be found in Huangdi Neijing, an ancient Chinese medical textbook also known as Yellow Emperor's Inner Canon dated back over 2000 years ago. However, the first mentioned PLC case could be dated as early as 1849 by Carl Rokitansky and the definition of PLC, as referenced to metastatic liver cancer, was only formally established in 1888 by Victor Hanot and Augustin Gilbert, and in 1889 independently by Moriharu Miura (Hanot and Gilbert 1888; Rokitansky 1849; Yamagiwa 1911). Traditionally, PLC was considered as an incurable disease due to an extremely poor outcome. Patients with PLC have been an underserved population since the beginning of its discovery and the disease is becoming a major health burden worldwide. Clearly, there is a strong need in expanding basic and translational research on PLC with an ultimate goal to reduce its severity. Recent studies on HCC genetic and genomic analyses feature important advances in the understanding of the complex biological processes underlying tumorigenesis and metastasis of PLC, and demonstrate how these insights might translate into clinical applications. As we approach a golden era in PLC research, we anticipate a significant advance in our understanding of this disease in near future. We are confident that the knowledge gain from continuing research efforts on PLC undoubtedly facilitates the understanding of molecular mechanism and tumor biology to provide the best therapy for each cancer patient and to improve patient management.

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Keywords Primary liver cancer · Hepatocellular carcinoma · Hepatitis B virus · Hepatitis C virus · Aflatoxin · TP53 mutation · Chronic liver disease · Cancer biomarker · Cancer screening · Therapy · Liver transplantation · Alpha fetoprotein · AFP-L3 · Tumor staging · Cirrhosis · HCC metastasis · Genetic signature · cDNA microarray · transcriptomics · Hepatocarcinogenesis · Tumor suppressor gene · Oncogene · microRNA · Molecular · Personalized cancer care

1 Historical Perspective

Hepatocellular carcinoma (HCC) represents a major form of PLC, specifically in high epidemic area where over 90% of PLC can be attributed to HCC (Carr et al. 1997). Several landmark studies in the last 50 years have significantly contributed to HCC diagnosis and prevention (Fig. 1.1). For example, the discovery of alpha-fetoprotein (AFP) by Yuri Tatarinov in 1964 as a tumor biomarker found in serum allows the development of surveillance programs to detect HCC at an early stage that can be effectively treated by surgery. This leads to an improved long-term survival for a subset of patients with early stage HCC (Zhou et al. 2001). Since the world's first liver transplant performed by Thomas Starzl, the liver transplant procedure when following a stringent Milan criterion for recruitment is an effective curative modality for HCC patients. The discoveries of hepatitis B virus (HBV) in 1967 by Baruch Blumberg, who was subsequently awarded the 1976 Nobel Prize in physiology or medicine, and hepatitis C virus (HCV) in 1989 by Harvey Alter and others, together with many subsequent studies to show these viruses as causative agents of chronic liver diseases (CLD) and HCC, have led the developments of strategies for HCC prevention (Blumberg and London 1981; Choo et al. 1989;

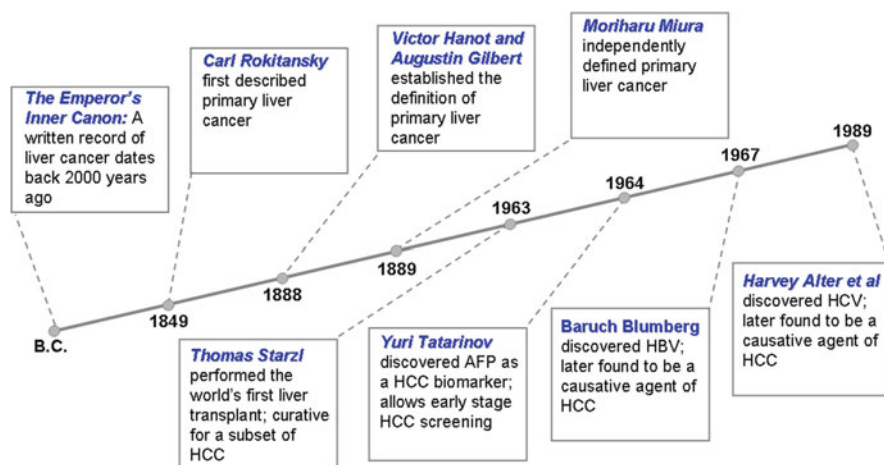


Fig. 1.1 Historical milestones of primary liver cancer studies

Kuo et al. 1989). Encouragingly, HBV vaccination has been shown to be effective in preventing HBV infection and the development of HCC (Chang et al. 1997). Since 2000, HBV vaccination programs have been implemented in over 130 countries and we expect in the near future a decline of HBV-related HCC. In contrast to HBV vaccines, numerous attempts have been made toward the development of new HCV vaccines; an effective vaccine program has yet to be developed to prevent HCV-related diseases. Taken together, the historical milestones have created tremendous opportunities for basic and translational studies on HCC and have provided a solid foundation to ensure our successful fight against this dismal disease in the future.

2 Current Standing

While hepatocarcinogenesis is a long-term process, HCC is considered a fatal disease because of its poor prognosis. The dismal outcome may be a result of the asymptomatic nature of the early disease (Curley et al. 1995). Most patients are thus diagnosed with advanced HCC. Currently, survival still remains gloomy for a majority of HCC patients and clinical complications such as tumor grade and compromised liver functions have hampered clinician's ability to give a sensible treatment recommendation. While about 10–20% HCC patients may be eligible for potentially curative therapies such as resection and liver transplantation, post-surgical survival is again complicated by recurrence since many patients eventually have relapse (Nakakura and Choti 2000). Despite many studies of HCC, information regarding phenotypic and molecular changes associated with the development of this disease is still limited (Kim and Wang 2003; Thorgeirsson and Grisham 2002). Such information is needed to develop methodology for early detection of HCC. Despite routine screening by ultrasonography and serum AFP of individuals at high risk, most patients are still diagnosed at late stages of HCC. Additional complications arise due the multinodular nature of HCC, portal vein invasion, intra-hepatic metastasis, as well as the recurrence of nodules at multiple distant sites of the liver. Due to the long waiting list for liver transplantation and the limited number of liver transplants, many patients fail the Milan criteria for transplant eligibility (Llovet et al. 2005). Therapies such as transcatheter arterial chemoembolization (TACE) and interferon-alpha (IFN) may prolong survival in some patients, however, prognosis is often less satisfactory due to the limited ability of stratifying/selecting appropriate patients who would most likely benefit from such targeted adjuvant therapies (Clavien 2007; Llovet and Bruix 2003; Lo et al. 2007; Sun et al. 2006). The recent SHARP trial has encouraging findings regarding sorafenib as a therapeutic agent, however, the survival benefit is modest (Llovet et al. 2008). Currently, there is an urgent need to develop sensible tools that may provide sufficient resolution to assist early HCC diagnosis, patient stratification for prognosis, and therapy.

The molecular mechanisms underlying the development of HCC are not well-understood. Many genes have been implicated in the pathology of HCC, including

those regulating DNA damage response pathways (e.g., p53), genes involved in regulating cell growth and apoptosis (e.g., TGF- β , SMAD2, SMAD4, M6P/IGF2R), cell cycle control genes (e.g., p16, Rb, cyclin D), and genes responsible for cell–cell interaction and signal transduction (e.g., e-cadherin, APC/ β -catenin) (Koike et al. 2002; Levy et al. 2002; Staib et al. 2003). Aneuploidy and multiple genetic alterations are often present in HCC (Feitelson et al. 2002). These findings provide some clues about the mechanisms leading to HCC. However, because of tumor heterogeneity, it is unclear how these molecular signaling pathways are interconnected in contributing to HCC development.

3 HCC Etiologies

HCC is one of the few human cancers where the underlying etiology can often be identified. Traditionally, HCC is mainly known to occur in the developing countries such as in certain parts of Asia and Africa where HBV is epidemic. HBV is a major etiological agent that contributes to the high incidence of HCC in China where over 50% of HCC worldwide are found. However, current data indicate that the HCC incidence is sharply increased in the developed countries including those from Australia, Europe, and North America (Altekruse et al. 2009; Deuffic et al. 1998; El-Serag and Mason 1999; Taylor-Robinson et al. 1997). The reason for such an increase in its incidence is unclear but HCV infection and possibly obesity may be the major culprits. While HBV vaccine has been effective in reducing virus spread, currently more than 350 million people worldwide are infected with chronic HBV and more than 20% of them will die from HCC or liver failure (Chen et al. 2006; Llovet et al. 2003). Therefore, it is crucial to develop additional effective anti-HBV agents to inhibit chronic virus replication.

In addition to viral hepatitis-mediated liver diseases, several other environmental factors as well as metabolic disorders have also been linked to HCC. For example, exposure to aflatoxin B1 (AFB), nonalcoholic steatohepatitis (NASH), cigarette smoking, and/or heavy alcohol consumption either alone or in synergy with viral infection contributes to HCC development (Kensler et al. 2003). The dietary aflatoxin AFB1 is an agent found in mycotoxin-contaminated foods and its uptake can lead to a G to T transversion at the third base of codon 249 of TP53. This mutation of TP53 causes inhibition of wild-type p53 and thereby increases cell survival. AFB1 uptake is the only etiology known to cause a distinct gene mutation leading to HCC (reviewed in Hussain et al. 2007). HCC also occurs more frequently in individuals with certain genetic disorders such as hemochromatosis (Powell et al. 1996), Wilson's disease (Berman 1988), porphyria (Huang et al. 1999), and α -anti-trypsin deficiency (Elzouki and Eriksson 1996). However, it is not clear whether individual etiological factors induce HCC directly or whether they act indirectly by producing chronic liver injury and regeneration through alteration of inflammatory status in the liver microenvironment (Budhu and Wang 2006).

4 HCC Diagnosis

Most symptomatic HCC patients are diagnosed at an advanced stage, thus precluding their chance for surgical intervention (Yuen et al. 2000). In contrast, HCC patients who were diagnosed at an early stage and received curative resection have a significantly improved survival time (Poon et al. 2002; Yamamoto et al. 2001). Thus, early detection and resection have been generally recognized as the most important factors to achieve long-term survival for HCC patients as patients with small HCC have a better outcome. The diagnostic tools, treatment modalities, and screening programs for HCC have improved in recent years, but early detection still remains a challenge. At the time of diagnosis, only about 20% of HCC are eligible for surgically resection and survival after this procedure is only 30–40% at 5 years. Staging systems have been created to define prognosis and treatment options for many diseases, including HCC (Wildi et al. 2004). Staging is essential, particularly in malignant diseases, to select and improve treatment. The requirements of a good staging system includes – simplicity and ease of use; reproducibility; provision of reliable information on the natural history of the disease; and categorization of patients into various treatment groups (e.g., sorafenib; IFN/5FU).

Well-defined and generally accepted staging systems are available for almost all cancers. HCC is an exception as many different staging systems have been introduced around the world and currently there is no clear consensus in tumor staging in clinical practice between east and west. This has led to considerable confusion in the literature. An improvement in treatment in association with better diagnostic techniques has changed the former rather fatalistic approach to HCC. The main factors affecting the prognosis of HCC are tumor stage, aggressiveness and growth rate of the tumor, general health of the patient, liver function, and choice of therapy. Many different prognostic models therefore need to be developed for each stratum of the disease because a single system cannot accurately establish the prognosis of all patients and help determine the efficacy of all available therapies.

Prognostic assessment and choice of treatment options in HCC is complex because they depend not only on the grade of cancer spread (tumor staging), but also on the grade of residual liver function (liver disease stage). A clinical staging system for cancer patients provides guidance for patient assessment and making therapeutic decisions. It is useful in deciding whether to treat a patient aggressively and in avoiding the over-treatment of patients who would not tolerate the treatment or patients whose life expectancy rules out any chance of treatment. Clinical staging is also an essential tool for comparison between groups in therapeutic trials and for comparison between different studies. Scoring systems arise as a compromise between simplicity and discriminatory ability.

Many systems have been developed to stage HCC and each is based on various patient populations, tumor characteristics, and treatment and inclusion criteria (Cillo et al. 2004). Individual systems may be applicable to only patients after resection, after transplantation, or with advanced tumors. The well-defined tumor

node metastasis (TNM) staging system has been widely utilized to stage liver cancer and has since been modified by several groups to fit subgroups of patients with HCC and cirrhosis. For those patients being evaluated for transplantation or resection, additional staging systems have been created. To confound matters, the definition of HCC itself seems to be inconsistent. Small well-differentiated HCCs identified in Asian countries are called regenerative nodules in the west. The lack of a consensus on the definition and staging of HCC combined with the wide heterogeneity of the disease has interfered with clinical recommendations and progress.

HCC mainly develops in a previously diseased liver so that both HCC and cirrhosis deeply influence survival and simultaneously determine the applicability and efficacy of therapy. Uni-dimensional prognostic systems accounting for only one of these hepatic diseases such as Child-Pugh score (Ueno et al. 2002), and TNM may result in inaccurate survival prediction of HCC patients. BCLC staging system may provide a better success in stratifying HCC patients in Europe for recommending specific treatments. The HCC population is characterized by a great heterogeneity since both tumor and cirrhosis may be diagnosed at different evolutionary stages each with different therapeutic perspectives and survival probabilities. Therefore, a staging system must be able to stratify HCC patients at these different categories reflecting this large range of potential survival figures.

Currently, AFP is the only widely used serum marker for HCC and allows for the identification of a subgroup of patients with small carcinomas. However, elevated serum AFP is only observed in 33–65% of patients with small HCC (Taketa 1990). Nonspecific elevation of serum AFP has been found in 15–58% of patients with chronic hepatitis and AFP levels highly vary between different ethnic backgrounds. Therefore, it is necessary to identify new serological HCC biomarkers that have a sufficient sensitivity and specificity for the diagnosis of HCC patients, especially in AFP normal and/or smaller HCC cases. Several candidates, including des- γ -carboxy prothrombin by a revised enzyme immunoassay kit and AFP-L3 [the Lens culinaris agglutinin (LCA) bound fraction of AFP] have been reported as potential diagnostic markers of HCC (Li et al 2001; Okuda et al. 1999; Shiraki et al. 1995). However, AFP remains the only universally accepted HCC biomarker in clinical practice. Therefore, improvement of the current screening system of high-risk patients is a major goal.

Surgery can be effective in HCC patients with small tumors. Interestingly, analyses of over 3000 cases with small tumors (<5 cm in diameter) that underwent curative resection in Liver Cancer Institute of Shanghai revealed a 5-year survival in about 60% patients, but no further survival improvement was observed in this current cohort when compared to resection of similar cases decades earlier (Tang, personal communication). These findings indicate that tumor biology is an important factor in determining tumor's aggression, i.e., a small tumor detected early could have the same aggressiveness as a large metastatic tumor. Thus, effective biomarkers used for early detection should be those that reflect tumor biology.

5 HCC Metastasis

A major complication of cancer-related mortality results from the development of metastases, the tumor colonies derived from the spread of cancer cells from a primary tumor through the blood or lymphatic system (Chambers et al. 2002). Local invasion and distant metastases are attributable to 90% of human cancer deaths (Hanahan and Weinberg 2000). Understanding the mechanisms involved in the process of tumor invasion and metastasis is a major challenge (Dong et al. 2009). The identification of biochemical factors augmented in invasive tumor cells may lead to improved methods to predict whether tumor cells have already metastasized and to stop them from spreads.

A metastasis model has emerged over many years of study using animal and cell culture models (Steege 2003). It is thought that cancer metastasis is a highly complex multistep process that involves alterations in growth, angiogenesis, dissemination, invasion, and survival, which leads to subsequent attachment and growth of new cancer cell colonies (Liotta 1985). Principally, a successful metastasis involves tissue invasion, intravasation, survival through circulation, extravasation, and outgrowth with angiogenesis. Interestingly, about 5000 to as much as 10^7 circulating cancer cells can be detected in the peripheral blood at any given time in a cancer patient, suggesting a constant dissemination of tumor cells. However, animal model studies indicate that the metastatic process is rather inefficient, which leads to the assumption that only a few rare cancer cells from primary lesions have acquired all the necessary steps with the right combination to form a metastatic colony. The dominance of this view, together with the nature of a multistep process during cancer initiation and progression, has led to a systematic effort to identify molecular events associated with the multiple steps of this process. Such an approach, while much less successful than the identification of cancer initiation genes, has identified many genes attributable to metastasis. However, due to limited technologies, it is still difficult to understand how these genes contribute to the key steps necessary for metastasis, thus precluding their potential clinical use. Recently, the metastasis paradigm has been challenged by the facts that most of the genetic and epigenetic changes necessary for metastasis appear to be the hallmarks of cancer. This raises a debate as to whether the tendency to metastasize is largely determined by the identities of mutant alleles that are acquired relatively early during multistep tumorigenesis (Bernards and Weinberg 2002).

Global molecular profiling by high-density cDNA microarray has become increasingly useful in identifying signatures that reflect the specific natures of diseases, such as cancer (Skena et al. 1995). A recent study indicates that a molecular signature can be identified in primary tumors and can be used to predict breast cancer patient survival and metastasis (Van de Vijver et al. 2002). Similarly, by comparing metastatic cells to primary tumors of lung, breast, and prostate, a molecular signature was uncovered from primary cancer lesions that could predict patient prognosis (Ramaswamy et al. 2003). Since microarrays detect signals contributed by the bulk of the tissues examined, the results suggest that most of these primary

tumor cells have acquired changes that favor metastasis. Recently, we found that the gene expression signature of primary HCCs with accompanying metastasis was very similar to that of their corresponding metastases (Ye et al. 2003). In contrast, the gene expression signature differs significantly between metastasis-free primary HCCs and HCCs with accompanying intrahepatic metastases. These results are consistent with our findings that the HCC metastasis signature is independent of tumor size, tumor encapsulation, and patient age. That a metastasis is more similar to its paired primary cancer compared with other metastases suggests that there may not be an integral set of changes that are selected for during the metastatic process. Rather, the genetics of the primary cancer may determine the capacity of the tumor to metastasize. Consistently, a recent study suggests that the genetic machinery that causes metastasis is hardwired into the primary tumor because metastatic foci harbor few genetic alterations compared with their corresponding primary cancer (Jones et al. 2008). Furthermore, clinical observations reveal that about 5–10% of patients with metastasis have a cancer of unknown primary site (van de Wouw et al. 2002), and recent experimental studies indicate that early disseminated cancer cells may account for metachronous metastases (Husemann et al. 2008), suggesting that systemic dissemination may be an early event in cancer development. These studies suggest that metastatic capacity is embedded in the majority of cells within the primary tumor and may be determined at an early stage of carcinogenesis. It also implies that one should not simply assume a favorable prognostic outcome if a small cancer lesion is detected. A clinical challenge is to be able to identify cancer patients with metastatic potential in advance so that an appropriate therapeutic regime can be applied. The identification of a molecular signature in primary tumors to predict metastasis and survival has provided an opportunity to classify these cancer patients in advance and thus allow clinicians to have ample time to manage them and to eliminate a false hope that may discourage them from seeking aggressive treatments.

Are metastatic signatures universal among cancer types? While the metastasis signature of adenocarcinomas of lung, breast, and prostate is similar to the gene expression profiles of murine metastatic tumors (Hunter et al. 2003), we have found no correlation between the signature of metastatic adenocarcinomas and the signature of metastatic HCC. These results suggest that the molecular program associated with HCC metastases may be unique to HCC patients.

Since Paget set forth the “seed vs. soil” hypothesis, most studies of metastasis have focused on the molecular analysis of the tumor and have largely ignored the heterogenic complexity of the surrounding tissue, which may also contribute to this process (Paget 1989). Are pro-metastatic changes inherent to the tumor cell (seed) or are they acquired through time and/or influenced by environmental status such as the conditions of the metastasis sites (soil)? Cancer cells are capable of altering their environment through a variety of mechanisms, including the production of growth factors and proteolytic enzymes, which allow for disruption of tissue homeostasis and the creation of pro-migratory and pro-invasive surroundings. However, tumors may also be directly affected by the tumor stroma itself, whereby tumor cells respond to certain factors present in the target organ that

are permissive to and promote tumor extravasation, aggregation, and metastasis (Mueller and Fusenig 2004). To determine whether metastatic ability is influenced by liver parenchyma, we have conducted gene expression profiling studies of non-cancerous liver parenchyma tissue from HCC patients with or without intrahepatic portal vein metastasis using cDNA microarray. We have identified a molecular signature that can significantly discriminate liver parenchyma from HCC patients with or without metastasis (Budhu et al. 2006). The lead genes in the metastasis signature from liver parenchyma are involved in the cellular immune and inflammatory response, suggesting that the inflammatory status of tumor stroma may play an important role in promoting HCC tumor progression and metastasis. Thus, the condition of liver parenchyma, whether influenced by viral hepatitis-mediated liver damage or individual genetic constitution could significantly contribute to intrahepatic metastatic potential.

6 HCC Genetics

Cancer is a genetic disease as genetic disruptions are commonly found in tumor cells. Unique genetic changes are expected to be found to contribute to the development of HCC. Viral integration into the genome is thought to play a role in hepatocarcinogenesis. Though the HBV integration into the genomic loci of the oncogenes cyclin A2, RAR β (retinoic acid receptor beta), hTERT (human telomerase reverse transcriptase), and SERCA1 encoding a sarco/endoplasmatic reticulum calcium ATPase has been reported, this seems to be a rare event and insertion is believed to occur randomly (Chami et al. 2000; Horikawa and Barrett 2001; Hytioglou and Theise 2006; Murakami et al. 2005). Viral integration may rather affect a non-coding RNA or microRNA and thus further investigations are needed. In about 10% of HCC cases no insertion of HBV could be found, suggesting that integration is either not required or that due to the genetic instability, the HBV genome has been lost together with parts of the host DNA before tumor onset (Feitelson and Lee 2007).

In addition to viral integration, the HBV encoded transcriptional modulator HBx appears to be involved in liver carcinogenesis, since most integration events maintain HBx, suggesting an important role. The expression of HBx in transgenic mice demonstrated that HBx has an oncogenic function in the liver (Kim et al. 1991). Consistently, expression of HBx has been linked to chromosome instability, centrosome amplification, senescence, cell cycle progression, and oxidative stress (Forgues et al. 2003; Wang et al. 2005, 2002). However, it is unclear whether the expression of HBx alone is sufficient for carcinogenesis or an additional etiology, for example, a dietary carcinogen, is required (Zheng et al. 2007).

In addition to expression profiling analysis, karyotyping studies have also been performed using array-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) arrays to assess chromosome instability (CIN) in HCC (Boige et al. 1997; Kim and Lee 2005; Nagai et al. 1997). The existence of

chromosome aberrations in common is widely assumed to imply that cancer-related genes exist within and around particular loci. Several studies have shown that gains of 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q and loss of heterozygosity (LOH) of 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, and 17p were associated with HCC (Farazi and DePinho 2006). In addition, some of these copy number alterations and allelic imbalances have been correlated with up- or down-regulated HCC related genes using expression imbalance maps (EIM) (Midorikawa et al. 2004). Furthermore, several studies have demonstrated that certain chromosome abnormalities are correlated with clinicopathological features such as tumor size, advanced tumor grade, and differentiation stage (Bando et al. 1999; Ohsawa et al. 1996; Tamura et al. 1997). A high number of chromosome abnormalities of a high-fractional allelic imbalance index correlates with a lower differentiation state, invasion and metastasis, and a shorter survival period (Nishida et al. 1992). These findings indicate that an increase in the overall chromosome aberrations in HCC may be linked to an unfavorable prognosis and the extent of aberrant chromosome regions could be useful for the prediction of prognosis and the selection of surgical therapy such as liver transplantation for HCC. Other studies have identified chromosome abnormalities in premalignant liver tissue such as chronic hepatitis and cirrhosis that may represent the early steps of hepatocarcinogenesis (Roncalli et al. 2000; Tsopanomichalou et al. 1999; Yamada et al. 1997; Yeh et al. 2001). Microsatellite instability, the mutation of short DNA repeats found evenly distributed throughout the genome normally repaired by the DNA repair mismatch system, has been observed in several types of cancers including breast, endometrial, and gastric carcinomas. However, a comparatively low level of MSI has been observed in HCC and thus the biological and clinicopathological significance of MSI in HCC remains to be determined. These studies suggest that multiple genetic alterations accumulate during the development of HCC, however, the exact sequence and number of events required for progression of this disease remains unclear.

In addition to HCC with obvious CIN, there are tumors with normal DNA content. In these cases, malignant transformation may occur through epigenetic mechanisms. One such mechanism is alterations in the methylation status of cancer-related genes such as tumor suppressors or oncogenes. Currently, the role of methylation in HCC carcinogenesis is not clear. Several studies also suggest that DNA hypermethylation of CpG islands is associated with HCC (Kanai et al. 2000; Kondo et al. 2000). Frequent promoter methylation and subsequent loss of protein expression has been demonstrated in HCC tumor suppressor genes p16, cadherin, and 14-3-3 σ (Herath et al. 2006). However, these studies have assessed these genes in isolation and therefore global studies of the methylation phenotype in malignant, adjacent microenvironments, and normal tissue need to be conducted to determine the role of methylation in hepatocarcinogenesis.

Most solid tumors are clinically and genetically heterogeneous due to genomic instability. This makes it difficult to identify driver mutations that functionally contribute to tumor development and to distinguish from passenger mutations that do not (Kops et al. 2005; Mitelman et al. 2007; Vogelstein and Kinzler 2004). Recent advances in using global genomic profiling coupled with functional analyses have

led to the identification of several potential driver genes that may be important in HCC development (Woo et al. 2009; Zender et al. 2008, 2006). These findings are very promising since defining critical step in hepatocarcinogenesis is the key to our success to develop effective therapeutic agents against HCC. Another important goal would be to classify HCC instances into subgroups based on the observed spectrum of genetic alterations and to determine treatment modalities that are most effective for each subgroup. Current high-resolution platform limitations include studies of patients with different etiologies, but few analyze different time points during the progressive sequence of the disease and the limited size of the studies which undermines the capability to produce a consistent genomic profile.

7 Future Perspectives

Recent progress in genetic and genomic studies on HCC is exciting and offers promises to translate bench-top discovery to clinical practice. Since viral loads are directly linked to HCC onset, development of effective anti-viral agents is a key step in chemoprevention to reduce HCC incidence. Identification of genetic variants that contribute to health disparity in HCC is another important area with a promise in reducing tumor burden. Identification of disease-associated polymorphisms can now be successfully explored by the genome-wide association (GWA) analysis. HCC heterogeneity should not be understated as it is critical to achieve an accurate prognosis prediction and patient stratification for an effective treatment. It is increasingly recognized that the underlying molecular activities are far superior to phenotypic surrogate parameters, such as age, gender, tumor size, and liver function in defining patient outcomes. Thus, understanding of tumor biology is a key future research area. We are in the midst of the most exciting time in HCC research with an extraordinary opportunity that may soon allow us to control this dreadful disease once for all.

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Chapter 2

Overview of Cholangiocarcinoma and Evidence for a Primary Liver Carcinoma Spectrum

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Abstract Intrahepatic cholangiocarcinoma, second in incidence to hepatocellular carcinoma among the primary liver carcinomas, has an even more dismal prognosis. Intrahepatic cholangiocarcinoma is difficult to diagnose at an early stage of development and advances aggressively, with widespread metastases. Molecular genetic features of intrahepatic cholangiocarcinoma have been partially elucidated, although the specific genetic lesions and molecular processes that drive its development, progression, and metastasis are still obscure. Evidence has accumulated from many sources suggesting that cholangiocarcinoma and hepatocellular carcinoma are components of a spectrum of primary liver carcinomas, including poorly and aberrantly differentiated varieties. Primary liver carcinomas arise from cells in different stages of development that encompass the entire lineage of liver epithelial cells generated from hepatoblasts and/or adult liver stem cells, and share critical genomic aberrations and phenotypes with these progenitor cells.

Keywords Primary liver cancer · Hepatocellular carcinoma · Intrahepatic cholangiocarcinoma · Overlap of primary liver cancers

1 Introduction

The primary tumors of the liver comprise a heterogeneous group of benign and malignant neoplasms that include representatives of each of the cellular elements of which the liver is composed – various epithelial, mesenchymal, and vascular cells and the multicellular structures that are made of combinations of these cells (Grisham 2009). Reflecting the cellular composition of the fully developed liver, the majority of the primary liver tumors are epithelial, the better-differentiated varieties resembling the cytology of either the more numerous hepatocytes of the metabolically complex hepatic parenchyma or the less numerous cholangiocytes of the bile ducts that connect the liver parenchyma to the gut. Clinical and pathological studies

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beginning in the mid-nineteenth century gradually defined the natural histories and the pathological features of the major malignant epithelial tumors of the liver, the primary liver carcinomas (PLC) – hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC).

Well-differentiated HCC contains histological structures that resemble hepatic plates and delicate microvessels with little supporting connective tissue, while well-differentiated ICC is composed of histological structures that mimic bile ducts and the generally dense connective tissue that encloses them (Goodman 2007). Since the better-differentiated PLC reflect cytological and histological features of mature hepatocytes and cholangiocytes, HCC and ICC are often considered to be neoplastic variants of the mature hepatic epithelial cells. However, many PLC are cytologically ambiguous and do not closely resemble either mature hepatocytes or cholangiocytes. Examination of the phenotypic properties of the component epithelial cells of PLC has disclosed that both well-differentiated and poorly differentiated tumors may be composed of cells that reflect mixed properties of both mature hepatocytes and cholangiocytes (Kim et al. 2004). Reflecting the embryonic development of hepatocytes and cholangiocytes (Lemaigre and Zaret 2004; Zaret and Grompe 2008; Zhao and Duncan 2005), current evidence suggests that PLC comprise a continuous spectrum of related malignant neoplasms that includes well-differentiated hepatocellular carcinoma and cholangiocarcinoma that closely mimic morphological and phenotypic properties of either hepatocytes or cholangiocytes, as well as cancers that express mixtures of the phenotypic properties of both mature cell types and of the immature cells that are their precursors.

In this chapter we present a brief overview of intrahepatic cholangiocarcinoma, or ICC, followed by a discussion of the evidence that PLC form a spectrum of closely related tumors derived from common precursors, together with implications of this relationship for further definition of the cellular origins and prognostic categories of individual PLC.

2 Overview of Cholangiocarcinoma

Cholangiocarcinoma or bile duct carcinoma can occur anywhere along the system of bile ducts, from the point at which the terminal (smallest) bile ducts connect to biliary canaliculi located between hepatocytes of hepatic plates, through progressively larger bile ducts within the liver, to a single common bile duct (extrahepatic) which connects intrahepatic ducts with the duodenum. Accompanied by branches of the portal vein and hepatic artery, the common bile duct divides into two branches (right and left hepatic bile ducts) at the entrance into the liver (the liver hilum) (Grisham 2009). Cholangiocarcinoma arising in the right and left hepatic ducts at the liver hilum and in the common bile duct and/or ampulla of Vater express growth patterns and genotypic/phenotypic properties that differ from ICC (Henson et al. 1992; Suto et al. 2000), likely reflecting differences in the embryonic origin and development of extrahepatic and intrahepatic bile ducts (Lemaigre

and Zaret 2004; Zaret and Grompe 2008). By convention extrahepatic and intrahepatic cholangiocarcinomas are considered to be separate neoplasms (Nakanuma et al. 2003).

Intrahepatic bile ducts form 8 to 10 branches within the liver that vary in size and structure (Grisham 2009). The largest intrahepatic bile ducts (septal and segmental ducts) contain cholangiocytes that show mucinous and serous differentiation and are associated with peribiliary glands embedded in a dense collagenous stroma. Smaller septal and interlobular branches are composed of a single layer of cuboidal cholangiocytes that lack apparent specific differentiation and are surrounded by a basal membrane and less dense collagenous connective tissue. Septal and interlobular ducts are usually located within portal tracts, where they are surrounded by a rich capillary plexus. The smallest branches, terminal bile ducts (ductules or cholangioles), emerge from portal tracts and extend into the liver parenchyma where they connect with bile canaliculi at the periphery of hepatic plates. The connections between cholangiocytes of terminal bile ductules and hepatocytes of hepatic plates form tubular structures composed of both small cholangiocytes and hepatocytes, called the Canal of Hering (Grisham 2009). The Canals of Hering are thought to be the major intrahepatic sites that enclose adult liver stem cells (Kuwahara et al. 2008).

ICC can arise from any part of the intrahepatic bile ducts (Nakanuma et al. 2003). ICC is less frequent than HCC by a ratio of 1 ICC to about 8–10 HCC (Bosch et al. 2004). However, the incidence of ICC varies geographically and is the most frequent PLC in parts of southern Asia (Bosch et al. 2004). ICC appears to be increasing in incidence world-wide (McGlynn et al. 2006; Schurr et al. 2006; West et al. 2006). In contrast to well-differentiated HCC composed of plates of large, eosinophilic cells that morphologically resemble hepatocytes together with capillary-sized vessels and sparse connective tissue, well-differentiated ICC contains smaller cuboidal or columnar cells with pale eosinophilic or clear cytoplasm that form duct-like structures (adenocarcinoma) enclosed in more-or-less dense arrays of matrix molecules (collagens and components of basal membranes) (Goodman 2007). ICC forms tumors that predominantly grow within the intrahepatic ducts, forms localized scirrhous nodules, or spreads widely through the liver mass (Nakanuma et al. 2003; Goodman 2007).

Clinical experience, confirmed by epidemiological studies, shows that ICC has a worse prognosis than does HCC (Blechacz and Gores 2008). As with HCC, diagnosis of ICC at an early stage of tumor development is difficult, impairing the success of potentially curative therapy. Neoplastic cholangiocytes may express mucin core protein (MUC), carcinoembryonic antigen (CEA), and other cancer-associated proteins, but these tumor-derived molecules have limited specificity and sensitivity as markers of early ICC and lack efficacy for early diagnosis (Blechacz and Gores 2008). ICC usually develops in a noncirrhotic liver, thus, is often advanced in development at the time of diagnosis, and metastasizes widely to organs outside the liver (notably to lymph nodes and bones). Tumor cells of ICC may express a variety of aberrant differentiations, including squamous, clear cell, and sarcomatous. Malignant tumors associated with terminal bile ductules (cholangiolocellular

or ductular carcinomas) often contain mixtures of neoplastic ductular epithelium and hepatocytes (Steiner and Higginson 1959; Komuta et al. 2008). Mixed HCC/ICC that contain both neoplastic hepatocytes and cholangiocytes also occur in situations in which an association with terminal bile ductules is uncertain (Allen and Lisa 1949; Goodman et al. 1985).

Major risk factors for the development of ICC have the common feature of producing chronic inflammation in and around bile ducts associated with recurring damage to cholangiocytes, with or without bile stasis. Particular factors long associated with high risk for ICC include chronic infections with the biliary flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, primary sclerosing cholangitis, hepatolithiasis, and congenital segmental dilation or cysts of bile ducts (Shaib and El-Serag 2004). Liver fluke infestation is particularly important in parts of southern Asia where ICC is a predominant PLC (Vatanasapt et al. 1995).

Chromosomal aberrations are frequent in ICC, with losses involving loci on 1p, 3p, 4q, 8p, 9p, 13q, 16q, and 17p, and gains involving loci on 1q, 3q, 5p, 6p, 7p, 8q, 12q, 15q, 17p, 18p, and 20q in more than 20% of 76 ICC examined by comparative genomic hybridization (CGH) (Koo et al. 2001; Wong et al. 2002; Lee et al. 2004; Uhm et al. 2005). Associated with these locus losses and gains are aberrations in expression of several genes that modulate the metabolism of cholangiocytes and participate in cellular processes that regulate birth, death, vascular supply, and invasion/metastasis of affected tumor cells. Included among these alterations are frequent mutations in *p53*, *p16^{INK4A}*, *p21^{WAF/CIP}*, *DCP4/Smad4*, *TGF β R*, and *Kras* genes (Fava et al. 2007). In many ICC these genetic aberrations are associated with up-regulation and over-expression of several regulatory molecules, including telomerase, Bcl-2, Bcl-X_L, Mdm-2, Mcl-1, IL-6 and IL6R/gp130, HGF/cmet, c-Erb-B2, COX-2, MMP, TGF- β , and VEGF in some combination (Nakanuma et al. 2003; Berthiaume and Wands. 2004; Sirica 2006; Fava et al. 2007). Nevertheless, the particular genetic changes and molecular pathways that drive the development of ICC are not yet understood, and the eradication of tumor development by molecular genetic methods is not yet possible. Most of the studies on which these data are based have examined only one or two genes or molecules in a relatively small number of ICC that likely represent a heterogeneous mixture of tumors representing different stages of development and progression. Only a few studies employing global analysis of gene expression of ICC have been published (Obama et al. 2005; Woo et al. 2009), precluding the precise delineation of the varieties of complex gene signatures that characterize these cancers.

3 Evidence that PLC Comprise a Spectrum of Closely Related Tumors

Mounting evidence indicates that HCC and ICC are components of a PLC tumor spectrum that includes well-differentiated hepatocellular carcinomas and cholangiocarcinomas, which express phenotypic properties that reflect major features of

the complex phenotypes of mature, fully differentiated hepatocytes or cholangiocytes, respectively. Mixed HCC/ICC may contain more-or-less well-differentiated examples of both hepatocytes and cholangiocytes (Allen and Lisa 1949; Goodman et al. 1985). Analysis of genetic aberrations in HCC and ICC components separated from a few mixed HCC/ICC suggests that both types of cells are genomically monoclonal, and arise from a common precursor cell (Imai et al. 1996; Fujii et al. 2000; Murata et al. 2001). Intermediate between the well-differentiated PLC are a variety of incompletely or aberrantly differentiated tumors in which individual tumor cells express different combinations, or mixtures, of the phenotypic properties of mature non-neoplastic hepatocytes and cholangiocytes (Kim et al. 2004), as well as tumors that express aberrant differentiations (squamous, clear cell, sarcomatous, etc.) not ordinarily found in either type of non-neoplastic cell.

The opinion that PLC form a unitary continuum of closely related tumors receives substantial conceptual support from new insights into the embryonic development of the liver, specifically the elucidation of cellular pathways by which differentiated hepatocytes and cholangiocytes are formed from primitive epithelial cells derived from the embryonic foregut. It is now well-established that both hepatocytes and intrahepatic cholangiocytes are derived from a common precursor cell, the hepatoblast, a direct descendant of epithelial cells that migrate from the foregut into the embryonic septum transversum (Zaret and Grompe 2008). Other important support comes from studies on the replacement of hepatocytes and cholangiocytes in livers injured by pathological processes (Fausto and Campbell 2003). Both mature hepatocytes and cholangiocytes can proliferate repeatedly to replace lost or damaged cells when proliferation of the residual mature cells is not impeded. Much evidence now demonstrates the ability of stem cells, located predominantly in the Canals of Hering, to generate new hepatocytes and cholangiocytes even when the residual populations of fully differentiated cells are unable to proliferate. During the process of replacing differentiated hepatocytes and cholangiocytes from stem cells, a transient population of phenotypically heterogeneous intermediate cells, which express various partial combinations of phenotypic properties of the fully differentiated cells and their cellular precursors, is generated (Fausto and Campbell 2003). Phenotypically heterogeneous intermediate cells undergo further differentiation to acquire the specific complex phenotypes that characterize fully differentiated hepatocytes and cholangiocytes.

It is likely that each of the different types of PLC arises from some of the cells composing this lineal mixture of closely related hepatic epithelial cells, and that all PLC ultimately have a common cellular precursor. Current understanding of the mechanisms of cancer development from non-neoplastic precursor cells indicates that any cell that can undergo consecutive proliferative cycles is highly susceptible to neoplastic transformation from the effects of various carcinogenic agents (Weinberg 2006). Thus, in the pathologically damaged liver multiple, phenotypically varied epithelial cells, including mature, fully differentiated hepatocytes and cholangiocytes, and a diverse group of less completely differentiated intermediate cells and their precursors are susceptible to neoplastic transformation from exposure to various carcinogenic factors in their environments. In the remainder of this chapter we briefly review published literature that supports this opinion.

Although not widely influential, the idea that HCC and ICC are closely related tumors is not new. The existence of mixed PLC that contain morphologically distinct neoplastic hepatocytes and cholangiocytes was first described more than 85 years ago (Wells 1903) and comprehensively reviewed more than 60 years ago (Allen and Lisa 1949). The latter study conclusively demonstrated that many of these morphologically mixed tumors develop in the absence of separate ICC and HCC that might have collided during their growth, suggesting a common cellular origin. More than 50 years ago a seminal analysis of 100 PLC that included 65 hepatocellular and 21 cholangiocellular carcinomas (Edmondson and Steiner 1954), concluded that both neoplastic hepatocytes and cholangiocytes could often be morphologically identified in both types of PLC, noted the difficulty to discern unmistakable morphological evidence of either hepatocytic or cholangiocytic origin of the more undifferentiated carcinomas, hypothesized the derivation of HCC and ICC from a common cellular precursor, and suggested that these tumors be grouped together as hepatobiliary cancers, rather than being more specifically defined (Edmondson and Steiner 1954).

The importance of phenotypic properties other than morphology in distinguishing HCC and ICC was clearly demonstrated nearly 25 years ago in a study that applied the histochemical detection of selected cytokeratin proteins to the analysis of morphologically mixed HCC/ICC (Goodman et al. 1985), since cytokeratins expressed by mature hepatocytes and cholangiocytes differ in molecular type (Moll et al. 1982). Individual cells of mixed HCC/ICC (termed transitional carcinomas by the investigators) expressed a mixture of cytokeratins that blended those of fully differentiated hepatocytes and cholangiocytes, suggesting a unitary cellular origin for HCC and ICC (Goodman et al. 1985). This viewpoint was explicitly restated more than 10 years ago on the basis of similar studies that histochemically assessed these and other proteins that are differentially expressed in fully differentiated hepatocytes and cholangiocytes (D'Errico et al. 1996). Many other studies have since used histochemistry to examine selected molecular phenotypic properties characteristic of both mature hepatocytes and cholangiocytes in mixed HCC/ICC to confirm and extend these findings (Tickoo et al. 2002; Kim et al. 2004; others not cited due to space limitations). Some of these studies demonstrated that the prognosis of mixed HCC/ICC more nearly reflects that of ICC occurring separately (Jarnigan et al. 2001; Yano et al. 2003; Koh et al. 2005; Aishima et al. 2006). More recently expanded studies show that poorly differentiated PLC often express a mixture of hepatocyte, cholangiocyte, and precursor cell phenotypes (Kim et al. 2004).

Histochemical studies also show that a significant fraction of morphologically typical HCC expresses histochemically detected proteins characteristic of cholangiocytes, and that these tumors have a worse prognosis than HCC that does not express cholangiocyte phenotypes (Wu et al. 1996; Durnez et al. 2006; Aishima et al. 2007). Recent evidence demonstrates that the prognosis of morphologically characteristic HCC is determined by several complex phenotypes, or signatures (defined by global gene expression arrays), expressed by tumor cells (Lee et al. 2004a,b, 2006), including a gene signature detected in ICC (Woo et al. 2010). These studies show the importance of phenotypic analysis for accurate assessment of prognosis of PLC. The difficulty accurately to categorize the prognosis of PLC (both

well and poorly differentiated) by morphology alone poses a major problem for predicting prognosis and defining therapeutic strategy for these cancers, a dilemma that may be addressed by the analysis of the molecular phenotype of individual tumors.

Although it is commonly believed that HCC and ICC do not share major risk factors, recent epidemiological studies have identified common risk factors for both types of PLC (Shaib and El-Serag 2004; Bosch et al. 2005; Seeff and Hoofnagel. 2006; Shaib et al. 2005), additional evidence that PLC are closely related tumors. Most prominently, epidemiological studies suggest that chronic infections by hepatitis viruses B (HBV) and C (HCV), long known to be major risk factors for HCC, are also important risk factors for ICC (Yamamoto et al. 2004; Hai et al. 2005; Shaib et al. 2005; Welzel et al. 2006). Concordant with these epidemiological studies, assessment of the prevalence of markers for HBV and HCV infections in clinical studies of more than 4500 patients with PLC (Maeda et al. 1995; Yano et al. 2003; Koh et al. 2005; Chantajitr et al. 2006; Lee W et al. 2006; Tang et al. 2006; Zuo et al. 2007), detected HBV surface antigen in $41\pm 30\%$ of 3233 patients with HCC and in $18\pm 10\%$ of 292 patients with ICC, as well as in $39\pm 18\%$ of 282 patients with mixed HCC/ICC. In the same studies the prevalence of HCV antibody was $44\pm 32\%$ in patients with HCC, $16\pm 10\%$ in patients with ICC, and $29\pm 26\%$ in patients with mixed HCC/ICC. The prevalence rates of chronic hepatitis virus infection in each of these PLC is much higher than is reported in the general populations of the countries from which the data were collected, showing that hepatitis viral infections are potent risk factors for both types of PLC. Furthermore, HBV and HCV gene sequences have been found in ICC (Perumal et al. 2006), as in HCC (Edamoto et al. 1996).

Other potent risk factors also increase risk for both HCC and ICC. For example, intrahepatic deposition of thorium dioxide (as thorotrast, formerly but no longer used as an angiographic contrast medium), is a potent hepatocarcinogen as a consequence of its radioactivity and its accumulation in the liver (Sharp 2002). The risk for development of both ICC and HCC is increased to about the same extent in patients who were exposed to thorotrast during angiographic procedures (Sharp 2002). Likewise, genetic hemochromatosis (Morcos et al. 2001) and Wilson's disease (Ponomarev et al. 1994; Walshe et al. 2003), congenital metabolic diseases characterized by accumulation in the liver of excessive amounts of iron and copper, respectively, are associated with increased risk to both types of PLC. Patients with nonalcoholic steatohepatitis also appear to be at increased risk for both ICC and HCC (Ichikawa et al. 2006; Hashizume et al. 2007). Confirming the general biological application of these observations, both types of PLC are also produced by exposure of experimental animals to various hepatocarcinogenic chemicals that act by different molecular mechanisms (National Toxicology Program (1980–2009), various years), and by engineered genetic changes that alter diverse but specific molecular pathways (Lin et al. 1995; Horie et al. 2004; Xu et al. 2006; Kim et al. 2007; Jang et al. 2007). Although certain risk factors are often associated with a dominant association with either HCC or ICC, the accumulated evidence suggests that virtually every known risk factor for PLC increases the risk of both HCC and IHC in both humans and in laboratory animals. The often larger number of

HCC relative to ICC may simply reflect the fact that hepatocytes far outnumber cholangiocytes in the total population of liver epithelial cells (Grisham 2009).

Substantial evidence also supports the opinion that ICC, HCC, and mixed HCC/ICC are closely related genomically. In studies employing identical methods to analyze loss of heterozygosity (LOH) at polymorphic microsatellite loci spanning the entire genome of typical HCC and typical ICC, unique LOH were found for each type of PLC, as well as LOH at specific loci that were shared by both ICC and HCC, suggesting significant overlap of genetic aberrations in these PLC (Momoi et al. 2001; Cazels-Hatem et al. 2004; Liu et al. 2004). Overlap of genomic aberrations in HCC and ICC is illustrated by comparison of the genomic locus losses and gains detected by CGH in 76 independent ICC included in four separate studies (Koo et al. 2001; Wong et al. 2002; Lee et al. 2004; Uhm et al. 2005) and in a meta-analysis of 785 HCC collected from 31 separate studies (Moinzadeh et al. 2005). Losses were found in more than 20% of HCC at loci on chromosome 4q, 8p, 13q, 16q, and 17p and gains were detected in more than 20% of HCC at loci on chromosome 1q, 6p, 8q, and 17q (Table 2.1). Locus losses were found in greater than 20% of ICC on 1p, 3p, 4q, 9p, and 17p, while locus gains were found in more than 20% of ICC on chromosome 1q, 3q, 5p, 6p, 7p, 8q, 12q, 15q, 17p, 17q, 18p,

Table 2.1 Comparison of chromosome losses and gains in HCC and ICC determined by comparative genomic hybridization (CGH)

Chromosome arm (Loss [-]/Gain [+])	HCC ^a (n=785) (%)	ICC ^b (n=76) (%)
1p-		30
3p-		20
4q-	34	26
8p-	38	
9p-		44
13q-	26	
16q-	36	
17p-	32	25
1q+	57	37
3q+		26
5p+		24
6p+	22	26
7p+		20
8q+	47	36
12q+		25
15q+		25
17p+		26
17q+	22	36
18p+		21
20q+		30

^aFrom Moinzadeh et al. (2005); ^bFrom Koo et al. (2001), Wong et al. (2002), Lee et al. (2004), Uhm et al. (2005)

and 20q (Table 2.1). Twenty locus losses and gains occurred in both HCC and ICC combined. HCC and ICC shared locus losses on chromosome 4q and 17p and locus gains on chromosome 1q, 6p, 8q, and 17q. For total aberrations (losses and gains combined), 6 of 9 found in HCC were shared with ICC (67%), while only 6 of 17 losses and gains found in ICC (35%) were shared with HCC. Three of eight locus losses (located at 8p, 13q, and 16q) were unique to HCC (38%), but there were no unique locus gains in HCC (thus three of nine aberrations [both locus losses and gains] in HCC were unique [33%]). ICC had unique locus losses on chromosome arms 1p, 3p, and 9p (38%) and unique locus gains on chromosome arms 3q, 5p, 7p, 12q, 15q, 17p, 18p, and 20q (67%), for a total of 11 unique locus aberrations out of a total of 17 (65%). Of 20 loci that showed aberrations (both locus losses and gains) in the combined typical ICC and HCC, 6 (30%) were shared by both tumors, 3 (15%) were unique to HCC, and 11 (55%) were unique to ICC.

These data show that ICC and HCC have distinct genomic similarities, as well as significant genomic differences. The overlap of genomic aberrations in ICC and HCC is compatible with the concept that all PLC arise by differentiation from a common precursor cell. Genomic evidence derived from dissected components of mixed HCC/ICC suggests (but does not prove) that the ICC and HCC elements are clonal progeny of a single precursor cell (Imai et al. 1996; Fujii et al. 2000; Murata et al. 2001). A common cellular origin of both types of PLC is also supported by observations that cell lines cloned from PLC of both humans (Murakami et al 1987; Yano et al. 1996; Parent et al. 2004) and laboratory animals (Tsao and Grisham 1987; Gil-Benso et al. 2001) have the capacity to produce both HCC and ICC when transplanted into suitable hosts. Furthermore, stem-like cells (Tsao et al. 1984) with bipotential differentiation capacity (Coleman et al. 1994, 1997; Couchie et al. 2002) have been clonally isolated from the livers of healthy adult rats. When neoplastically transformed and re-cloned in vitro and subsequently transplanted into isogenic hosts, single clones of these neoplastic cells produce both HCC and ICC, as well as other types of PLC that express aberrant phenotypes, such as squamous (Tsao and Grisham 1987). Taken together these results reflect both the multipotential differentiation capacity of adult liver stem/progenitor cells and their neoplastic counterparts (Sell and Dunsford 1989).

These results can be explained most simply by the origin of PLC from a phenotypically plastic liver progenitor (stem) cell that can be isolated directly from the liver (in the instance of rodent liver epithelial cells) and/or from tumor stem cells (rodent and human liver tumor-derived cell lines) and that have multipotential differentiation capacities. Such results strongly support the idea that hepatocellular and cholangiocellular carcinomas are closely related tumors formed of cells with differentiation flexibility to express both hepatocellular and cholangiocellular phenotypes, and, therefore, the concept of the continuity of the liver epithelial lineage centered on bi- (multi-) potential stem cells. Although not discussed here, fibrolamellar carcinoma, a variant of HCC found mostly in young adults (Tanaka et al. 2005), hepatoblastoma, a PLC that occurs predominantly in children (Zimmermann 2003), clear cell carcinoma of the liver, sometimes considered to be a variant of either HCC or ICC (Adamek et al. 1998; Tihan et al. 1998; Olivera et al. 2000), and

rare tumors composed of epithelial cells that resemble liver stem/progenitor cells (Robrechts et al. 1998; Theise et al. 2003; Durnez et al. 2006) or intermediate cells (Kim et al. 2004) are PLC that show mixed hepatocyte/cholangiocyte phenotypes and also likely arise from the hepatic epithelial cell lineages originating from fetal hepatoblasts and adult liver stem cells.

4 Conclusions

The prognosis of both ICC and HCC is grim. New methods to diagnose PLC at an earlier stage of development and to establish precise prognosis are needed to enable development of more adequate therapeutic strategies. Concepts and diagnostic/clinical practices pertaining to PLC need to be modified. The combined results of different types of studies that range from clinical to experimental suggest a new way to define PLC that may lead to more accurate diagnosis and more precise prognosis. Plausible changes would be to abandon the idea that ICC and HCC are distinctly different cancers and to base prognosis of both PLC on the phenotypic properties expressed by individual tumors. Much evidence now indicates that the complex phenotypes (gene signatures) expressed by individual PLC determine prognosis more accurately than does morphology.

The studies reviewed support the idea that cells of the entire hepatic epithelial lineage, originating from hepatoblast or adult liver stem cell, and including various intermediate cells, as well as mature hepatocytes and cholangiocytes, are susceptible to neoplastic change. The development of PLC is a cellularly and metabolically complex process that may originate from progeny of liver epithelial stem cells at different stages of differentiation and maturity to yield tumors that express phenotypes typical of mature, differentiated hepatocytes and cholangiocytes, and mixed phenotypes that blend elements of the phenotypes of hepatocytes, cholangiocytes, and precursor cells. Neoplastic transformation of cells in the hepatocyte/cholangiocyte lineage appears to involve various combinations of genes and molecular regulatory pathways that characterize each fully differentiated cell and their precursors. Both well-differentiated HCC and ICC and a variety of tumors that express properties that differ from either of the major PLC, tumors either less completely differentiated or aberrantly differentiated, can result. Most important, prognostic characterization of the malignant neoplasms depends on the particular combination of genes and molecules expressed by individual tumors, and definition of relevant tumor-specific gene signatures is required. Gene signatures that contribute to the most adverse prognoses are already known to include those of the precursor cells of hepatocytes and cholangiocytes and some elements of the cholangiocyte phenotypes.

Although histochemical analysis of a limited number of molecular phenotypes may identify some of the phenotypic properties that are important for establishing prognosis of PLC, widespread use of this technique is impaired by methodological limits to specificity and sensitivity. Poor cellular differentiation further reduces

the sensitivity with which proteins characteristic of differentiated cells can be histochemically detected. Moreover, histochemical techniques are labor intensive and require relatively large amounts of tissue; the small size of tumor specimens typically available for diagnosis practically limits the number of phenotypes that can reasonably be analyzed in each tumor specimen by histochemical methods, preventing delineation of complex phenotypic signatures. Global analysis of gene expression by gene expression profiling (GEP) has supplanted histochemical methods as the most powerful and efficient technology currently available with which to assess the molecular phenotype and, thereby, the cellular origin and developmental stage of PLC. Most of this work has thus far been applied to analysis of HCC. Future application of GEP and other high-throughput techniques of genomic and phenotypic analysis to different tumors of the PLC spectrum may enable the identification of expressed gene signatures that characterize the prognosis of individual tumors throughout the entire spectrum of PLC.

(Note: Space limits prevented a comprehensive citation of the voluminous literature on these subjects. The authors apologize to the many investigators whose relevant work is not cited here.)

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Part II
Liver Cancer Development
and Pathogenesis

Chapter 3

Pathology of Hepatocellular Carcinoma

Masamichi Kojiro

Abstract According to extensive studies on resected and biopsy materials of small hepatocellular carcinoma (HCC) of the early stage, a lot of new information about the morphologic characteristics of minute early-stage HCCs and the morphologic evolution of HCC from early to progressed stages have been obtained. Small HCC up to around 2 cm in size is categorized to two major types; distinctly and vaguely nodular type. Most of small HCC of distinctly nodular type are encapsulated and moderately differentiated, and are detected as hypervascular lesion. On the other hand, small HCC of vaguely nodular type is indistinctly nodular and well-differentiated, and contains portal tracts within the nodule and most of them are hypovascular. Despite small tumor size, the distinctly nodular type HCC is considered as progressed cancer but the vaguely nodular type is interpreted as an early HCC. Well-differentiated early HCCs are gradually de-differentiated and start to proliferate. Eventually, most of them become moderately differentiated when they reach to around 3 cm in size and show the clinicopathological features of classical HCC.

Keywords Hepatocellular carcinoma · HCC · Dysplastic nodule · De-differentiation · Nodule-in-nodule appearance

1 Introduction

According to the remarkable advances in various imaging techniques and the establishment of high-risk populations have led to the detection of small hepatic nodules in patients with cirrhosis or chronic hepatitis caused by hepatitis B or C viruses. Some of these nodules are benign and some have malignant potential or are already malignant. Extensive survey of those small nodular lesions strongly suggests the existence of sequential events from precursor lesions to hepatocellular carcinoma (HCC) (Arakawa et al. 1986; Sakamoto et al. 1991; Borzio et al. 1995; Le Bail et al.

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1997; Libbrecht et al. 2001; Hytioglou 2004; Libbrecht et al. 2005; Theise et al. 2002; Plentz et al. 2007). However, since there had been considerable confusion in the nomenclature of those nodular lesions, International Working Party (IWP) of the World Congresses of Gastroenterology proposed a consensus nomenclature and diagnostic criteria for hepatocellular nodular lesions to solve terminological confusions of nodular lesions in chronic liver disease in 1995 (International Working Party 1995). The IWP classified nodular lesions found in chronic liver disease into large regenerative nodule (LRN), low-grade dysplastic nodule (L-DN), high-grade DN (H-DN), and HCC; this nomenclature has been widely adopted, and L-DN and H-DN were regarded as precursor lesions of HCC. Regarding the pathologic diagnosis of DN and small well-differentiated HCC of the early stage, there had been a discrepancy between western and Asian pathologists (Kojiro 2007). However, pathologic diagnostic consensus has recently been obtained (International Consensus Group for Hepatocellular Neoplasia 2009) (Fig. 3.1).

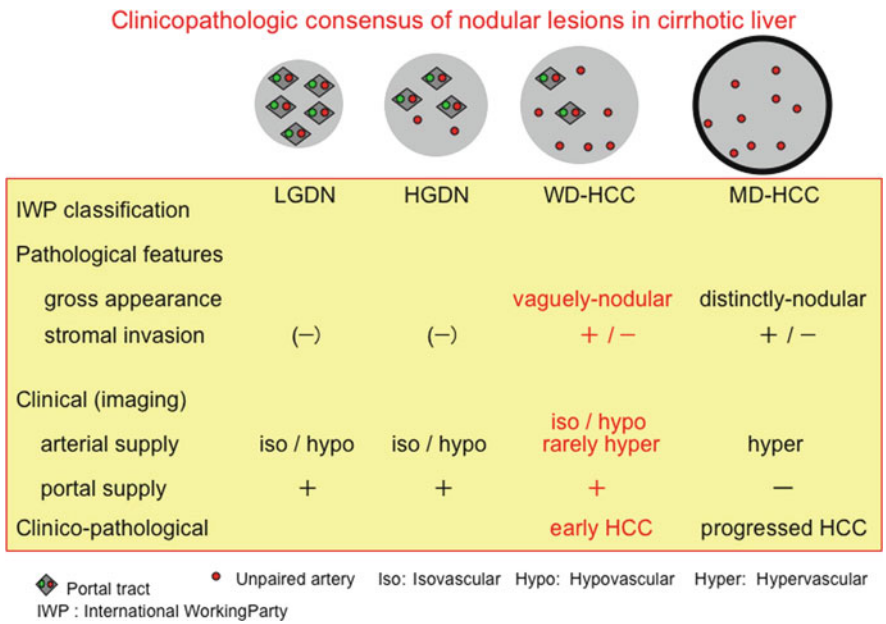


Fig. 3.1 International consensus on small nodular lesions in cirrhotic liver. Diagram summarizing clinical and pathological correlations. The cartoons in the top row show the anatomic changes that are found with the evolution of fully malignant HCC. Because early HCCs grow in a replacing pattern at the boundary, with tumor cells replacing the surrounding liver cell cords, they show a vaguely nodular appearance. When the tumors reach 1.5–2 cm in diameter, they tend to de-differentiate, becoming moderately differentiated and proliferating with formation of a fibrous capsule. Hypovascularity, hypervascularity, and isovascularity are understood to mean the signal intensity in the arterial phase of contrast-enhanced imaging relative to the nontumorous liver. Hypervascularity is related to the development of unpaired arteries, the absence of portal vein supply, and the distinctly nodular growth. The diagnosis must consider the context of the lesion, especially the presence of cirrhosis, the imaging findings, and the growth rate. In the appropriate context, a lesion with decreased portal vein supply without hypervascularity is suggestive of early HCC. (Quoted from Hepatology 49, 2009)

In this chapter, the author will describe the pathologic characteristics of HCC, in particular regarding early-stage HCC and its morphologic evolution to progressed HCC.

2 Precursor Lesions of HCC

2.1 *Dysplastic Nodule*

Dysplastic nodule (DN) is defined as a small hepatocytic nodular lesion without definite histological criteria of malignancy according to the IWP classification. Many of DNs are found as distinct or indistinct nodular lesions ranging from 5 to 10 mm in diameter mostly in cirrhotic liver. According to the degree of dysplasia, DNs are subdivided into low- and high-grade DN. It has been reported that DNs are monoclonal in origin based on the studies by using phosphoglycerokinase gene-based method and DNA fingerprinting (Aihara et al. 1996; Paradis et al. 1998). Premalignant nature of DNs is also suggested by the presence of DNs containing clinicopathological evidence such as the presence of DNs containing HCC focus (Arakawa et al. 1986; Ohno et al. 1990) and frequently evolution to HCC during follow-up study (Takayama et al. 1998; Sakamoto and Hirohashi 1998; Takayama et al. 1998) followed up 18 biopsy-proven cases of low-grade DN and found that 9 cases (50%) transformed to HCC in 6–50 months (mean 21 months) after biopsy. Sakamoto and Hirohashi reported that 13 (72.2%) out of 18 biopsy-proven low-grade DNs transformed to HCC in 3 years (Sakamoto and Hirohashi 1998). The fact that DNs sometimes contain obvious HCC focus or foci within the nodules and this finding also supports the possibility of malignant transformation of DN.

2.1.1 Low-Grade DN

Low-grade DNs are distinct or indistinct nodules around 1.0 cm in diameter, not encapsulated, and slightly more yellowish than the surroundings. The frequency of low-grade DN is reported as 22–25% in explant cirrhotic livers (Ferrell et al. 1992; Theise et al. 1992). Portal tracts are present in the nodules, but the number of portal tracts is fewer than that of the surrounding liver tissue. The hepatocyte atypia is minimal and cell density is slightly increased but no more than two times and a trabecular arrangement is more distinct compared to the surrounding liver tissue (Fig. 3.2). Marked iron deposit is sometimes observed (Terada and Nakanuma 1989). Low-grade DNs are hypovascular on contrast CT, and the sinusoidal endothelial cells are only sporadically positive to CD34 immunohistochemically, suggesting weak and incomplete capillarization. Unpaired arteries which are isolated small arteries are few or absent.

2.1.2 High-Grade DNs

High-grade DN is considered as a borderline lesion. Macroscopically, many of high-grade DNs are vaguely nodular without capsule and range from 1.0 to 2.0 cm in

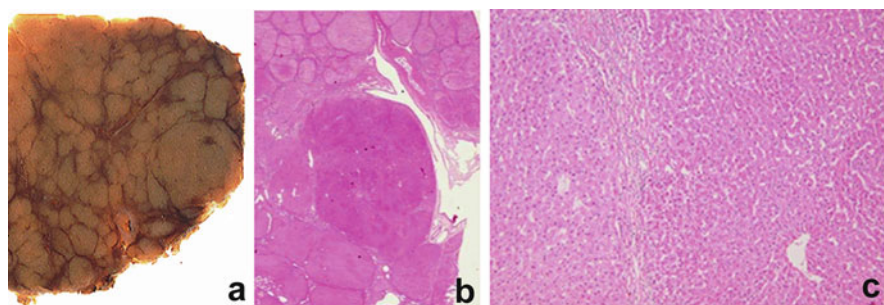


Fig. 3.2 Low-grade dysplastic nodule. (a) A nodule measuring 1 cm in diameter in hepatitis C virus (HCV)-related cirrhosis is indistinctly nodular. (b) Histologically, the nodule shows slightly increased eosinophilic staining. (c) Slight increase in cell density and a more distinct trabecular pattern compared with the surrounding liver tissue (*left side*)

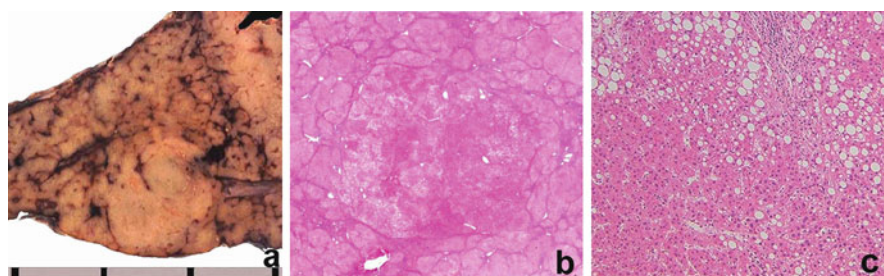


Fig. 3.3 High-grade dysplastic nodule. A nodule measuring 1.3 cm in diameter in HCV-related cirrhosis is indistinctly nodular. (a,b) A tumor shows marked fatty change in parts. (c) In the area without fatty change, irregular thin-trabecular pattern is observed

diameter. Grossly, it is impossible to differentiate high-grade DN from small HCC. Histologically, they show more cytological and architectural atypia than low-grade DNs but not sufficient for the diagnosis of HCC. Cell density is usually 2–3 times higher than the surrounding tissues and an irregular trabecular arrangement with 2–3 cell-thick plates (Fig. 3.3). The only way to differentiate high-grade DN from well-differentiated HCC is the absence of “stromal invasion” that is tumor cell invasion into the intratumoral portal tracts (Tomizawa et al. 1995; Nakano et al. 1997) (Fig. 3.4). When stromal invasion is observed in the nodules, the nodules should be diagnosed as well-differentiated HCC because stromal invasion is considered as a destructive growth by the tumor cells. Most of high-grade DNs are hypo- or iso-vascular on contrast CT, and unpaired arteries are occasionally present and the area of capillarization is more wide-spread compared with that of low-grade DNs (Park et al. 1998; Krinsky et al. 2001).

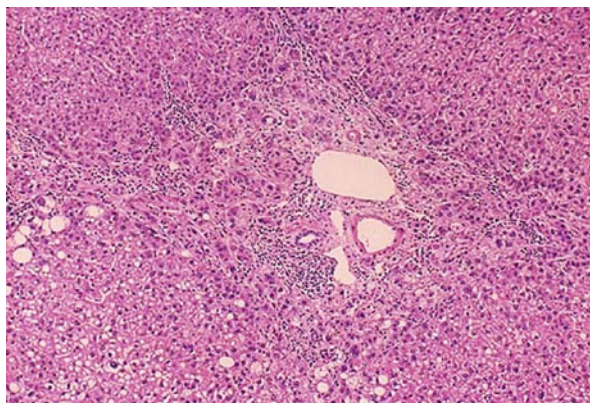


Fig. 3.4 Tumor cell invasion into the intratumoral portal tract (stromal invasion) in well-differentiated HCC of vaguely nodular type. Although portal veins, hepatic artery, and bile duct are preserved, portal tract is almost occupied by tumor cells

3 Hepatocellular Carcinoma

In the past, HCC was a major problem only in Asian and African countries, but it has become a common problem in western countries as well because of the increasing incidence of HCC in the west. Until 30 years ago, most HCCs were detected in an advanced stage in Japan and the average survival time after diagnosis was only 3–6 months. However, according to remarkable development of various imaging modalities and the establishment of a follow-up system of the high-risk populations such as the patients with chronic hepatitis and cirrhosis in the past 2 decades, increasing numbers of HCC are detected in an early stage and are successfully treated medically and surgically. Based on the extensive studies of small-sized resected HCCs of the early stage and biopsy materials from minute HCCs, pathologic characteristic of early-stage HCC that are markedly different from those of classical HCC and morphologic evolution of HCC from early to advanced stage have been explored.

3.1 Small HCC of the Early Stage

It is often possible to make a diagnosis of small HCC when a lesion is distinctly nodular and is hypervascular on contrast-enhanced imaging in the setting of chronic liver disease (Matsui et al. 1985; Kudo et al. 1992a, b; Hayashi et al. 2002; Takayasu et al. 1995). However, when small tumor is hypovascular, it cannot be accurately diagnosed by imaging and such lesions should undergo guided needle core biopsy (Bruix and Sherman 2005). Macroscopically, small HCCs up to around 2 cm in diameter are divided to two types; distinctly nodular type and vaguely nodular type

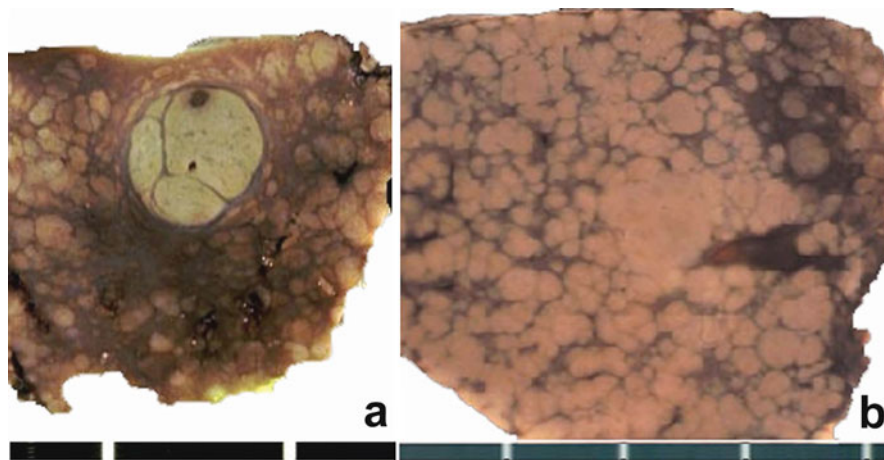


Fig. 3.5 Gross appearance of early-stage small HCC. (a) Distinctly nodular type. The tumor is 1 cm in diameter and has a thin fibrous capsule and fibrous septa as seen in classical HCC. (b) Vaguely nodular type. The lesion is 1 cm in diameter and indistinctly nodular. Both tumors are associated with HCV-related cirrhosis

(Fig. 3.5). On contrast imaging, small HCC of the distinctly nodular type is detected as a hypervascular nodule. However, most of small HCCs of the vaguely nodular small HCC are detected as hypovascular nodule. Small HCC of the vaguely nodular type has been believed to be the smallest HCC that could be clinically detected at present and it has been designated as “early HCC” (International Consensus Group for Hepatocellular Neoplasia 2009).

3.1.1 Small HCC of the Vaguely Nodular Type (Early HCC)

Vague nodular appearance is explained by the histological characteristics that well-differentiated HCC proliferate as if they replace normal liver cell cords of the surrounding liver tissue at the boundary without showing an expansile growth (Fig. 3.6). Histologically, small HCC of the vaguely nodular type contains portal tracts within the tumor and it means that early HCC received portal blood supply as well as arterial blood supply. In many cases, tumor cell invasion into the intratumoral portal tracts is observed (stromal invasion) and it is the most helpful clue in differentiation of early HCC and H-DN (Fig. 3.4). The number of portal tracts within the tumor is markedly reduced to around one-fourth compared with that of the surrounding tissue due to destruction caused by stromal invasion (Nakashima et al. 1999). Accordingly, although the tumor receives portal blood supply through the intratumoral portal tracts, it is suggested that portal blood volume seems to be reduced, and marked decrease of portal blood flow has been observed on contrast-enhanced imaging (Matsui et al. 1985; Kudo et al. 1992).

Small HCCs of the vaguely nodular type are characterized by various combinations of the following five major histologic features (Kojiro 2006; Hytioglou

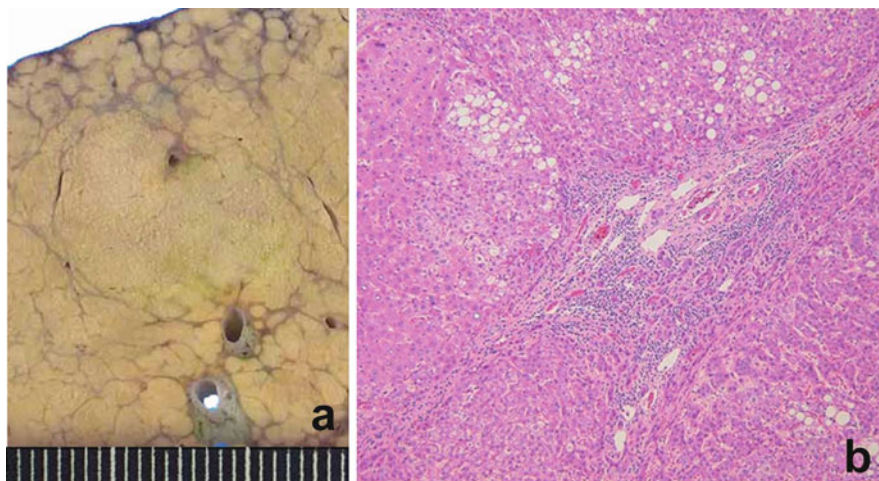


Fig. 3.6 Small HCC of the vaguely nodular type (early HCC). **(a)** A tumor measuring 1.5×1.0 cm in size in HCV-related cirrhosis is indistinctly nodular. **(b)** The tumor is well-differentiated with fatty change and stromal invasion

et al 2007; Kojiro and Nakashima 1999; Kutami et al. 2000): (1) increased cell density more than two times that of the surrounding tissue, with an increased nuclear/cytoplasm ratio and irregular thin-trabecular pattern with frequent small pseudoglands (Fig. 3.7a), (2) varying numbers of portal tracts within the nodule, (3) frequent diffuse fatty change (Fig. 3.7b), and (4) varying numbers of unpaired arteries. Among these features, diffuse fatty change is observed in 30–40% of cases and its frequency declines along with increase of the tumor size (Kutami et al. 2000), and fatty change is uncommon in moderately differentiated HCCs. It is important to note that stromal invasion remains most helpful in differentiating early HCC from H-DNs since all of these features may be found in H-DN as well.

The reasons why the majority of small HCCs of the vaguely nodular type are hypovascular could be explained by incomplete vascularization of the tumor (Park et al. 1998; Krinsky et al. 2001).

3.1.2 Small HCC of Distinctly Nodular Type

It is frequent that small HCCs around 2 cm in diameter are distinctly nodular and are encapsulated by a thin fibrous capsule. Histologically, most of distinctly nodular small HCCs are moderately differentiated and contain no portal tract at all (Fig. 3.8). Microscopically, tumor cell invasion into the blood vessels around the tumor and intrahepatic metastasis in the vicinity of the tumor are occasionally found in the distinctly nodular small HCC.

Most of them are detected as a hypervascular lesion and vascular imaging are not much different from those of classical HCC. Although small HCC of the distinctly nodular type is only around 2 cm in size, they could be interpreted as progressed HCC already (Fig. 3.1).

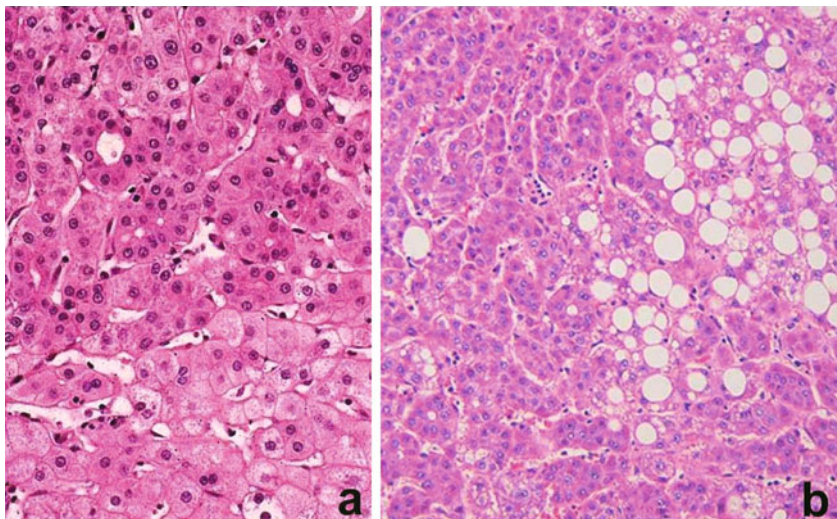


Fig. 3.7 Well-differentiated HCC. (a) Tumor boundary of well-differentiated HCC of vaguely nodular type. The tumor (*lower half*) shows markedly increased cell density, an irregular thin-trabecular pattern, occasional small pseudoglands, and increased eosinophilic staining. The tumor cells are proliferating as if they are replacing the non-cancerous liver cell cords. (b) Well-differentiated HCC with fatty change. In the area where fatty change is mild, and irregular thin-trabecular pattern is remarkable

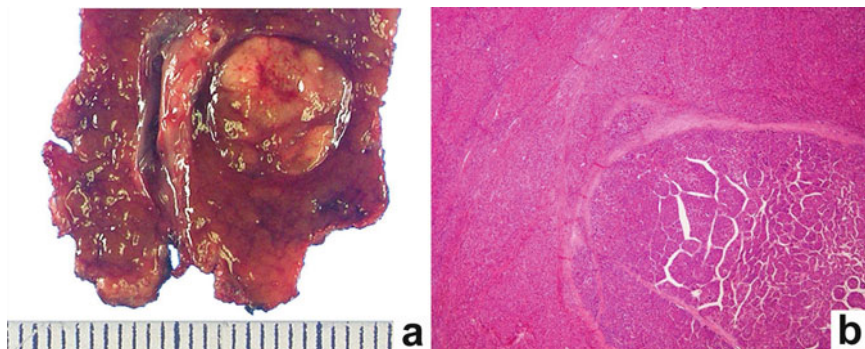


Fig. 3.8 Small HCC of distinctly nodular type. (a) A tumor measuring 1.5 cm in diameter in HCV-related cirrhosis is distinctly nodular. (b) The tumor is composed of moderately differentiated HCC tissue and is encapsulated by a thin fibrous capsule

3.2 Evolution From Early to Advanced HCC

In about one-thirds of HCCs less than 3 cm in diameter, the tumor consists of more than two cancerous tissues of different histologic grades from well-differentiated to moderately differentiated, and moderately differentiated cancerous tissues are located inside of well-differentiated cancerous tissues (Kenmochi et al. 1987). The

areas of well-differentiated cancerous tissues diminish in size along with tumor growth and the areas of moderately differentiated cancerous tissues increase in size. Eventually, when those tumors grow over 3 cm in diameter, the well-differentiated cancerous tissues are completely replaced by moderately differentiated cancer tissues in most cases, and it is rather rare to find well-differentiated cancer tissues in the tumors larger than 3 cm in diameter. Such an evolution was also confirmed by the comparative study of the tumor histology of biopsy specimens from minute HCCs of the early stage and autopsy specimens in the individual cases (Sugihara et al. 1992).

3.2.1 A “Nodule-in-Nodule” Appearance

It is sometimes observed that early HCC contains a distinct subnodule inside, and it is called as a “nodule-in-nodule” appearance (Fig. 3.9). The majority of tumors presenting a nodule-in-nodule appearance are well-differentiated HCC containing a less differentiated subnodule growing in an expansive fashion, and it can be interpreted as a morphologic expression of dedifferentiation process from well-differentiated to moderately differentiated HCC. A “nodule in nodule” appearance is also observed by imaging modalities such as ultrasonography and CT. In the

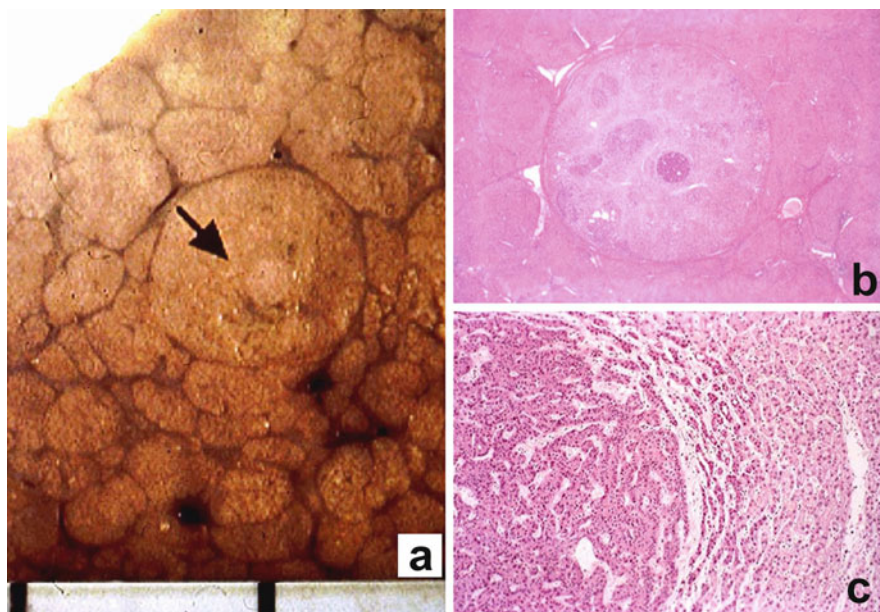


Fig. 3.9 Small HCC with a “nodule-in-nodule” appearance. (a) A tumor measuring 1 cm in diameter contains a minute subnodule (arrow). Histologically, subnodule consists of moderately differentiated HCC with a trabecular pattern and is growing in an expansive fashion to the surrounding well-differentiated HCC tissue

tumors with a “nodule-in-nodule” appearance, the subnodules of moderately differentiated HCC show the higher labeling index of Ki67 compared to the surrounding well-differentiated HCC tissues. Tissue culture study also demonstrated higher proliferative activity of HCC cells in the less differentiated inner nodule and such an evolution occurs in a monoclonal fashion (Yano et al. 1993). Over expression of p53 protein is observed in the subnodule in about 30% of the cases with a “nodule-in-nodule” appearance (Oda et al. 1994). On the other hand, well-differentiated cancerous tissues of the outer side frequently show over expression of transforming growth factor- β (TGF- β) and its receptor; epidermal growth factor receptor (EGFR) (Morimitsu et al. 1995); and cyclooxygenase-2 (COX-2), which is a key enzyme in the synthesis of prostanoids (Koga et al. 1999). These findings suggest that increased expression of those factors seems to play a certain role in cell proliferation and dedifferentiation in the early stages of hepatocarcinogenesis.

3.2.2 Clonal Dedifferentiation of HCC Cells in a Single Tumor Nodule

HCC frequently consists of more than two tumor tissues with different histologic grade in the individual tumor and it was a matter of controversy whether those tumor tissues are monoclonal or polyclonal in origin. The author and colleagues have established a well-differentiated HCC cell line (HAK-1A) and a poorly differentiated HCC cell line (HAK-1B) from a surgically resected HCC tumor with a “nodule in nodule” appearance (Yano et al 1993). These two distinct cell lines show morphologically and biologically different characteristics. Doubling time of well-differentiated HAK-1A is approximately three -times longer than poorly differentiated HAK-1B, and DNA ploidy pattern is diploid in HAK-1A but aneuploidy in HAK-1B. HAK-1B is easily transplantable to nude mice but HAK-1A is not despite of repeated trials because of its weak proliferative activity. Although the two cell lines show completely different morphology and biological characteristics, there are identical structural abnormalities on chromosomes 2 and 17, and nucleotide sequence analysis of exon 7 of the p53 gene shows an identical point mutation of p53 at codon 242. These findings prove that the two cell lines have the same clonal origin, and that the HAK-1B cell, which is poorly differentiated, might have developed from dedifferentiation of well-differentiated HAK-1A cell. Therefore, in HCC nodule consisting of cancerous tissues at varying histologic grades, it is conceivable that less differentiated cancer cells would be generated via the dedifferentiation of well-differentiated cells due to various mechanisms.

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Part III
Genetics and Epidemiology
of Liver Cancer

Chapter 4

Epidemiology of Hepatocellular Carcinoma

Donna L. White and Hashem B. El-Serag

Abstract Hepatocellular carcinoma (HCC) is the sixth leading cause of cancer worldwide and the third leading cause of cancer-related deaths. Most HCC cases (<80%) are attributable to hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, explaining the distinct geographic distribution of HCC with most cases found in developing countries with endemic infections. Strong and continuing recent increases in HCC rates have been observed in Western countries where from 15–50% of HCC cases are cryptogenic. Together with findings from recent epidemiologic research, this suggests the potential importance of additional non-viral or alcohol-related etiologic factors for HCC in these populations, including non-alcoholic steatohepatitis, obesity and Type 2 diabetes mellitus.

Keywords Primary liver cancer · Diabetes mellitus · Non-alcoholic steatohepatitis · Obesity · Risk

1 Global Incidence of Hepatocellular Carcinoma

1.1 Overview

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality (Parkin 2000). Globally, over 560,000 people develop liver cancer each year and an almost equal number, 550,000, die of it. Liver cancer burden, however, is not evenly distributed throughout the world (Fig. 4.1). Most HCC cases (>80%) occur in either sub-Saharan Africa or in eastern Asia. China alone accounts for more than 50% of the world's cases (age-standardized

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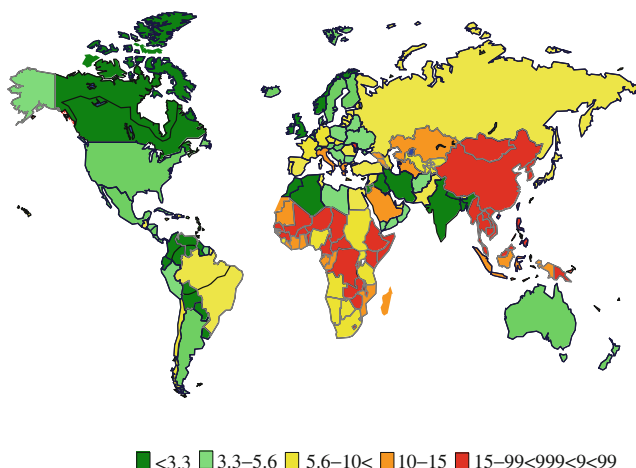


Fig. 4.1 Regional variations in the incidence rates of hepatocellular carcinoma expressed as age-adjusted incidence rates

incidence rate (ASR) male: 35.2/100,000; female: 13.3/100,000). Other high-rate (>20/100,000) areas include Senegal (male: 28.47/100,000; female: 12.2/100,000), The Gambia (male: 39.67/100,000; female: 14.6/100,000), and South Korea (male: 48.8/100,000; female: 11.6/100,000).

North and South America, northern Europe, and Oceania are low-rate (<5.0/100,000) areas for liver cancer among most populations. Typical incidence rates in these areas are those of the United States (male: 4.21/100,000; female: 1.74/100,000), Canada (male: 3.2/100,000; female: 1.1/100,000), Colombia (male: 2.2/100,000; female: 2.0/100,000), United Kingdom (male: 2.2/100,000; female: 1.1/100,000), and Australia (male: 3.6/100,000; female: 1.0/100,000). Southern European countries, typified by rates in Spain (male: 7.5/100,000; female: 2.4/100,000), Italy (male: 13.5/100,000; female: 4.6/100,000), and Greece (male: 12.1/100,000; female: 4.6/100,000) are medium-rate (5.0–20.0/100,000) (Ferlay et al. 2001).

HCC accounts for between 85 and 90% of primary liver cancer. One noteworthy exception is the Khon Kaen region of Thailand, which has one of the world's highest rates of liver cancer (ASR_{1993–1997} male: 88.0/100,000; female: 35.4/100,000) (Parkin et al. 2002). However, due to endemic infestation with liver flukes, the major type of liver cancer in this region is intrahepatic cholangiocarcinoma rather than HCC (Okuda et al. 2002).

Encouraging trends in liver cancer incidence have been seen in some of these high-rate areas (McGlynn et al. 2001). Between 1978–1982 and 1993–1997, decreases in incidence were reported among Chinese populations in Hong Kong, Shanghai, and Singapore (Parkin et al. 2002). In addition to these areas, Japan also began to experience declines in incidence rates among males for the first time between 1993 and 1997 (Fig. 4.2).

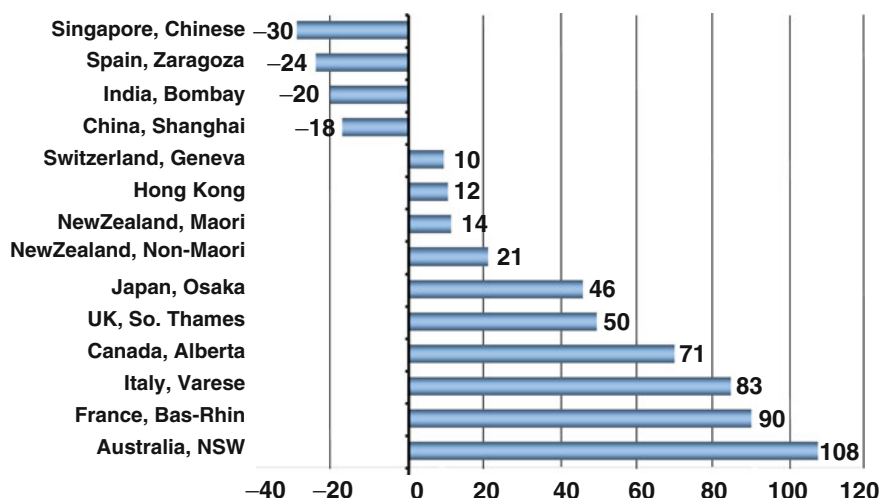


Fig. 4.2 Recent changes in the incidence of HCC. The incidence of HCC has been declining in some “high-incidence” areas, such as China and Hong Kong. This is partly related to HBV vaccination of children, and to reduction of aflatoxin exposure in grains. On the other, HCC incidence in several “low and intermediate incidence” areas, have been increasing. For example, hepatitis C related HCC has been responsible for most the observed recent rise in the United States (McGlynn et al. 2001; Lok 2004; Chang et al. 2000)

Many high-rate Asian countries now vaccinate all newborns against HBV and the effect on HCC rates has already become apparent. In Taiwan, where national newborn vaccination began in 1984, HCC rates among children aged 6–14 years declined significantly from 0.70/100,000 in 1981–1986 to 0.36/100,000 in 1990–1994 (Chang et al. 1997). It is too soon yet for HBV vaccination to have had an effect on adult rates, but other public health measures may have contributed to declines in HCC incidence in high-risk areas of China. A Chinese government program started in the late 1980s to shift the staple diet of the Jiangsu Province from corn to rice may have limited exposure to known hepatocarcinogen aflatoxin B1 (AFB1) in this area (Yu 1995). Similarly, another Chinese public health campaign initiated in the early 1970s to encourage drinking of well rather than pond or ditch water, may have decreased consumption of microcystins, cyanobacteria-produced compounds demonstrated to be hepatocarcinogenic in experimental animals.

In contrast, registries in a number of low-rate areas reported increases in HCC incidence between 1978–1982 and 1993–1997. Included among these registries are those in the United States, United Kingdom, and Australia. Reasons for both the decreased incidence in high-rate areas and the increased incidence in low-rate areas are not yet clear, suggesting that each area will be an important case study. It has been widely hypothesized, however, that increased incidence in low-rate areas may be related to greater prevalence of HCV infection and of obesity and Type II diabetes mellitus within these areas.

1.2 Race/Ethnicity

HCC incidence rates also vary greatly among different populations living in the same region. For example, ethnic Indian, Chinese, and Malay populations of Singapore had age-adjusted rates ranging from 21.21/100,000 among Chinese males to 7.86/100,000 among Indian males between 1993 and 1997 (Parkin et al. 2002). The comparable rates for females were 5.13/100,000 among ethnic Chinese and 1.77/100,000 among ethnic Indians. Another example is the United States where, at all ages and among both genders, HCC rates are two times higher in Asians than in Blacks, which are two times themselves higher than those in Caucasians. The reason(s) for this interethnic variability likely include differences in prevalence and acquisition time of major risk factors for liver disease and HCC.

1.3 Gender

In almost all populations, males have higher liver cancer rates than females, with male:female ratios usually averaging between 2:1 and 4:1. At present, the largest discrepancies in rates ($>4:1$) are found in medium-risk European populations. Typical among these ratios are those reported from Geneva, Switzerland (4.1:1), and Varese, Italy (5.1:1). Among ten French registries listed in Volume VIII of Cancer in Five Continents, nine report male:female ratios $>5:1$. In contrast, typical ratios currently seen in high-risk populations are those of Qidong, China (3.2:1), Osaka, Japan (3.7:1), The Gambia (2.8:1), and Harare, Zimbabwe (2.4:1). Registries in Central and South America report some of the lowest sex ratios for liver cancer. Typical ratios in these regions are reported by Colombia (1.2:1) and Costa Rica (1.6:1).

The reasons for higher rates of liver cancer in males may relate to gender-specific differences in exposure to risk factors. Men are more likely to be infected with HBV and HCV, consume alcohol, smoke cigarettes, and have increased iron stores. Higher levels of androgenic hormones, body mass index, and increased genetic susceptibility may also adversely affect male risk.

1.4 Age

The global age distribution of HCC varies by region, incidence rate, gender, and, possibly, by etiology (Parkin et al. 2002). In almost all areas, female rates peak in the age group 5 years older than the peak age group for males. In low-risk populations (e.g., United States, Canada, United Kingdom), the highest age-specific rates occur among persons aged 75 and older. A similar pattern is seen among most high-risk Asian populations (e.g., Hong Kong, Shanghai). In contrast, male rates in high-risk African populations (e.g., The Gambia, Mali) tend to peak between ages 60 and 65 before declining; while female rates peak between 65 and 70 before declining. These variable age-specific patterns are likely related to differences in the dominant

hepatitis virus in the population, the age at viral infection and the existence of other risk factors. Notably, while most HCV carriers became infected as adults, most HBV carriers became infected at very young ages.

Exceptions to these age patterns occur in Qidong, China, where liver cancer rates are among the world's highest. Age-specific incidence rates among males rise until age 45 and then plateau, while among females, rates rise until age 60 and then plateau. The explanation for these younger peak ages is unclear, but may be due to existence of other hepatocarcinogenic exposures.

1.5 Distribution of Risk Factors

Major risk factors for HCC vary by region. In most high-risk areas, the dominant risk factor is chronic HBV infection. In Asia, HBV infection is largely acquired by maternal–child transmission, while sibling-to-sibling transmission at young ages is more common in Africa. Consumption of aflatoxin B₁-contaminated foodstuffs is the other major HCC risk factor in most high-rate areas.

Unlike the rest of Asia, the dominant hepatitis virus in Japan is hepatitis C (HCV). HCV began to circulate in Japan shortly after the World War II (Yoshizawa 2002). Consequently, HCC rates began to sharply increase in the mid-1970s with an anticipated peak in HCV-related HCC rates projected around 2015, though recent data suggests the peak might have already been reached.

In low-rate HCC areas, increasing numbers of persons living with cirrhosis is the likely explanation for rising HCC incidence. This has resulted from a combination of factors including: rising incidence of cirrhosis due to HCV and, to a lesser extent, HBV infection, as well as a general improvement in survival among cirrhosis patients. It has been estimated that HCV began to infect large numbers of young adults in North America and south and central Europe in the 1960s and 1970s as a result of intravenous drug use (Armstrong et al. 2000). The virus then moved into national blood supplies and circulated until a screening test was developed in 1990, after which time rates of new infection dropped dramatically. Currently, it is estimated that HCV-related HCC in low-rate countries will peak between 2020 and 2030 (Davis et al. 2010).

1.5.1 HCC in the United States

Age-adjusted HCC incidence rates increased more than twofold between 1985 and 2002 (El-Serag 2004) (Fig. 4.3). Average annual, age-adjusted rate of HCC verified by histology or cytology increased from 1.3 per 100,000 during 1978–1980 to 3.3 per 100,000 during 1999–2001 (El-Serag et al. 2003). The increase in HCC started in the mid-1980s with greatest proportional increases occurring during the late 1990s. The largest proportional increases occurred among whites (Hispanics and non-Hispanics), while the lowest proportional increases occurred among Asians. The mean age at diagnosis is approximately 65 years, 74% of cases occur in men, and the racial distribution is 48% white, 15% Hispanic, 13% African-American, and 24% other race/ethnicity (predominantly Asian). During recent years as incidence

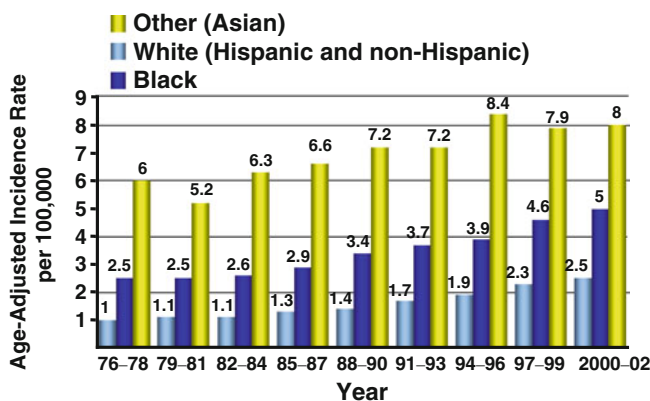


Fig. 4.3 Racial Incidence Rates for HCC in the United States. The average annual age-adjusted incidence rates for HCC shown for three-periods between 1976 and 2002 and broken down by racial groups (source: SEER: Surveillance Epidemiology and End Results). Although the highest incidence rates are observed in Asians, the highest proportional increase was observed among Whites (non Hispanic as well Hispanic). Black African Americans have intermediate rates (El-Serag et al. 2003; El-Serag and Mason 1999)

rates increased, the age-distribution of HCC patients has shifted towards relatively younger ages, with greatest proportional increases between ages 45 and 60.

Four published studies examined secular changes in HCC risk factors in the United States (Davila et al. 2004; El-Serag and Mason 2000; Hassan et al. 2002; Kulkarni et al. 2004). Two studies were from large, single referral centers where viral risk factor ascertainment was based on serology findings, while the other two were from national databases in which risk factors were ascertained from ICD-9 codes in billing or discharge records. In all four studies, the greatest proportional increases occurred in HCV-related HCC, while HBV-related HCC had the lowest and most stable rates. Overall, between 15 and 50% of HCC patients in the United States have no established risk factors.

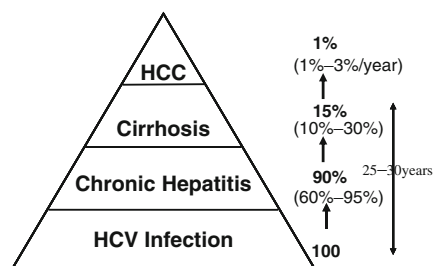
2 Risk Factors of Hepatocellular Carcinoma

HCC is unique in that it largely occurs within an established background of chronic liver disease and cirrhosis (~70–90% of all detected HCC cases) (Fig. 4.4). Major causes of cirrhosis in patients with HCC include: hepatitis B, hepatitis C, alcoholic liver disease, and possibly, non-alcoholic steatohepatitis.

2.1 Hepatitis B Virus

Globally, HBV is the most frequent underlying cause of HCC with an estimated 300 million persons with chronic infection worldwide. Case-control studies

Fig. 4.4 Estimated progression rates to cirrhosis and hepatocellular carcinoma in hepatitis C infection



have demonstrated chronic HBV carriers have a 5–15-fold increased risk of HCC compared to the general population.

The great majority, between 70 and 90%, of HBV-related HCC develops in a background of cirrhosis. HBV DNA is found in the host genome of both infected and malignant hepatic cells. HBV may therefore initiate malignant transformation through a direct carcinogenic mechanism by increasing likelihood of viral DNA insertion in or near proto-oncogenes or tumor-suppressor genes. However, despite initial excitement accompanying this discovery, subsequent research has failed to show a unifying mechanism by which integration of HBV DNA leads to HCC.

The increased HCC risk associated with HBV infection particularly applies to areas where HBV is endemic. In these areas, it is usually transmitted from mother to newborn (vertical transmission) and up to 90% of infected persons follows a chronic course. This pattern is different in areas with low-HCC incidence rates where HBV is acquired in adulthood through sexual and parenteral routes (horizontal transmission) with >90% of acute infections resolving spontaneously. The annual HCC incidence in chronic HBV carriers in Asia ranges between 0.4 and 0.6%. This figure is lower in Alaskan natives (0.26%/year) and lowest in Caucasian HBV carriers (McMahon et al. 1990).

Several other factors have been reported to increase HCC risk among HBV carriers including: male gender; older age (or longer duration of infection); Asian or African race; cirrhosis; family history of HCC; exposure to aflatoxin, alcohol or tobacco; or co-infection with HCV or HDV. HCC risk is also increased in patients with higher levels of HBV replication, as indicated by presence of HBeAg and high HBV DNA levels. In addition, it has been suggested in Asian studies that genotype C is associated with more severe liver disease than genotype B (Kao et al. 2002).

In the natural history of chronic HBV infection, spontaneous or treatment-induced development of antibodies against HBsAg and HBeAg leads to improved clinical outcomes. A meta-analysis of 12 studies with 1,187 patients who received interferon and 665 untreated patients followed for 5 years found lower HCC incidence in treated 1.9% (95% CI, 0.8–3.0%) than untreated patients 3.2% (95% CI, 1.8–4.5%). However, this difference was not statistically significant (Camma et al. 2001).

Using sensitive amplification assays, many studies have demonstrated that HBV DNA persists as “occult HBV infection” for decades among persons with serological recovery (HBsAg negative) from acute infection. Occult HBV is associated with anti-HBc and/or anti-HBs (Torbenenson and Thomas 2002). However, in a significant proportion of individuals, neither anti-HBc nor anti-HBs can be detected. A single multinational investigation found prevalence of occult HBV in liver tissue to be 11% in Italy, 5–9% in Hong Kong, and 0% in the United Kingdom. Supporting an association with occult HBV, a high proportion of individuals with HCV infection who develop HCC have demonstrable HBV DNA and proteins in their neoplastic and adjacent non-neoplastic liver tissue. However, although some studies have linked development of HCC in individuals with chronic HCV infection to occult HBV, others have not found an association.

2.2 Hepatitis C Virus

Chronic HCV infection is a major risk factor for development of HCC. Markers of HCV infection are found in a variable proportion of HCC cases; for example, 44–66% in Italy (Fasani et al. 1999; Stroffolini et al. 1999), 27–58% in France, 60–75% in Spain, and in 80–90% of HCC cases in Japan (Yoshizawa 2002). A higher but undefined proportion of HCC patients might have had HCV detected by PCR testing of liver tissue and/or serum, even if antibody to HCV (anti-HCV) was non-detectable. In a meta-analysis of 21 case-control studies in which second generation enzyme immunoassay tests for anti-HCV were used, HCC risk was increased 17-fold in HCV-infected patients compared with HCV-negative controls (95% confidence intervals (CI): 14–22) (Donato et al. 1998).

The likelihood of development of HCC among HCV-infected persons is difficult to determine due to the paucity of adequate long-term cohort studies; however, the best estimate is from 1 to 3% after 30 years (Fig. 4.5). HCV increases HCC risk by promoting fibrosis and eventually cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of 1–4%; though rates up to 7% have been reported in Japan. Rates of cirrhosis 25–30 years post-infection range between 15 and 35% (Freeman et al. 2001). The highest incidence rates were observed in

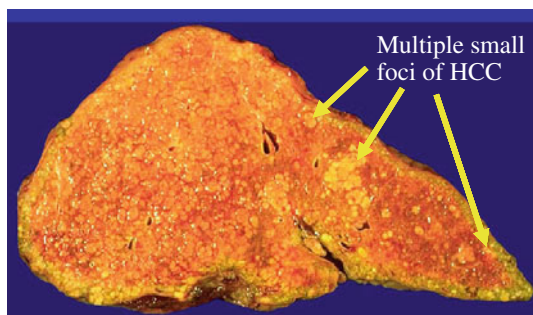


Fig. 4.5 Cirrhosis and hepatocellular carcinoma. Explanted liver showing features of cirrhosis and multiple small foci of HCC throughout the liver in a miliary pattern (arrows)

HCV-contaminated blood or blood products recipients (14 and 1 per 1000 person-years for cirrhosis and HCC, respectively) and in hemophiliacs (5 and 0.7 per 1000 person-years). The lowest rates have been reported in women who received a one-time contaminated anti-D immune globulin treatment (1 and 0 per 1000 person-years, respectively).

In HCV-infected patients, factors related to host as well as environment and lifestyle appear to be more important than viral factors in determining progression to cirrhosis. These factors include: older age, older age at the time of acquisition of infection, male gender, heavy alcohol intake (>50 gm/day), diabetes, obesity, and co-infection with HIV or HBV (Cramp 1999). There is no strong evidence that HCV viral factors like genotype, viral load, or quasispecies are important in determining the risk of progression to cirrhosis or HCC.

Successful antiviral therapy in patients with HCV-related cirrhosis may reduce future risk of HCC, but the evidence is weak. There is only one prospective, randomized, controlled trial that examined the effects of antiviral therapy on HCC, a Japanese trial in which 100 patients were randomized to receive either 6 million units of interferon-alfa thrice weekly for 3–6 months or were followed without treatment (Nishiguchi et al. 1995). After a 2–7-year follow-up period, HCC was significantly reduced in the treated (4%) compared to the non-treated control group (38%), a 93% reduction in adjusted risk. However, much of this risk reduction was a result of the unusually high-HCC rate among these controls. Other studies, mostly retrospective and non-randomized, suggested moderately decreased HCC risk among HCV-infected patients treated with interferon (International Interferon-alpha Hepatocellular Carcinoma Study Group 1998; Bressac et al. 1991; Bruno et al. 2001; Garner et al. 1992; IARC Monographs 1987; Ikeda et al. 1999; Imai et al. 1998; Niederau et al. 1998; Okanoue et al. 1999; Serfaty et al. 1998; Turner et al. 2002; Valla et al. 1999).

In general, reported preventive effects of interferon therapy were less marked in European compared to Japanese studies. However, the lack of randomization in most of these studies may exaggerate treatment benefits as it is likely healthier patients tend to get treated more frequently than those with advanced liver disease (who are known to be more likely to develop HCC). In addition to a role in primary prevention of HCC among HCV-infected patients, a few Japanese reports suggest interferon may also be effective for secondary prevention in individuals who have previously undergone resection for HCC.

2.3 Alcohol

Heavy alcohol intake, defined as ingestion of >50–70 gm/day for prolonged periods, is a well-established HCC risk factor. It is unclear whether risk of HCC is significantly altered in those with low or moderate alcohol intake. Although heavy intake is strongly associated with development of cirrhosis; there is little evidence of a direct carcinogenic effect of alcohol otherwise.

There is also evidence for a synergistic effect of heavy alcohol ingestion with HCV or HBV, with these factors presumably operating together to increase HCC risk by more actively promoting cirrhosis. For example, Donato et al. 2002 reported that among alcohol drinkers, HCC risk increased in a linear fashion with daily intake >60 gm. However, with concomitant presence of HCV infection, there was an additional twofold increase in HCC risk over that observed with alcohol usage alone (i.e., a positive synergistic effect).

2.4 Aflatoxin

Aflatoxin B₁ (AFB₁) is a mycotoxin produced by the *Aspergillus* fungus. This fungus grows readily on foodstuffs like corn and peanuts stored in warm, damp conditions. Animal experiments demonstrated that AFB₁ is a powerful hepatocarcinogen leading the International Agency for Research on Cancer (IARC) to classify it as carcinogenic (IARC Monographs 1987).

Once ingested, AFB₁ is metabolized to an active intermediate, AFB₁-*exo*-8,9-epoxide, which can bind to DNA and cause damage, including producing a characteristic mutation in the p53 tumor-suppressor gene (p53 249^{ser}) (Garner et al. 1992). This mutation has been observed in 30–60% of HCC tumors in aflatoxin endemic areas (Bressac et al. 1991; Turner et al. 2002).

Strong evidence that AFB₁ is a risk factor for HCC has been supplied by person-specific epidemiological studies performed in the last 15 years. These studies were permitted by development of assays for aflatoxin metabolites in urine, AFB₁-albumin adducts in serum, and detection of a signature aflatoxin DNA mutation in tissues.

Interaction between AFB₁ exposure and chronic HBV infection was revealed in short-term prospective studies in Shanghai, China. Urinary excretion of aflatoxin metabolites increased HCC risk fourfold while HBV infection increased risk sevenfold. However, individuals who both excreted AFB₁ metabolites and were HBV carriers had a dramatic 60-fold increased risk of HCC (Qian et al. 1994).

In most areas where AFB₁ exposure is a problem, chronic HBV infection is also highly prevalent. Though HBV vaccination in these areas should be the major preventive tactic, persons already chronically infected will not benefit from vaccination. However, HBV carriers could benefit by eliminating AFB₁ exposure. Efforts to accomplish this goal in China (Yu 1995) and Africa (Turner et al. 2002) have been launched.

2.5 Non-alcoholic Fatty Liver Disease (NAFLD) and Non-alcoholic Steatohepatitis (NASH)

Studies in the United States evaluating risk factors for chronic liver disease or HCC have failed to identify HCV, HBV, or heavy alcohol intake in a large proportion of patients (30–40%). It has been suggested that many cryptogenic cirrhosis and HCC cases in fact represent more severe forms of non-alcoholic fatty liver disease

(NAFLD), namely non-alcoholic steatohepatitis (NASH). Potential risk factors such as diabetes, obesity, and possibly HCV are likely to increase HCC risk at least partly by promoting NAFLD and NASH.

One difficulty in epidemiological studies attempting to elucidate the association between NASH and risk of HCC in humans, however, is that once either cirrhosis or HCC are established, it is difficult to identify pathological features of NASH. Several clinic-based case-control studies have in fact indicated that HCC patients with cryptogenic cirrhosis tend to have clinical and demographic features suggestive of NASH (predominance of women, diabetes, obesity) than age- and sex-matched HCC patients of well-defined viral or alcoholic etiology (Ferlay et al. 2001; Okuda et al. 2002; Parkin et al. 2002). For example, Regimbeau et al. (2004) examined 210 patients who underwent resection for HCC of whom 18 (8.6%) had no identifiable cause for chronic liver disease and found higher prevalence of obesity (50% vs. 17% vs. 14%) and diabetes (56% vs. 17% vs. 11%) compared to patients with alcoholic and viral hepatitis, respectively (Regimbeau et al. 2004). Evidence of progression from NAFLD to HCC from prospective studies is scant. There are case reports (Chang et al. 1997; McGlynn et al. 2001) and a small case series describing development of HCC several years following NASH diagnosis (Shimada et al. 2002). In a community-based retrospective cohort study, 420 patients diagnosed with NAFLD in Olmsted County, MN, were followed for a mean duration of 7.6 years (Adams et al. 2005). In that study, liver disease was the third leading cause of death (as compared with the 13th leading cause of death in the general Minnesota population), occurring in seven (1.7%) subjects. Twenty-one (5%) patients were diagnosed with cirrhosis of whom two developed HCC.

2.6 Diabetes

Diabetes, particularly Type II diabetes, has been proposed to be a risk factor for both chronic liver disease and HCC through development of NAFLD and NASH. It is known to contribute significantly to hepatic steatosis (Armstrong et al. 2000; Davila et al. 2004) with development of increased levels of steatosis associated with more severe necro-inflammatory activity (Davila et al. 2004; El-Serag et al. 2003) and fibrosis (Camma et al. 2001; Kao et al. 2002; McMahon et al. 1990). Fibrosis progression rates have also appeared to be higher when marked steatosis was present (Torbenenson and Thomas 2002), with some studies suggesting that the increase in steatosis itself may be an indicator of fibrosis progression (El-Serag and Mason 2000). Additionally, liver disease occurs more frequently in those with more severe metabolic disturbances with insulin resistance itself demonstrated to increase as liver disease progresses (Fasani et al. 1999).

Several case-control studies from the USA, Greece, Italy, Taiwan, and Japan examined the association between diabetes, mostly type II, and HCC. Among thirteen case-control studies, nine found a significant positive association between diabetes and HCC, two found a positive association that did not quite reach

significance, and three found a negative association (El-Serag et al. 2006). A potential bias in cross-sectional and case-control studies, however, is difficulty in discerning temporal relationships between exposures (diabetes) and outcomes (HCC). This problem is relevant in evaluating HCC risk factors because 10–20% of patients with cirrhosis have overt diabetes and a larger percentage have impaired glucose tolerance. Thus, diabetes may also be the result of cirrhosis.

Cohort studies, which are intrinsically better suited to discern temporal relationships between exposure and disease, have also been conducted. All compared HCC incidence in cohorts of diabetic patients to either the expected incidence given HCC rates in the underlying population or to the observed HCC incidence among a defined cohort without diabetes (El-Serag et al. 2006). Three studies conducted among younger or smaller cohorts found either no or low number of HCC cases (Ragozzino et al. 1982; Hjalgren et al. 1997; Zendejdel et al. 2003). At least five other cohort studies examined large number of patients for relatively long-time periods (Kessler 1970; Adami et al. 1996; Wideroff et al. 1997; El-Serag et al. 2004; Coughlin et al. 2004), with three studies finding significantly increased risk of HCC with diabetes (risk ratios ranging between 2.5 and 4) (Adami et al. 1996; Coughlin et al. 2004; Wideroff 1997; El-Serag et al. 2004). We recently conducted a study of HCC incidence in a large cohort of VA patients ($n = 173,643$ with and $n = 650,620$ without diabetes). The findings of this study indicate HCC incidence doubled among patients with diabetes and was higher among those with longer duration of follow-up (El-Serag et al. 2004) (Fig. 4.9).

While most studies have been conducted in low-HCC rate areas, diabetes has also been found to be a significant risk factor in areas of high-HCC incidence like Japan. Further, although other underlying risk factors like HCV may confound the association between diabetes and HCC, they do not seem to fully explain it. Taken together, a systematic review and meta-analysis of available data suggests diabetes is a moderately strong risk factor for HCC (El-Serag et al. 2006). However, additional research is needed to more fully examine how any excess risk conveyed by diabetes is mediated by such potentially confounding factors as duration and treatment of diabetes, family history of diabetes, and current and historical levels of obesity and physical activity.

2.7 Obesity

Obesity, especially abdominal obesity, is strongly correlated with insulin resistance and Type II diabetes, a state of clinically diagnosable advanced insulin resistance that has itself been associated with HCC risk. Some evidence in support of a direct contribution of obesity-mediated metabolic errors in hepatocarcinogenesis comes from experimental research in a genetically obese ob/ob knockout mouse model of NAFLD that demonstrated hepatic hyperplasia even at very early stages of disease and without evidence of cirrhosis (Nishiguchi et al. 1995).

The effect of obesity on HCC risk has been examined in several cohort studies. In a large prospective cohort study of more than 900,000 individuals from around the USA followed for a 16-year-period, liver cancer mortality rates were five times greater among men with the greatest baseline BMI (35–40) compared to those with normal BMI (Calle et al. 2003) (Figs. 4.6 and 4.7). In the same study, the risk of liver cancer was not as elevated in women with a relative risk of 1.68 (0.93–3.05). Two other population-based cohort studies from Sweden and Denmark found excess HCC risk (elevated relative risk of two- to three-fold) in obese men and women compared to those with normal BMI (Moller et al. 1994; Wolk et al. 2001). In a large prospective cohort study in Taiwan, obesity (BMI 30+) conveyed excess risk of HCC even after controlling for other metabolic risk factors including presence of diabetes mellitus (International Interferon-alpha Hepatocellular Carcinoma Study

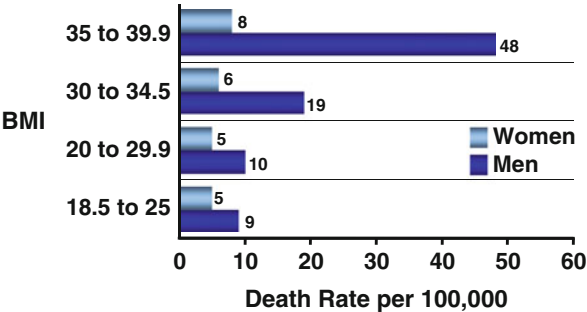


Fig. 4.6 Obesity and liver cancer. In both men and women, a higher body-mass index (BMI) is significantly associated with higher rates of death due to cancer of the liver. Modified from Calle et al. (2003)

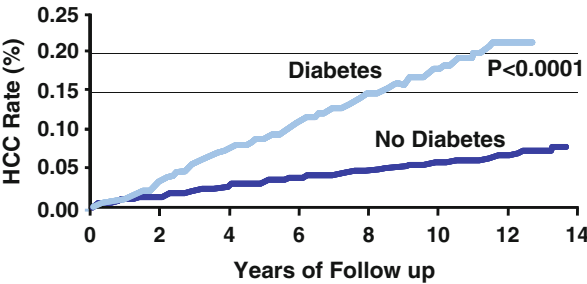


Fig. 4.7 Diabetes is associated with a two-fold increase in risk of HCC. In this study, all patients with a hospital discharge diagnosis of diabetes between 1985 and 1990 using the computerized records of the Department of Veterans Affairs. Three patients without diabetes for every patient with diabetes. Patients with concomitant liver disease were excluded. The remaining cohort was followed through 2000 for the occurrence of hepatocellular carcinoma (HCC). The study cohort comprised 173,643 patients with diabetes and 650,620 patients without diabetes. Most were men (98%). The incidence of HCC was significantly higher among patients with diabetes (incidence rate: 2.39 vs. 0.87 per 10,000 person-years, respectively, $P < 0.0001$). Diabetes was associated with an HRR of 2.16 (1.86–2.52, $P < 0.0001$) of HCC (El-Serag et al. 2004)

Group 1998). The greatest increase in risk with obesity was observed in the context of HCV infection (HR = 4.10, 95% CI 1.38–12.4). While a 2.4-fold excess risk that approached significance was also observed among persons who were negative for both HBV and HCV infection, obesity conveyed only a very modest and non-significant 1.4-fold excess risk among persons with HBV infection. There was, however, evidence of very strong synergism between obesity and diabetes which, when both conditions occurred together, conveyed a 100-fold excess HCC risk with obesity in the context of either HBV or HCV infection. In a retrospective study of over 19,000 registry-listed individuals in the United States with cirrhosis who received a liver transplant, the effect of obesity on HCC risk also varied according to disease etiology (Nair et al. 2002). Specifically, obesity conveyed strong and significant excess risk of HCC even after controlling for presence of diabetes among transplant recipients with cryptogenic or alcoholic cirrhosis (OR = 11.1, 95% CI 1.5–87.4 and OR = 3.2, 95% CI 1.5–6.6, respectively). However, obesity was not a significant independent predictor of HCC risk among those with other disease etiologies including HCV or HBV infection, biliary cirrhosis, or autoimmune hepatitis.

Several case-control studies have also evaluated the association between BMI and risk of HCC. In a study in Japan conducted in chronically HCV-infected patients, the incidence of HCC was significantly increased among those with a higher BMI. Further, there was also evidence of a dose-dependent relationship with a significant 1.8-fold excess HCC risk in HCV+ cases who were overweight (BMI 25–<30) that increased to a 3.1-fold excess in those who were obese (BMI 30+) in comparison to lean HCV+ cases (Niederau et al. 1998). Another case-control study conducted in a regional medical center in the United States compared the prevalence of obesity among 70 HCC cases to that observed among 140 age- and gender-matched controls ($n = 70$ with cirrhosis and $n = 70$ without liver disease) (Marrero et al. 2005). HCC cases were significantly more likely to be obese than either cirrhotics or normal controls (OR = 4.3, 95% CI 2.1–8.4 and OR = 47.8, 95% CI 9.6–74.5). Further, there was evidence of significant synergism or particularly increased risk of HCC among those with obesity (BMI 30+) who also drank more than 100 drinks and smoked more than 100 cigarettes during their lifetime (OR = 7.4, 95% CI 2.1–14.6). Although this study did not include adjustment for presence of diabetes, the overall prevalence of diabetes was similar among the HCC case, cirrhotic case, and normal control groups.

Taken together the data suggest obesity conveys excess risk of HCC beyond that conveyed by diabetes. However, the actual magnitude of risk and the specific sub-groups of chronic liver disease patients in whom its presence may be most salient in promoting HCC risk varied across studies. Future research with evaluation of additional factors that may influence obesity-mediated risk of HCC including timing and duration of obesity as well as family history of obesity and diabetes may be helpful in identifying sub-groups of obese chronic liver disease patients who may particularly benefit from enhanced surveillance and therapeutic interventions.

In conclusion, many developing countries are in the midst of a burgeoning obesity epidemic. This is particularly apparent in the United States where a recent

national study found that 30% of all adults (60+ million) are obese (i.e., BMI 30+) (2009) and 16% of all children (9+ million) are overweight (i.e., BMI-for-age $\geq$ 95th percentile per CDC Growth Charts) (Hedley et al. 2004). Although the exact magnitude and mechanisms of obesity-mediated HCC risk are currently unknown, even small increases in obesity-mediated risk could translate into a large number of HCC cases.

2.8 Tobacco

The relationship between cigarette smoking and HCC has been examined in more than 50 studies in both low- and high-rate areas. In almost all countries, both positive association and lack of association findings have been reported. Among studies reporting positive associations, several found effects were limited to population sub-groups defined by HBV status, HCV status, genetic polymorphism, or other exposure. Taken together, available evidence suggests that any effect of smoking on HCC is likely to be weak and limited to a subset of the general population. However, because two studies conducted exclusively among women reported positive associations, it has been suggested that attributable risk among women may be higher than that in men (Evans et al. 2002; Tanaka et al. 1995).

2.9 Oral Contraceptives

The association between oral contraceptives use and HCC risk was examined in at least twelve case-control studies ($n = 740$ cases and $n = 5,223$ controls) (Maheshwari et al. 2007). The pooled estimator was $OR = 1.43$ (95% CI = 0.90–2.26, $p = 0.13$). Six studies showed a significant 2–20-fold increase in HCC risk with longer durations (>5 years) of oral contraceptives use. Whether newer, low-dose oral contraceptives convey similar potential risks is currently unknown.

2.10 Diet

The role of diet, except for alcohol drinking and aflatoxin contamination, in the etiology of HCC in human populations is largely unknown. Dietary antioxidants including selenium as well as retinoic acid, and beta-carotene have been shown to inhibit hepatocarcinogenesis in animals. However, epidemiologic data are fairly limited and in some places conflicting. In a cohort study of men in Taiwan, higher baseline levels of serum retinol were associated with a decreased risk of developing HCC in HBV carriers (Yu 1995b). In the same cohort, a lower vegetable intake was significantly associated with an increased risk of HCC; however, this effect was limited to individuals who were both chronic hepatitis B carriers and cigarette smokers (Yu et al.). In a subsequent report from the same cohort, low baseline serum levels of selenium were also predictive of increased HCC risk (Yu et al. 1995b). In another large cohort study in Japan, the only foods whose consumption conveyed significantly decreased risk of HCC in subjects without a known history of liver

disease was fish, while the only food that conveyed decreased risk in subjects with a history of liver disease was coffee. Another study among Japanese atomic bomb survivors reported an approximately 50% reduction in HCC risk among those with high consumption of miso soup and tofu, both rich in the antioxidant isoflavones, after adjusting for HBV and HCV viral infections (Sauvaget et al. 2003).

Several studies performed in southern Europe, predominantly in Italy, have also evaluated various dietary factors as potential risk or protective factors for HCC. A favorable effect of high intake of specific foods (including milk and yogurt, white meats, eggs, and fruits) and of selected macro-nutrients including beta-carotene was reported by a multi-center hospital-based case-control study in Italy (Talamini et al. 2006). A similar inverse association between vegetable and fruit consumption and risk of HCC was also demonstrated in another much smaller case-control study in Italy. On the other hand, a smaller case-control conducted in Athens, Greece did not support an association between vegetable intake or any other specific foods or nutrients with risk of HCC, with the possible exception of milk/dairy products which conveyed a modestly decreased risk that closely approached significance (Kuper et al. 2006).

2.10.1 Coffee Drinking

One of the most extensively studied dietary factors studied in relation to HCC risk in human populations is coffee drinking. Several epidemiological studies have previously reported coffee drinking reduces risk of elevated liver enzymes and of cirrhosis, while animal studies suggest that coffee reduces liver carcinogenesis. Further, coffee drinking has also been associated with reduced insulin levels as well as reduced risk of type 2 diabetes, itself considered to be a risk factor for HCC (El-Serag et al. 2006). At least nine epidemiological studies conducted in Japan and southern Europe specifically evaluated the relationship between coffee consumption and HCC risk. Coffee drinking was associated with reduced HCC risk in at least five case-control studies (25–75% risk reduction with 2–4 cups of coffee per day as compared to none) (El-Serag et al. 2006; Gallus et al. 2002; Gelatti et al. 2005a; Ohfuji et al. 2006; Tanaka et al. 2007). Three cohort studies have also reported on the association between coffee intake and subsequent risk of HCC (Shimazu et al. 2005; Inoue et al. 2005; Kurozawa et al. 2005). Of those, two studies showed significant reduction in HCC risk with coffee intake of one or more cups of coffee (Shimazu et al. 2005; Kurozawa et al. 2005), and of those, with one further showing a dose-response relationship (20% reduction with 1–2 cups and 75% reduction with 5 or more cups (Inoue et al. 2005)). Although the third publication reporting on two cohorts also showed reduced HCC risk with coffee drinking, its findings were only of borderline significance (Shimazu et al. 2005). One potential limitation of most of these studies is that they used general population controls, which may not be the most appropriate comparator group given their low background risk for HCC. However, the inverse association between coffee consumption and HCC persisted in the studies that presented results stratified by liver disease (Montella et al. 2007; Ohfuji et al.

2006; Shimazu et al. 2005) or used a second control group of patients with liver disease (Tanaka et al. 2007). Taken together these data suggest a modest reduction of HCC risk with coffee drinking. However, the specific components of coffee and the exact mechanisms by which they act to reduce HCC risk are not well-established.

Overall, there is increasing evidence suggesting dietary factors may play a role in promoting hepatocarcinogenesis. However, there are important gaps in the epidemiologic literature that limit broad generalizations about the role specific dietary factors may play in HCC risk both within and across populations. First, studies published to date have used a variety of instruments to assess dietary intake. Even with use of validated instruments, there is well-known difficulty in reliably measuring dietary intake which is further complicated by differences in the relevant time-period for which dietary intake was assessed. Second, many studies did not adequately account for factors that may confound the relationship between actual and biologically effective intake of specific micro- and macro-nutrients including obesity and physical activity. Finally, most studies have been performed in Southeast Asian and southern European populations. It is unclear whether results obtained solely within those populations would generalize to other populations including those of northern Europe and North America where there are differences in the underlying risk factors for HCC as well as in dietary patterns and in prevalence of potentially confounding factors like obesity and diabetes.

3 Genetic Epidemiology of HCC

Although a very small minority HCC cases are associated with familial disorders of Mendelian inheritance like hereditary hemochromatosis, alpha-1-antitrypsin deficiency, or porphyrias, epidemiological research has convincingly demonstrated that the great majority of adult-onset HCC cases are sporadic (i.e., have no similarly affected first-degree relative) and that many have at least one established non-genetic risk factor like habitual alcohol abuse or chronic infection with hepatitis B or C viruses. However, most people with these known environmental risk factors for HCC never develop cirrhosis or HCC, while a sizable minority of HCC cases develops among individuals without any known environmental risk factors.

Genetic variation has long been suspected to influence the variable risk for HCC observed both within and across populations. Familial clusters of disease have been observed in HCC in the context of HBV infection (Yu et al. 2000a; Yu et al. 2002) as well as among those without established risk factors (Donato et al. 1999). However, as most HCC cases are sporadic or have no similarly affected first-degree relative, interest in the role commonly inherited genetic variants may play as potential risk factors for HCC has grown.

Currently far fewer genetic epidemiological studies have been reported for HCC than for other more common cancers in developed countries, like lung, prostate, or breast cancers. Most studies in area of HCC have been case-control studies conducted in populations with high (Asian, African) or medium (European) HCC rates. Typically, they have examined only a limited number of polymorphisms within a

few genes selected because of: (1) their role in the key liver function of detoxification including Phase I and II enzymes like Cytochrome P-450 s (CYPs), *N*-acetyl transferases (NATs), and Glutathione S-transferases (GSTs), (2) their role in biological pathways potentially relevant in chronic liver disease and carcinogenesis including inflammatory response (e.g., interleukins (ILs) 1 β , IRN) and DNA repair (e.g., XRCC1), or (3) their role in mitigating or exacerbating the effects of exposure to specific etiologic risk factors for HCC like alcohol or aflatoxin (e.g., ADH3, ALDH2, EPHX1).

Results from the genetic epidemiology studies evaluating varied polymorphisms, including CYPs (e.g., Wong et al. 2000; Yu et al. 1995a; Yu et al. 1999a), NATs (e.g., Gelatti et al. 2005b; Yu et al. 2000b), GSTs (e.g., Long et al. 2006; Sun et al. 2001), ILs (Migita et al. 2005; Nieters et al. 2005), and ALDH2 (e.g., Kato et al. 2003; Sakamoto et al. 2006), as risk factors for HCC have largely been equivocal, with findings of a positive association, association only within a limited subset of the population, or no or negative association all reported. The lack of reproducibility is a phenomenon widely reported in the broader field of genetic epidemiology. It has been widely attributed to inadequate sample sizes to reliably detect the likely small effects of common genetic variants on risk, particularly within a background of strong environmental risk factors and with likely polygenic influences on development of disease (Cordell and Clayton 2005; Hattersley and McCarthy 2005). Furthermore, virtually all of these studies have lacked power to detect interactions; it is estimated that thousands of cases and controls are typically required to adequately assess the effects of gene–gene or gene–environment interactions. Other contributing factors include population stratification or population-based differences in the relative distribution of alleles (e.g., among different racial groups), use of non-representative control groups, variable genetic penetrance, and potential differences in relevant genes based on underlying etiology of liver disease (e.g., alcohol- or hepatitis-related, etc.).

Given genetic epidemiology studies are often highly under-powered, meta-analysis has been recognized as an important tool to more precisely define the effect of individual polymorphisms on relative risk of disease and to identify potentially important sources of between-study heterogeneity (Khouri and Little 2000; Little et al. 2003). We recently completed a meta-analysis evaluating the effect of the two most frequently evaluated polymorphisms for HCC risk to date, the dual deletion GST polymorphisms *GSTM1* ($n = 14$ studies) and *GSTT1* ($n = 13$ studies) (White et al. 2008). Heterogeneity among individual studies for both polymorphisms necessitated use of random effects models. Pooled estimators suggested a possible small excess risk with either *GSTM1* or *GSTT1* null genotypes, though findings approached significance only for *GSTT1* ($OR_{GSTM1} = 1.16$, 95% CI 0.89–1.53, $OR_{GSTT1} = 1.19$, 95% CI 0.99–1.44). Exploratory meta-regressions suggested that the source of the controls was a possible source of observed between-study heterogeneity, with greater risk observed among studies using hospital-based controls for both polymorphisms. Year of publication was an additional source of between-study heterogeneity for *GSTM1* only. Although overall pooled estimators for *GSTM1* and *GSTT1* suggest a possible small excess of HCC with the null genotype, additional

studies with larger samples and conducted in other populations are needed to further clarify the role of both polymorphisms in the etiology of HCC and to investigate gene–environment interaction.

The epidemiologic literature evaluating selected SNPs as HCC risk factors is currently limited to case-control studies of only small to modest size. Therefore, a particularly noteworthy recent advance in field of genetic epidemiology is the development of large-scale cohorts or DNA “biobank” cohorts that will be prospectively followed for development of disease (e.g., biobanks in the United Kingdom ($n = 500,000$) and Mexico ($n = 200,000$)) (Davey et al. 2005). These large-scale genetic cohort studies offer many important advantages over traditional case-control studies including the ability to validly discern temporal relationships between exposure and disease and the availability of an appropriate control group. However, in spite of their impressive sample size, given the rarity of HCC and the considerable latency until disease onset, they are unlikely to generate enough HCC cases to fully replace genetic case-control and disease-based registry studies.

Overall, as in other areas of genetic epidemiology, results of studies in HCC have fallen short of early expectations that they would rapidly and unequivocally result in identification of genetic variants conveying substantial excess risk of disease and thereby establish the groundwork for effective genetic screening for primary prevention. However, recent identification of genetic risk factors for some chronic diseases such as Alzheimer’s disease and breast cancer, development of multidisciplinary efforts to address the considerable complexity in identifying genetic risk factors and the increasing accessibility to technology to concomitantly evaluate over a million SNPs across the genome (i.e., genome-wide association studies or GWAS) have contributed to a “cautious optimism” (Davey et al. 2005) that genetic epidemiology will ultimately provide important information on etiopathogenesis of many chronic diseases. Efforts within the field of gastroenterology to promote use of best practice in genetic epidemiologic research may facilitate identification of genetic risk factors for particular diseases of interest including HCC (El-Serag et al. 2008).

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Chapter 5

Genetics and Epidemiology of Cholangiocarcinoma

Boris R.A. Blechacz and Gregory J. Gores

Abstract Cholangiocarcinoma (CCA) is the most common biliary tract cancer and the second most common primary hepatic malignancy. Its incidence has significantly increased in Western countries and in areas of Asia it is the most common hepatic malignancy. Its prognosis is dismal with a median survival of 4.6 months following diagnosis. The only potentially curative surgery is resection or liver transplantation; however, only the majority of patients qualify for surgical management at the time of diagnosis. Hence, there is an urgent need to identify new therapeutic approaches. In the recent past, our understanding of this disease, its etiology and especially its molecular pathogenesis and genetic alterations have increased, thereby potentially helping us to identify patients at risk, prognostic factors and new therapeutic targets. This chapter aims in providing a concise overview of the current knowledge of the etiology and genetics of this disease.

Keywords Biliary tract cancer · Cholangiocarcinoma · Genetics · Mutagenesis

1 Introduction

Cholangiocarcinoma (CCA) is an epithelial malignancy of the biliary tree. Based upon its anatomic location, CCA is classified into intra- and extrahepatic CCA with the branches of the segmental bile ducts being the anatomic cutoff between the two forms. Extrahepatic CCA is the predominant form and is further subclassified into perihilar and distal CCA. The distinction between these forms is significant as management as well as biologic behavior is distinct (Blechacz and Gores 2008). CCA develops through malignant transformation of cells with cholangiocyte differentiation, and it is the most common biliary cancer and the second most common primary

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hepatic malignancy. Its incidence has significantly increased in western countries and is one of the most common cancers in parts of Asia (Welzel et al. 2006). In the majority of cases, CCA develops in the absence of identifiable risk factors. However, there are certain conditions with significantly increased risk for the development of CCA, such as primary sclerosing cholangitis (PSC) and liver fluke infection. These conditions cause chronic inflammation and cholestasis, factors promoting genetic aberrations, and tumor formation. The prognosis of this cancer is devastating with median survival rates of less than 24 months following diagnosis. CCA is resistant to non-surgical therapies and the majority of patients are not surgical candidates due to their advanced stage. Research over the last two decades has provided significant insights into the genetic basis of this malignancy and its development.

2 Epidemiology

Historically, CCA is considered a rare malignancy thought to constitute less than 2% of human cancers (Parker et al. 1996). However, it is the most common cancer of the biliary system, and globally the second most common primary hepatic cancer. Ten to twenty percent of hepatobiliary malignancy-caused mortality is caused by CCA. In the United Kingdom, intrahepatic CCA is the leading cause of death from primary hepatic malignancy (Khan et al. 2002). These numbers likely underestimate the true incidence of this malignancy as intrahepatic CCA are frequently mistaken for metastases. Intrahepatic CCA is the most common cause of solitary, intrahepatic adenocarcinoma mass lesions in the absence of other primary malignancies or cirrhosis. Statistical data on CCA in the past were frequently inaccurate due to grouping of CCA with gallbladder and ampullary cancers, misclassification of CCA, and the inclusion of intrahepatic CCA with other primary hepatic malignancies. The consequences of these inaccuracies were previously shown by Welzel et al. for intra- and extrahepatic CCA. The misclassification of 91% of hilar CCA in the surveillance, epidemiology, and end results (SEER) ICD-O coding system as intrahepatic resulted in an overestimation of intrahepatic CCA rates by 13% and an underestimation of extrahepatic CCA incidence rates by 15% (Welzel et al. 2006). Even 4 years later, only 67% of CCA were anatomically specified (45% as extrahepatic, including perihilar CCA, and 22% as intrahepatic), while the remaining 33% were coded without an anatomic specification (Everhart and Ruhl 2009). The most recent analyses identified significantly increasing trends in CCA incidence, thereby stressing the need to define the molecular basis of this cancer and develop rationale-based therapeutic approaches. Incidence rates are globally heterogeneous with large variations between continents, within geographic regions, and even within countries. The lowest age-adjusted incidence rates (AAIR) are described in Australia with 0.1/100,000 in women and 0.2/100,000 in men. In contrast, the highest incidence rates are observed in Asia, especially northeast Thailand with an AAIR of up to 96/100,000 in men (Shaib and El-Serag 2004). In the USA, most recent incidence rates are reported as 6,200 new cases for the year 2004 (Everhart and Ruhl 2009).

Interestingly, also ethnic heterogeneity has been observed within the US population. Hispanics and Asians had the highest AAIR with 2.8 and 3.3 per 100,000. The lowest AAIR is seen in non-hispanic whites and blacks with 2.1/100,000 for each group. Over the course of the last four decades, the incidence of CCA in the US has significantly increased by 22% – predominantly after 1985 and mainly due to increased incidence of intra- but not extrahepatic CCA (Shaib et al. 2004; Welzel et al. 2006; Everhart and Ruhl 2009). Similar trends have been reported in parts of western Europe and Japan. In Greece, annual incidence rates had increased from 0.998/100,000 between 1992 and 1994 to 3.327/100,000 in 1998 and 2000, and in Japan intrahepatic CCA rates diagnosed during autopsy had increased from 0.31 to 0.58% between 1976–1977 and 1996–1998 (Mouzas et al. 2002; Okuda et al. 2002). The causes of the globally increasing incidence rates of CCA are not known.

Globally, the median age at presentation is 50. However, in Western countries the median age at presentation is >65 years. CCA is only rarely diagnosed in patients younger than 40 years with the exception of PSC patients (Shaib and El-Serag 2004). In regard to gender differences, there is a slight male predominance in incidence rates with global male-to-female ratios in the range of 1.3–3.3. Interestingly, estimated annual percentage changes in mortality are higher in women with 6.9 ± 1.5 than in men with 5.1 ± 1.0 (Patel 2001, 2002; Shaib and El-Serag 2004). Mortality rates of CCA, particularly intrahepatic CCA, have globally increased with continental heterogeneity, e.g., higher mortality rates were observed in western Europe versus central or northern Europe (Taylor-Robinson et al. 2001; Patel 2002).

3 Genetics

CCA develops through malignant transformation of biliary tract epithelia. Although these cancers are thought to arise from cholangiocytes, they may also arise from biliary tract glands and perhaps a stem cell compartment formed in the canals of Hering (Nomoto et al. 2006; Komuta et al. 2008). Based upon epidemiologic evidence as well as in vitro and in vivo animal data, CCA carcinogenesis is associated with inflammation and cholestasis. In this environment, increased concentrations of cytokines, growth factors, and bile acids promote carcinogenesis, genetic defects, and tumor growth.

3.1 Mechanisms of Mutagenesis in CCA

3.1.1 iNOS

Inducible nitric oxide synthase (iNOS) generates nitric oxide (NO) enzymatically from L-arginine. NO can directly react with DNA causing base deamination, nitration, and oxidation, and is thereby mutagenic (Sawa and Ohshima 2006; Kundu

and Surh 2008). It can also nitrosylate proteins resulting in their functional alterations (Jaiswal et al. 2001). Abundant expression and high activity of iNOS has been demonstrated in CCA. The transcriptional regulation of iNOS in CCA cells is dependent on cytokine stimulation. Cytokine induced iNOS in CCA was shown to produce high levels of NO causing single- and double-strand DNA breaks as well as oxidative lesions (Jaiswal et al. 2000). In addition, NO has been shown to inhibit the DNA repair machinery in CCA (Jaiswal et al. 2000, 2001b). Human 8-oxoguanine glycosylase (hOgg1) is an important repair enzyme involved in correction of 8-oxodG lesions, which accumulate with iNOS expression. These lesions are highly mutagenic by predisposing to GC→TA transversions. In CCA, hOgg1 is inactivated by direct NO-mediated protein nitrosylation (Jaiswal et al. 2001a). NO also interferes with the apoptotic machinery in CCA through nitrosylation of procaspase 9, thereby inhibiting its activation (Torok et al. 2002). Another mechanism by which iNOS interferes with apoptosis in CCA is Notch-signaling. Dysregulated Notch-signaling can result in developmental abnormalities and carcinogenesis (Miyamoto et al. 2003). Immunohistochemical studies showed that Notch-1 is upregulated in cholangiocytes of PSC patients and in CCA (Ishimura et al. 2005). In addition, iNOS also upregulates inflammatory proteins such as cyclooxygenase-2 (COX-2). In immortalized murine cholangiocytes, iNOS was found to transcriptionally upregulate COX-2 via p38 MAPK and JNK-1/2 pathways (Ishimura et al. 2004). COX-2 elicits a wide spectrum of effects on cell proliferation and apoptosis pathways involved in CCA carcinogenesis (vide infra). In summary, iNOS is likely a key mediator contributing to bile duct oncogenesis.

3.1.2 DNA Repair Genes

Genome stability is an important feature in cellular prevention of carcinogenesis. Efficient DNA repair mechanisms comprise a critical component in the protection against human cancer, as indicated by the high predisposition to cancer of individuals with germ-line mutations in DNA repair genes (Hoeijmakers 2001). In CCA, several defects of DNA repair enzymes have been identified; these will be discussed in the following sections.

OGGI-1

Human 8-oxoguanine glycosylase (hOgg1) is a base excision repair protein involved in correction of 8-oxodG DNA lesions secondary to oxidative stress. Human CCA cell lines were shown to harbor Ogg1 mutations (Ku et al. 2002). In 22 human CCA samples, genetic alterations of the Ogg1 gene have been detected in 42% (Cong et al. 2001). The samples were of heterogeneous origin with 13 being of intrahepatic and 11 of extrahepatic origin. However, no subgroup analysis was performed; therefore, no conclusions can be drawn in regard to anatomy-related differences in Ogg1 mutations status. Interestingly, hOgg1 protein function is in addition directly inhibited by nitric oxide and reactive nitrogen oxide species in CCA cells, thereby further promoting mutagenesis (Jaiswal et al. 2001a).

MGMT

O6-methylguanine-DNA methyltransferase (MGMT) is a repair enzyme involved in repair of alkylated DNA, thereby also protecting cells from carcinogenic, alkylating agents (Dempfle et al. 1982). Decreased MGMT expression has been reported in several malignancies and its genetic silencing shown to result in tumor formation (Sakumi et al. 1997; Kohya et al. 2002). Hypermethylation of the MGMT in CCA promoter has been reported in 33–49%, although in one of the two MGMT-reporting studies CCA was grouped together with gallbladder carcinoma (Koga et al. 2005; Yang et al. 2005). Subgroup analysis of 72 CCA cases showed higher MGMT-methylation frequencies in extra- versus intrahepatic CCA with 40% versus 27% although statistical significance was not achieved (Yang et al. 2005). Decreased MGMT expression has been reported in 60% of extrahepatic CCA by immunohistochemical analysis (Kohya et al. 2002). MGMT methylation was significantly associated with methylation of other tumor suppressor genes such as APC, hMLH1, and RASSF1A in extrahepatic CCA (Yang et al. 2005). MGMT methylation and decreased protein expression are negative prognostic factors in extrahepatic CCA (Kohya et al. 2002; Koga et al. 2005).

Human mutL Homologue

Human mutL homologue 1 and 2 (hMLH1, hMLH2) are mismatch repair genes located on chromosome 3p21. In thorotrast-associated CCA, hMLH1, and hMLH2 promoter hypermethylation was observed in 46 and 25% of 29 examined cases. Comparison between thorotrast to non-thorotrast-associated CCA, indicated statistically significant differences in hMLH1 but not hMLH2 methylation status (Liu et al. 2002). Studies evaluating genetic changes in liver fluke-associated CCA found microsatellite instabilities in 7% for hMLH1 and 20% for hMLH2, and LOH in 19% of MLH1, but none in hMLH2 (Limpaiboon et al. 2002). Interestingly, in an immunohistochemical study MLH1 and MLH2 immunoreactivity was decreased in only 6.9 and 13.8% indicating that MLH silencing is not a significant contributor to liver fluke-associated CCA (Liengswangwong et al. 2006).

3.1.3 Epigenetic Changes by DNA Methylation

DNA methylation – defined as the addition of methyl groups to cytosine residues in CpG dinucleotides of DNA – is an epigenetic regulatory mechanism of protein expression. In carcinogenesis as well as in other conditions, aberrant DNA methylation can either be due to hyper- or hypomethylation (Tischoff et al. 2006). During DNA hypermethylation, DNA methyltransferases reversibly methylate cytosine residues within so called CpG-islands allowing binding of methyl-specific DNA-binding proteins such as MeCP1 or MeCP2 to regulatory elements. These events result in transcriptional repression. Further, these binding proteins can promote histone deacetylase-mediated remodeling of chromatin into a highly repressed state (Stutes et al. 2007). In CCA, epigenetic regulation of multiple genes has

been shown. Yang et al. found in a set of 72 CCA cases that 85% had promoter methylation of tumor suppressor genes versus 0–10% in benign biliary epithelium (Yang et al. 2005). Similar methylation frequencies were reported by other groups including genes involved in cell cycle regulation, DNA repair, and protection, and metastases (Lee et al. 2002). Interestingly, IL-6 has been shown to regulate the promoter of the DNA methyltransferase DNMT-1 and its resulting enzyme activity (Hodge et al. 2001). IL-6 is also a significant mediator of CCA proliferation and anti-apoptotic properties (Blechacz and Gores 2008). Patel and colleagues have shown that IL-6 mediates methylation of several genes in CCA cells such as the epidermal growth factor receptor (EGFR gene). While treatment with the methylation inhibitor 5-aza-2'-deoxycytidine decreased cell proliferation, IL-6 overcame this effect and altered promoter methylation, indicating the role of IL-6 in DNA methylation in CCA (Wehbe et al. 2006). Epigenetic regulation of oncogenes and tumor suppressor genes is common in CCA.

SOCS-3

Signal transducers and activators of transcription 3 (SOCS-3) are important negative regulators of IL-6 signaling. Their expression is induced by IL-6 signaling and they inhibit the gp130 subunit of the IL-6 receptor, thereby building a negative feedback loop (Heinrich et al. 2003). IL-6 signaling is constitutively active and increased in CCA. IL-6 induced JAK/STAT signaling induces transcription of important cell cycle regulatory and anti-apoptotic proteins such as Mcl-1, which render these cells resistant to apoptosis (Isomoto et al. 2005; Kobayashi et al. 2005). Sustained IL-6 signaling is in part mediated by epigenetic silencing of SOCS-3 and treatment with demethylating agents of CCA cells was able to interrupt IL-6 signaling with subsequent downregulation of Mcl-1 (Isomoto et al. 2007). The inverse correlation was also confirmed by immunohistochemical staining of 22 human intrahepatic CCA samples in the same study.

p15INK4a and p16INK4a

The tumor suppressor genes p15INK4a and p16INK4a are both located on chromosome 9p21. They elicit their tumor suppressive effects through inhibition of cyclin-dependent kinases (CDK) 4 and 6 by blocking phosphorylation of retinoblastoma protein (Rb) resulting in G1 arrest (Huschtscha and Reddel 1999; Sherr 2004). The 16INK4a gene is frequently inactivated and associated with k-ras mutation in CCA (Tannapfel et al. 2000a). In samples of 51 CCA patients – presumably intrahepatic CCA – 76–83% of patient samples were found to have p16INK4a promoter hypermethylation with concomitant transcriptional downregulation and loss of immunohistochemical positivity (Tannapfel et al. 2000a, 2002). LOH was found in 20% and homozygous deletions in 5%. Missense mutations were not found in this patient group (Tannapfel et al. 2000a). Interestingly, frequencies of the genetic p16INK4a alterations were differently distributed in patients with CCA in the background of PSC. Allelic loss was observed in 90%, methylation in 25%, and immunohistochemical loss in 57% (Ahrendt et al. 1999). In liver fluke-associated

CCA, methylation frequencies of 28.3% were described. However, genetic biallelic inactivation of p16INK4a was observed in 81.5% (Chinnasri et al. 2009). These data indicate that mechanisms between these ethnically and etiologically different CCA groups share the same tumor suppressor downregulation but differ in its silencing mechanism. Based upon expression data and in vitro gene silencing experiments, the chromatin-modifying enzyme EZH2 had been implied as a significant contributor to p16INK4a promoter methylation in CCA with associated hepatolithiasis (Sasaki et al. 2008). While p16INK4a expression was not correlated with prognosis in European patients, a statistical significant correlation with survival was described in Asian patients with liver fluke-associated CCA (Tannapfel et al. 2000a; Chinnasri et al. 2009). The p16INK4a methylation status was also examined in endoscopically obtained bile samples showing a methylation frequency of 6% in benign biliary diseases versus 54% in biliary malignancies, indicating a potential diagnostic value of this test for CCA (Klump et al. 2003).

Genetic alterations of p15INK4a have only been evaluated in two studies from Asia, therefore, not allowing conclusions about ethnical or etiologic differences (Ku et al. 2002; Chinnasri et al. 2009). In patients with liver fluke-associated CCA, p15INK4a methylation frequencies were reported in 40.2% and loss of expression in 58%. p15INK4a methylation was statistically significantly correlated to higher tumor stages and loss of expression to neural invasion. Interestingly, expression of p15INK4a and p16INK4a was inversely correlated in CCA indicating a potential compensatory mechanism (Chinnasri et al. 2009).

p14ARF

p14ARF is a tumor suppressor gene also located at chromosome 9p21. It differs from p16INK4a through a distinct exon 1 and an alternative reading frame secondary to splicing into exon 2 of the p16INK4a gene and a different promoter (Quelle et al. 1995). It is also functionally different, as p14ARF functions as a tumor suppressor gene through inhibition of MDM2-dependent p53 degradation, resulting in p53 stabilization and activation of the p53 pathway. Frequently, p14ARF is activated in response to oncogenic stimuli such as c-myc or activated ras. In samples of 51 CCA patients – presumably intrahepatic CCA – promoter methylation of p14ARF in tumor tissue was found in 25% of samples; in all of which reduction of mRNA was observed. There was no statistically significant correlation to any clinical parameters. Co-hypermethylation of p14ARF and p16INK4a was detected in only 10%. LOH of the p14ARF and p16INK4a was observed in 16% of patients and homozygous deletion in only 4%. Mutational analysis of exon 1 and 2 failed to identify any specific mutations in these regions (Tannapfel et al. 2002). Higher frequencies were observed in Asian patients with liver fluke-associated CCA with p14ARF methylation rates of 40.2% (Chinnasri et al. 2009).

14-3-3 σ

The 14-3-3 σ gene product is one of seven isoforms of the 14-3-3 gene family and has been linked to tumor development. It is a p53-inducible, negative regulator of

cyclin-dependent kinases (CDK) and prevents the cyclin B1/cdc2 complex from entering the nucleus, thereby preventing cell cycle progression. Even in the absence of oncogenic stimuli, its downregulation can result in indefinite growth of epithelial cells, and 14-3-3 σ is frequently lost in epithelial cancers (Dellambra et al. 2000; Osada et al. 2002). Its frequency of hypermethylation in intrahepatic CCA has been reported as 59.5% and decreased 14-3-3 σ expression by immunohistochemistry was seen in 32% of tumors (Lee et al. 2002; Kuroda et al. 2007). No correlation was found between 14-3-3 σ and clinical parameters such as tumor grade, stage, invasion, or metastases. However, patients with 14-3-3 σ positive CCA had 5-year survival rates of 35.7% versus 20.9% in 14-3-3 σ negative patients, and multivariate analysis identified 14-3-3 σ as an independent prognostic factor (Kuroda et al. 2007).

APC

The adenomatous polyposis coli gene (APC) is a tumor suppressor gene located on chromosome 5 at 5q21-q22. It regulates several cellular events such as cell division and migration, as well as cell–cell interactions. In intrahepatic CCA, APC hypermethylation was detected in 22–46% of 79 samples (Lee et al. 2002; Yang et al. 2005). Methylation frequencies did not differ significantly between intrahepatic (47.2%) and extrahepatic CCA (44%) (Yang et al. 2005). Mutational analysis found no APC mutations in extrahepatic CCA, but LOH in 38.5%. LOH rates in intrahepatic CCA were lower at 24% (Kang et al. 1999). No statistical significant correlation was observed between APC LOH status and survival (Suto et al. 2000). However, APC gene hypermethylation was statistically significant correlated with poorer survival (Lee et al. 2002).

Ras-Association Domain Family 1

RASSF1A is the major transcript of the Ras-association domain family 1 (RASSF1) gene located on chromosome 3p21.3 and is frequently silenced through promoter methylation in cancer (Dammann et al. 2000). It functions as a tumor suppressor through different mechanisms including genomic stabilization, induction of cell cycle arrest, and apoptosis (van der Weyden and Adams 2007). There are only few studies evaluating allele loss and epigenetic inactivation of RASSF1A in CCA. Interestingly, mutational analysis in 48 extrahepatic CCA revealed that mutations in the RASSF1A gene are rare with only 6% (Chen et al. 2005). However, LOH and epigenetic silencing are frequent events in CCA. In studies from Asia, allelic loss within the 3p21.3 region was observed in 40–69%, and RASSF1A promoter hypermethylation in 58–69% (Shiraishi et al. 2001; Wong et al. 2002; Chen et al. 2005). In European studies, allelic loss of 3p21.3 was observed in 20%, and hypermethylation of within the RASSF1A promoter in 64–68% (Foja et al. 2005; Tischoff et al. 2005). Frequency rates of RASSF1A gene LOH and hypermethylation were similar in intra- and extrahepatic CCA, although samples numbers were too small to make final conclusions.

3.2 Gene Defects in CCA

3.2.1 Oncogenes

K-ras

K-ras is one of the most frequently activated oncogenes in human malignancies with 17–25% of all human cancers harboring an activating gene mutation. K-ras is a GDP/GTP-binding protein activated through cell surface receptors. Activation of K-ras is normally transient and self-inactivating through its GTP-hydrolyzing properties. In malignancies, this GTPase activity of K-ras is frequently inhibited through point mutations in its gene resulting in a constitutively active state. K-ras mutations were found in 54–57% of 51 intrahepatic CCA tumor samples in European studies; 77% of these patients had mutations in codon 12, 23% in codon 13, and none had multiple mutations. All patients with K-ras mutations were also found to be positive for promoter methylation of the tumor suppressor gene p16. In Asian patients with intrahepatic CCA, K-ras mutations were observed in 5–50% (Tada, Omata and Ohto 1992; Ohashi et al. 1995; Furubo et al. 1999; Momoi et al. 2001a; Isa et al. 2002) (Table 5.1). Mutations were predominantly found in codon 12, but none in codons 13 and 61. Interestingly, K-ras mutations are less frequent in hepatolithiasis (17%), chronic hepatitis, and cirrhosis-associated (0%), and liver fluke-associated CCA than in unassociated CCA (82%) (Kiba et al. 1993; Ohashi et al. 1995). Similarly, K-ras mutation frequency was observed in only 33% of patients with PSC-associated CCA (Ahrendt et al. 2000; Boberg et al. 2000). Point mutations in PSC-associated CCA were predominantly located in codon 12 (27%) while codon 13 mutations were observed in only 6% (Boberg et al. 2000). In extrahepatic CCA, K-ras mutations were found in 9.6% of patients; similar to intrahepatic CCA, mutations were predominantly in codon 12 (Suto et al. 2000).

Table 5.1 Genetic defects of K-ras in CCA

Reference	Continent	Intrahepatic CCA [n]	Extrahepatic CCA [n]	Undefined CCA [n]
Isa et al. (2002)	Asia	20%[3/15]	75%[6/8]	–
Momoi et al. (2001a)	Asia	5%[3/65]	–	–
Tannapfel et al. (2000a)	Europe	54%[22/41]	–	–
Ahrendt et al. (2000)	America (N)	100%[1/1]	33%[3/9]	–
Boberg et al. (2000)	Europe	–	–	33%[11/33]
Suto et al. (2000)	Asia	–	9.6%[5/52]	–
Furubo et al. (1999)	Asia	20%[3/15]	–	–
Kang et al. (1999)	Asia	23%[9/40]	–	–
Sturm et al. (1998)	America (N)	–	–	22%[6/27]
Ohashi et al. (1995)	Asia	48%[10/21]	–	–
Lee et al. (1995)	Asia	–	–	33%[2/6]
Watanabe et al. (1994)	Asia	–	–	–
Ohashi et al. (1994)	Asia	50%[7/14]	67%[6/9]	–
Tada et al. (1992)	Asia	–	–	50%[9/18]

Clinically, K-ras mutations have been associated with advanced stages in extra-hepatic CCA (Suto et al. 2000). The correlation of K-ras mutational status and 5-year survival is controversial with some studies reporting statistically significant and other studies reporting no significant correlation (Suto et al. 2000; Isa et al. 2002).

Class I Receptor Tyrosine Kinases

Class I receptor tyrosine kinases are constituted by the ErbB-family of receptor tyrosine kinases which includes four members termed ErbB1 through ErbB4. Their activation is induced by direct ligand interaction or can be constitutive through mutational activation of the receptor or its overexpression. Activation of members of this family results in downstream activation of the Ras-Raf-MEK-ERK-pathway and the PI3K/Akt-pathway. The former predominantly mediates cell proliferation and migration, while the latter is involved in cell survival. The majority of studies have examined ErbB1 and ErbB2 in CCA, while ErbB3 and ErbB4 have not been a major focus of studies.

ErbB1

ErbB1 – also known as the epidermal growth factor receptor (EGFR) – is a membrane receptor with an extracellular domain, a single α -helix transmembrane domain and an intracellular tyrosine kinase domain. Several ligands activate this receptor such as epidermal growth factor (EGF), heparin-binding epidermal growth factor-like growth factor, and transforming growth factor- α . Ligand binding to the receptor results in its homodimerization, and heterodimerization with other members of the ErbB-family, followed by activation of its tyrosine kinase through autophosphorylation. Several mechanisms, the majority of these being signaling pathway cross-talk result in constitutive and enhanced signaling activity of this receptor kinase. ErbB1 mutations have been observed in 14% of human CCA; deletional mutations were exclusively located on exon 19 (Gwak et al. 2005).

ErbB2

ErbB2 (=HER2 [in humans] = neu [in rodents]) is a 185 kDa transmembrane glycoprotein encoded by the proto-oncogene c-erbB-2 on chromosome 17q. Its activation results in phosphorylation of major tyrosine residues such as Tyr 1248 which couple the kinase to the downstream effectors Ras-Raf-MAPKp42/44. In contrast to other members of the ErbB-family, ErbB2 does not have a specific ligand and cannot undergo homodimerization. However, recent crystallographic studies showed ErbB2 to be in a constitutively activated conformation (Burgess et al. 2003; Garrett et al. 2003). The constitutive exposure of its dimerization domain allows heterodimerization with other ligand-activated ErbB-family members, resulting in constitutive activation. ErbB2 overexpression is frequently observed in CCA (Sirica 2008). Genetic studies confirmed ErbB2 gene amplification and its association with

ErbB2 protein overexpression (Ukita et al. 2002). Its role in CCA carcinogenesis is further supported by rodent *in vivo* models, in which stable transduction of cholangiocytes with the ErbB2 gene results in CCA formation (Kiguchi et al. 2001; Lai et al. 2005; Sirica et al. 2007). ErbB2 gene amplification has been reported in 18.1% of human CCA (Kim et al. 2007). However, analyses of 22 CCA samples for activating mutations in the ErbB2 gene were negative (Bekaii-Saab et al. 2006). Hence, further studies are required for evaluation of ErbB2 receptor mutation status and their effect on tyrosine kinase effect.

3.2.2 Tumor Suppressor Genes

Tumor suppressor genes have key functions in controlling cellular fate. Their inactivation can result in carcinogenesis. Eighty-five percent of CCA were found to have methylated tumor suppressor genes. While single tumor suppressor gene methylation can occasionally be seen in benign cholangiocytes, methylation of several tumor suppressor genes is restricted to CCA. Approximately 70% of CCA were found to have >3 tumor suppressor genes methylated, and >52% had four tumor suppressor genes methylated (Yang et al. 2005). The following section will discuss the genetic status of several tumor suppressor genes in CCA.

p53

p53 mutations are one of the most common genetic defects in malignancies. The p53 protein is a key cell cycle regulator arresting cells with damaged DNA in the G1 phase of the cell cycle. It is also a strong inducer of apoptosis via NOXA and PUMA. Under physiologic conditions, p53 protein is unstable and does not accumulate in the nucleus, thereby preventing unintentional binding to p53 control elements. However, DNA damage causes its stabilization and accumulation resulting in transcription of the cyclin kinase inhibitor p21CIP/WAF1. This inhibitor binds to and inhibits the cell cycle regulator mammalian cyclin-dependent kinase (CdK) complex resulting in cell cycle arrest in G1/2. Once the triggering DNA damage has been repaired, p53 levels decrease with a subsequent decrease in p21CIP/WAF1 allowing the cell to enter the S-phase of the cell cycle. However, extensive, unreparable DNA damage results in p53 induced expression of pro-apoptotic proteins (Harris 1996). Mutations in the p53 gene abolish its DNA-binding ability allowing cells to replicate even in the presence of significantly damaged DNA, potentially resulting in malignant cell-transformation.

The majority of studies evaluating p53 in CCA used immunohistochemistry for its analysis. Physiologically, p53 has a short half-life of 6–30 min and is, therefore, normally not detectable. Mutations in p53 increase the half-life of its protein up to 4 h resulting in its cellular accumulation. Hence, detection by immunohistochemistry is interpreted as a surrogate marker for p53 mutations (Harris 1996). Frequencies of reported p53 overexpression in CCA vary between 11 and 86% with the majority indicating overexpression in approximately one-third of the cases (Table 5.2). The high variability might in part be explained with inconsistencies in

Table 5.2 Genetic defects of p53 in CCA

Reference	Continent	Technique	Intrahepatic CCA [n]	Extrahepatic CCA [n]	Undefined CCA [n]
Shen et al. (2009)	Asia	IH	20%[20/74]	36.7%[18/49]	–
Iguchi et al. (2009)	Asia	IH	36.1%[22/61]	–	–
Karamitopoulou et al. (2008)	Europe	IH	0%[0/27]	32%[20/62]	–
Kuroda et al. (2007)	Asia	IH	37.6%[35/93]	–	–
Liu et al. (2006)	Asia	IH	–	–	52.8%[19/36]
		DS			61.1%[22/36]
Jarnagin et al. (2006)	America (N)	IH	31.8%[7/23]	26%[19/73]	–
Jhala et al. (2005)	America (N)	IH	10%[3/30]	20%[6/30]	–
Jan et al. (2004)	Asia	IH	30%[9/30]	–	–
Kang et al. (2002)	Asia	IH	35.7%[15/42]	–	–
Tannapfel et al. (2002)	Europe	IH	37%[15/42]	–	–
		DS			
Momoi et al. (2001a)	Asia	DS	10.7%[3/28]	–	–
Horie et al. (2000)	Asia	IH	57%[27/47]	–	–
Boberg et al. (2000)	Europe	IH	–	–	32.3[10/33]
Tannapfel et al. (2000b)	Europe	IH	34%[14/41]	–	–
		DS	36%[15/41]		
Batheja et al. (2000)	America (N)	IH	–	–	94%[17/18]
Furubo et al. (1999)	Asia	IH	78.9%[15/19]	73.7%[14/19]	–
Arora et al. (1999)	Europe	IH	–	–	85.7%[24/28]
Tannapfel et al. (1999)	Europe	IH	–	–	34%[14/41]
		DS			36%[15/41]
Kang et al. (1999)	Asia	IH	25%[10/40]	–	–
		DS	30%[12/40]		
Shrestha et al. (1998)	Asia	IH	50%[6/12]	72.7%[8/11]	–
Ashida et al. (1998)	Asia	IH	–	–	57%[27/47]
Washington and Gottfried (1996)	America (N)	IH	33%[2/6]	38%[8/21]	–
Rizzi et al. (1996)	Europe	IH	–	–	78.5%[11/14]
Ohashi et al. (1995)	Asia	IH	19%[4/21]	–	–

DS=DNA sequencing, IH=immunohistochemistry (included are all studies reporting p53 positive in >10% of tumorcells)

immunohistochemical techniques (e.g., antigen retrieval, antibodies), or criteria and grading of p53 positivity. However, studies using immunohistochemistry as well as DNA analysis report consistent p53 mutation rates confirming the accuracy of the immunohistochemistry in this setting (Kang et al. 1999; Tannapfel et al. 2000b; Liu et al. 2006). In addition to the above-discussed mutagenic events, other mechanisms of p53 inactivation have been proposed. Recently, activation-induced cytosine deaminase (AID) was found to induce p53 mutations in CCA, thereby providing an additional potential mechanism linking chronic inflammation to CCA carcinogenesis (Komori et al. 2008). Another mechanism of p53 inactivation is mediated by MDM2. MDM2 transcription is induced by p53 protein and forms a negative feedback loop inhibiting p53 (Wu et al. 1993). Based upon immunohistochemical data,

MDM2 was found to be overexpressed in 38% of 47 cases of intrahepatic CCA, and was correlated with enhanced p53 expression as well as advanced tumor stage and the presence of metastases (Horie et al. 2000). Gene amplification of *mdm-2* was reported in 32% of CCA (Momoi et al. 2001a). Although, etiology and epidemiology of CCA differs between different regions of the world, frequencies reported from different continents do not indicate major differences in p53 overexpression rates (Tables 5.2 and 5.3) (Blechacz and Gores 2008). In Europe, p53 overexpression is reported in 0–87% with a median frequency of 34%. Studies from the North American continent report p53 overexpression in 10–94%, and in Asia frequencies of 5–67% are reported. Intracontinental comparison found no difference in p53 overexpression frequency between northeast Thailand and Japan (Suzuki et al. 2000). Frequencies of p53 overexpression in CCA show small differences in frequency dependent on the etiology. In liver fluke-associated CCA, p53 overexpression has been reported in 11–37% (Hughes et al. 2006; Tangkawattana et al. 2008). In PSC-associated CCA, p53 overexpression was observed in 32–94% (Rizzi et al. 1996; Ahrendt et al. 2000; Boberg et al. 2000). Only a limited number of studies compared p53 overexpression in intra- versus extrahepatic CCA. However, there appears to be a trend to higher p53 overexpression frequencies in extrahepatic CCA with a median

Table 5.3 Genetically aberrant tumor suppressor genes in CCA

Gene	Frequency (%)	Reference
p14ARF	4–40	Tannapfel et al. (2002), Chinnasri et al. (2009)
p15INK4a	40–58	Ku et al. (2002), Chinnasri et al. (2009)
p16INK4a	5–90	Ahrendt et al. (1999), Tannapfel et al. (2000a), Tannapfel et al. (2002), Klump et al. (2003), Sasaki et al. (2008), Chinnasri et al. (2009)
14-3-3σ	21–59	Lee et al. (2002), Kuroda et al. (2007)
APC	22–47	Kang et al. (1999), Suto et al. (2000), Lee et al. (2002), Yang et al. (2005)
p53	0–94	Ohashi et al. (1995), Rizzi et al. (1996), Washington and Gottfried (1996), Ashida et al. (1998), Shrestha et al. (1998), Arora et al. (1999), Furubo et al. (1999), Kang et al. (1999), Tannapfel et al. (1999), Batheja et al. (2000), Boberg et al. (2000), Horie et al. (2000), Tannapfel et al. (2000b), Momoi et al. (2001a), Kang et al. (2002), Tannapfel et al. (2002), Jan et al. (2004), Jhala et al. (2005), Jarnagin et al. (2006), Liu et al. (2006), Kuroda et al. (2007), Karamitopoulou et al. (2008), Iguchi et al. (2009), Shen et al. (2009)
p63	5–100	Nomoto et al. (2006), Ramalho et al. (2006)
p73	36–55	Momoi et al. (2001b), Yang et al. (2005)
RASSF1A	40–69	Shiraishi et al. (2001), Wong et al. (2002), Chen et al. (2005), Foja et al. (2005), Tischoff et al. (2005)
FIHT	14–42	Koch et al. (2003), Foja et al. (2005)
Smad4/PTEN	45/0	Kang et al. (2002), Pineau et al. (2003)
DMBT-1	20–79	Sasaki et al. (2003)
WWOX	30–67	Wang et al. (2009)

frequency of 71% (33–90%) versus 36% (5–100%) in intrahepatic CCA. Within the extrahepatic CCA group, one study reported higher p53 expression in distal versus hilar CCA (50% versus 19.6%) (Jarnagin et al. 2006).

Data evaluating the prognostic value of p53 are controversial. Per univariate analysis, significant survival advantage was reported for p53 negative CCA patients (Briggs et al. 2009; Iguchi et al. 2009). However, the majority of studies found no significant correlation between p53 status and survival (Washington and Gottfried 1996; Shrestha et al. 1998; Tannapfel et al. 2000b; Momoi et al. 2001a; Karamitopoulou et al. 2008).

p63 and p73

p63 and p73 are highly similar in structure to p53. The p73 gene is located on chromosome 1p36, a region with frequent deletions in human malignancies (Kovalev et al. 1998; Kang et al. 2000). Studies identified a transactivation domain-deficient isoform of p73 with inhibitory function on p53 (Flores et al. 2005; Rosenbluth and Pietenpol 2008; Tomasini et al. 2008). The p63 gene is localized on chromosome 3q27. Its mechanisms as a tumor suppressor gene is controversial. Similar to p73, it has a transactivation domain-deficient isoform, which is thought to inhibit the proapoptotic p73 transactivation, thereby promoting tumorigenesis (Tomkova, Tomka and Zajac 2008).

Few studies evaluated the expression of these p53 family members in CCA. The majority of these studies were conducted in intrahepatic CCA. Initial studies were restricted to immunohistochemistry and reported p73 overexpression in 32% of 41 patients. Mutational analysis in intrahepatic CCA studies from Asia revealed high incidence of loss of heterozygosity on chromosome 1.36 with the highest LOH rates in p73 with up to 54.5% (Momoi et al. 2001b). In a North American study, p73 promoter methylation was observed in 36% of 72 CCA cases (Yang et al. 2005).

p63 expression has been observed in 100% of 16 CCA cases from South America. Intratumoral p63 positivity ranged between 5 and 40% and expression was correlated with differentiation grade; highest rates were observed in poorly differentiated CCA (Ramalho et al. 2006). Interestingly, in intrahepatic CCA in the setting of cirrhosis 80% of tumor cells were p63 positive but only 23% in the absence of cirrhosis (Nomoto et al. 2006). Mutational analysis has not been undertaken yet for the p63 gene.

Clinically, LOH on chromosome 1.36 were associated with tumor progression and metastases (Momoi et al. 2001b). However, a significant correlation of p73 expression and survival was found by univariate but not multivariate analysis (Tannapfel et al. 1999).

Smad4, PTEN

Smad4 and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) are known tumor suppressor gene silenced in a variety of human malignancies. PTEN

functions as a tumor suppressor through induction of cell cycle arrest in G1. In established CCA cell lines, studies evaluating homolog deletions in PTEN were negative (Pineau et al. 2003). In intrahepatic CCA, loss of Smad4 was reported in up to 45% (Kang et al. 2002). There are no other studies evaluating other genetic aberrations of PTEN in CCA. However, combined silencing of PTEN and Smad4 resulted in orthotopic CCA tumor formation in a murine knockout model (Xu et al. 2006). Although, these data were derived in artificial models, they still might be indicative for a role of these tumor suppressor genes in CCA carcinogenesis warranting further studies.

3.2.3 Other Genes

There are reports of a variety of other genes altered in CCA. However, the majority of these reports are restricted to single reports and the majority of these studies were conducted on small sample numbers. Therefore, conclusions or generalizations cannot be made based upon these data. The following section will group the results of some, but not all of these studies and briefly discuss their results.

Fragile histidine triad (=FIHT). FIHT is located on chromosome 3p14.2 and encodes a tumor suppressor protein. In intrahepatic CCA, LOH of the FIHT gene was identified in 14–20% (Koch et al. 2003; Foja et al. 2005). Methylation was observed in 42% of intrahepatic CCA (Foja et al. 2005). These data indicate role of this tumor suppressor gene in CCA carcinogenesis. However, given the limited number of samples, further studies are necessary to define the role of FIHT in CCA.

Deleted in malignant brain tumor-1 (DMBT-1). DMBT-1 is a tumor suppressor gene frequently deleted in human cancers (Mollenhauer et al. 1997; Mori et al. 1999). Homozygous deletion within DMBT-1 was observed in 20% of intrahepatic CCA and in 50% of established CCA cell lines. Immunohistochemical analysis indicated weak expression in 30–79% of intrahepatic CCA. Interestingly, DMBT-1 overexpression was seen in 76% of 25 cases of hepatolithiasis (Sasaki et al. 2003).

WW domain-containing oxidoreductase (WWOX). WWOX is a tumor suppressor gene located at chromosome 16q23 preventing nuclear translocation of several transcriptionally active proteins. Genetic silencing of WWOX has been shown in a variety of human malignancies (Aqeilan and Croce 2007). In extrahepatic CCA samples of 30 patients, LOH within WWOX was observed in 50%. In comparison to non-malignant biliary ducts, WWOX mRNA in CCA was less than 30% in 67% of samples and immunohistochemical loss of WWOX was observed in 53%. A statistically significant correlation was only found between WWOX loss and tumor grade with the lowest intratumoral WWOX expression in poorly differentiated CCA (Wang et al. 2009).

Trefoil Factor Family. Trefoil Factor Family (TFF) comprises three members, which are encoded on chromosome 21q22.3. TFFs and 11p5.5 mucins (MUC2, MUC5AC, and MUC6) are secreted coordinately in a site-specific fashion in the human GI tract and are involved in the maintenance of mucosal barriers. TFF1 is especially associated with MUC5AC overexpression which has been associated with CCA progression (Boonla et al. 2005). Under normal conditions, TFFs are not

significantly expressed in the biliary tree (Sasaki et al. 2007). However, it is frequently overexpressed in inflammatory conditions such as hepatolithiasis (Sasaki et al. 2003). TTF expression and genetic alterations have especially been conducted in liver fluke-associated CCA (Sasaki et al. 2003; Muenphon et al. 2006; Thuwajit et al. 2007). Gene amplification for TFF1, TFF2, and TFF3 was observed in 22.5, 7.5, and 28.8% of 80 liver fluke-associated CCA samples. Immunohistochemical data reported overexpression of TFF1 in 66–91.8% of cases (including hepatolithiasis and liver fluke-associated as well as unassociated CCA), with coexpression with MUC5AC in 80% of the cases; however, a correlation to survival did not reach statistical significance (Thuwajit et al. 2007). Using mutational analysis in a small number of study samples, somatic missense mutations in the TFF1 gene were identified in 11–29% of cases (Sasaki et al. 2003). Data on correlation between TFF1 expression and survival in liver fluke-associated CCA are controversial with one study reporting prognostic value on multivariate analysis, while in another study statistical significance was not reached between TFF1 overexpression and survival (Muenphon et al. 2006; Thuwajit et al. 2007).

3.3 Microarray Data

Few studies have employed cDNA microarray analysis for CCA. The principle of this technique is the hybridization of free, labeled cDNA targets of biological samples to specific gene DNA probes immobilized on a matrix, and the subsequent relative quantification of transcription of specific genes based upon the labeling. It allows simultaneous transcription analysis for a high number of different genes and has been used successfully in a multitude of human diseases for identifying overexpressed genes (Shackel et al. 2002; Quackenbush 2006). However, it is important when using this technique to be aware of its restrictions (Russo et al. 2003). Factors possibly influencing the reliability of microarray data include tissue RNA quality, origin of tissue mRNA (tumor cells versus mesenchymal cells), efficiency of reverse transcription of tissue mRNA, clinical background of “normal” tissue used for comparison, and the lack of a gold standard of clustering microarray data. Obama et al. (2005) identified 52 upregulated and 421 downregulated genes by microarray analysis of 25 intrahepatic CCAs. Samples were processed by laser microbeam microdissection and compared to biliary epithelium of ten patients with metastatic liver tumors. Upregulated genes were known regulators of signal transduction, transcription, DNA synthesis, apoptosis, angiogenesis, and adhesion. Downregulated genes included genes known to be involved in growth suppression. Data were confirmed by real-time PCR and for randomly selected seven upregulated and three downregulated genes. Thereby, upregulation of survivin (BIRC5), P-cadherin (CDH3), forkhead box M1 (FOXM1), fascin homolog 1 (FSC1), dead ringer-like 1 (DRIL1), collagen 7A1 (COL7A1), and topoisomerase 2A (TOP2A) was confirmed, as well as downregulation of early growth response-1 (EGR1), AXIN1-upregulated 1 (AXUD1), and deleted in liver cancer (DLC1). Immunohistochemistry was used for confirmation of overexpression of survivin and P-cadherin. Using supervised cluster analysis, transcription of tissue inhibitor of

metalloproteinase 3 (TIMP3) and epithelial membrane protein 2 (EMP2) was shown to be inversely correlated to lymph node metastases, and the Ras oncogene family member RAB27B to be positively correlated for lymph node metastases (Obama et al. 2005). In a follow-up study of the same group, upregulation of RAD51 associated protein-1 (RAD51AP1) was confirmed in 61% of intrahepatic CCA samples. Its functional significance was shown by siRNA mediated gene silencing resulting in CCA cell growth suppression; in addition, it was implied as part of a DNA repair complex for γ -irradiation mediated DNA double-strand breaks (Obama et al. 2008). In a recent European study, oligonucleotide microarray analysis was used for gene profiling of intrahepatic CCA tumor samples of ten patients (Hass et al. 2008). For comparison, adjacent non-malignant tissue was used from eight of these patients. Two hundred twenty-one genes were found to be upregulated and 331 to be downregulated. The majority of the upregulated genes were involved in metabolic and signaling pathways; others included similar to Obama et al.'s study genes involved in cell cycle regulation, DNA synthesis and transcription. Downregulated genes were particularly ones involved in regulation of apoptosis. Using real-time PCR, osteopontin – a secreted adhesive glycoprotein overexpressed in a variety of human cancers – was identified to be consistently overexpressed in intrahepatic CCA (Hass et al. 2008). In an Asian study, gene profiling was used for identification of genes correlated to recurrence in 46 patients having undergone resection for intrahepatic CCA (Tonouchi et al. 2006). Using DNA microarray and confirmation by real-time PCR and immunohistochemistry, overexpression of pancreatic secretory trypsin inhibitor (PSTI) was found to be statistically significant correlated to early recurrence. Median survival after resection in patients with high levels of PSTI expression was 9 months versus 29.5 months in patients with low PSTI expression (Tonouchi et al. 2006). Comparison between gene expression in *Opisthorchis viverrini* and non-*Opisthorchis viverrini*-associated CCA found increased expression of growth factor signaling and cytoskeleton-related genes in the former, and elevated expression of genes involved in cell growth regulation, mitochondrial energy transfer, ion channel and transport, and metabolism in the latter one. While this study was technically well-conducted and the results were logical, it is limited by the sample origin: the *Opisthorchis viverrini*-associated samples were from patients from Thailand, while the non-*Opisthorchis viverrini*-associated CCA were from Japanese patients. Therefore, a geographic and ethnic variability cannot be ruled out. In summary, microarray analysis has provided interesting insights in the molecular profile of CCA and will provide the basis for further functional studies. However, more studies will be necessary and technical standardization should be used in order to minimize variability of results.

3.4 Micro-RNAs

Micro-RNA (miRNA) are short, non-coding RNA molecules of ~21 nucleotides. They are encoded in genomes of plants and animals, and highly conserved. Their significance lies in their ability to specifically regulate expression of their corresponding target gene by binding to the '3-untranslated region of mRNA. Each

miRNA is thought to regulate several different genes. miRNAs have been implicated as key regulators of cell differentiation, proliferation, and apoptosis (Stutes et al. 2007). They also can function as key factors in carcinogenesis (Kent and Mendell 2006). Several miRNAs have been identified influencing the pathogenesis of CCA. One of the first studies evaluating miRNA in CCA, using microarray technique with subsequent confirmation by northern blotting and real-time PCR, found significant differences in expression patterns of miRNAs in malignant versus non-malignant cholangiocytes (Meng et al. 2006). miR-21 and miR-200b were found to be involved in the chemotherapy sensitivity, and miR-141 in cell proliferation (Meng et al. 2006). Further studies identified PTEN as a miR-21 target through which PI3K activity is enhanced, contributing to chemotherapy resistance. Later, miR-21 was also found to suppress protein levels of programmed cell death 4 (PDCD4) and tissue inhibitor of metalloproteinases 3 (TIMP3) (Selaru et al. 2009). These data show the regulatory function of miR-21 in tumor cell apoptosis and invasion, as well as the ability of each miRNA to regulate several different genes. Additional anti-apoptotic functions mediated by miRNAs were identified for the anti-apoptotic Bcl-2 protein Mcl-1. miR-29 target sites were identified in Mcl-1 mRNA, and miR-29b was found to be suppressed in CCA cells versus non-malignant cholangiocytes. Expression of miR-29b in CCA cells resulted in Mcl-1 downregulation and TRAIL sensitization in these otherwise apoptosis-resistant cells (Mott et al. 2007). However, miRNA also underlie regulatory mechanisms as shown for miR-370. IL-6 was shown to suppress miR-370 expression through methylation resulting in upregulation of the oncogene MAP3K8 (Meng et al. 2008). Conversely, IL-6 signaling is also under miRNA regulation. miRNA let-7a suppresses the neurofibromatosis gene (NF2), which is a negative regulator of STAT3 – an essential component of IL-6 induced JAK/STAT3 signaling – thereby allowing constitutive IL-6 signaling (Meng et al. 2007).

3.5 Summary

CCA is the most common biliary malignancy. The incidence rates have significantly increased in northern America and western Europe. In Asia and some western counties, CCA is the most common primary hepatic malignancy. Modern molecular analytic techniques have provided significant insights helping us in gaining better insights in the genetics of this disease. The most thoroughly studied genes are p53 and k-Ras. Interestingly, these studies indicate the distinct characteristics between intra- and extrahepatic CCA, as well as geographic and etiologic differences in CCA genetics. There is a multitude of other genes studied. However, expansion of these studies is necessary. Shortcomings of current studies include the restriction of mutagenesis studies to restricted number of exons, small sample sizes, and single reports on genetic aberrations obtained through high-throughput array techniques. Further, evaluations of functional significance of these genetic aberrations have to be explored in more detail.

Hence, we have gained significant insight into the genetics and epidemiology of this malignancy. However, many questions remain to be answered, warranting further research in this field. The results of these future studies might guide us in the prevention, early detection, and targeted treatment of CCA.

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Part IV
Molecular Basis of Cancer Susceptibility

Chapter 6

Signaling Pathways in Viral Related Pre-neoplastic Liver Disease and Hepatocellular Carcinoma

Jack R. Wands and Miran Kim

Abstract Hepatocellular carcinomas (HCC) demonstrates substantial genetic heterogeneity. Recent studies support the concept that such tumors exhibit cellular phenotypes that may correlate with tumor recurrence and overall survival. For example, the proliferative phenotype is characterized by the poor prognosis and is associated with growth factor signal transduction pathway activation whereas the “stem cell phenotype” portends activation of WNT/ β -catenin signaling and generally has a better long term survival rate. Thus, most HCC tumors are associated with activation of the insulin/IGF-1/IRS-1/Ras/Raf/MAPK/Erk and WNT/Frizzled receptor/ β -catenin signaling cascades which provide molecular targets for innovative therapy. Both pathways may be activated by genetic mutations (e.g. β -catenin), overexpression of upstream signaling components (e.g. WNTs, Frizzled receptors, IRS-1, etc.), or loss of regulatory proteins such as Ras or Raf kinase inhibitors. Evidence is presented that constitutive activation of the insulin/IGF-1/IRS-1/MAPK and WNT/ β -catenin cascades are necessary and sufficient to transform normal liver to HCC in the context of hepatitis viral protein expression.

Keywords Hepatocellular carcinoma · Signal transduction pathways · Malignant transformation

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and is responsible for a large proportion of cancer deaths (Okuda 2000; Wands 2004). The incidence ranges from <10 cases per 100,000 population in North America and western Europe to 50–150 cases per 100,000 population in parts of

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Africa and Asia. A rise in HCC incidence and mortality has been recently observed in the United States (Wands 2004) and other industrialized countries (El-Serag and Mason 1999) likely reflecting the increased prevalence of HCV infection (Bosch et al. 1999; Okuda et al. 1987). In addition, with the obesity epidemic and increased incidence of Type II diabetes and insulin resistance, the emergence of non-alcoholic steatohepatitis (NASH) has begun to assume a larger pathogenic role. There is widespread concern among health care professionals regarding the increase in HCC and the lack of optimal screening techniques leading to delay in diagnosis and treatment. It is hoped that a better understanding of the molecular pathogenesis and the identification of cellular targets for therapy may improve the prognosis of this devastating liver disease.

The major etiologies of HCC are now well-defined and include chronic hepatitis B (HBV), hepatitis C (HCV), hepatic delta virus (HDV), toxins and drugs (e.g., alcohol, aflatoxins, anabolic steroids), and metabolic liver diseases (e.g., hereditary hemochromatosis, α 1-antitrypsin deficiency). On a global scale, persistent HBV and HCV infection account for well over 90% of HCCs (Wands and Moradpour 2006). Indeed, the risk of HCC development is about 25–35 times higher among those patients with chronic HBV infection compared to uninfected persons; co-infection with HBV and HCV increases this risk by 130-fold (Tagger et al. 1999). Another major clinical risk factor for HCC development is liver cirrhosis. In fact, 70–90% of HCCs develop in a cirrhotic liver (Wands and Moradpour 2006). The HCC risk in patients with liver cirrhosis depends upon the activity, duration, and etiology of the underlying liver disease. It is particularly high in cirrhosis resulting from chronic viral hepatitis and hemochromatosis, followed in descending order by alcoholic cirrhosis, autoimmune hepatitis, and primary biliary cirrhosis. The risk of HCC is low in Wilson's disease. Coexistence of etiologies, such as HBV and HCV co-infection, HBV and aflatoxin B1 (El-Serag and Mason 1999), or HCV and alcohol, increases the relative risk of developing HCC (El-Serag and Mason 2000).

The molecular factors and interactions involved in hepatocarcinogenesis are still poorly understood. This is particularly true with respect to genomic mutations, as it has been difficult to identify common genetic changes in more than 20–30% of tumors. Indeed, it is becoming clear that HCCs are genetically heterogeneous tumors. Regardless of the etiology of liver disease, malignant transformation of hepatocytes may result from a sequence of increased liver cell turnover induced by chronic liver injury and regeneration in the context of inflammation and oxidative DNA damage. This raises the question as to what the processes are that regulate hepatocyte proliferation, migration, and survival. Advances in our understanding of the molecular pathogenesis of HCC have led to the identification of signal transduction pathways activated during cellular transformation (Wands and Moradpour 2006). Two-signal transduction cascades that appear to be very important are insulin/IGF-1/IRS-1/MAPK and Wnt/FZD/ β -catenin pathways as shown in Fig. 6.1. This review will focus on the characteristics and biological consequences of enhanced activity of these pathways in viral related HCC. We will present emerging evidence to suggest that a HBV non-structural protein interacts with and promotes these cascades, ultimately leading to increased cell growth. A comprehensive review of HCV viral and cellular protein interactions is provided by de Chassey et al. (2008).

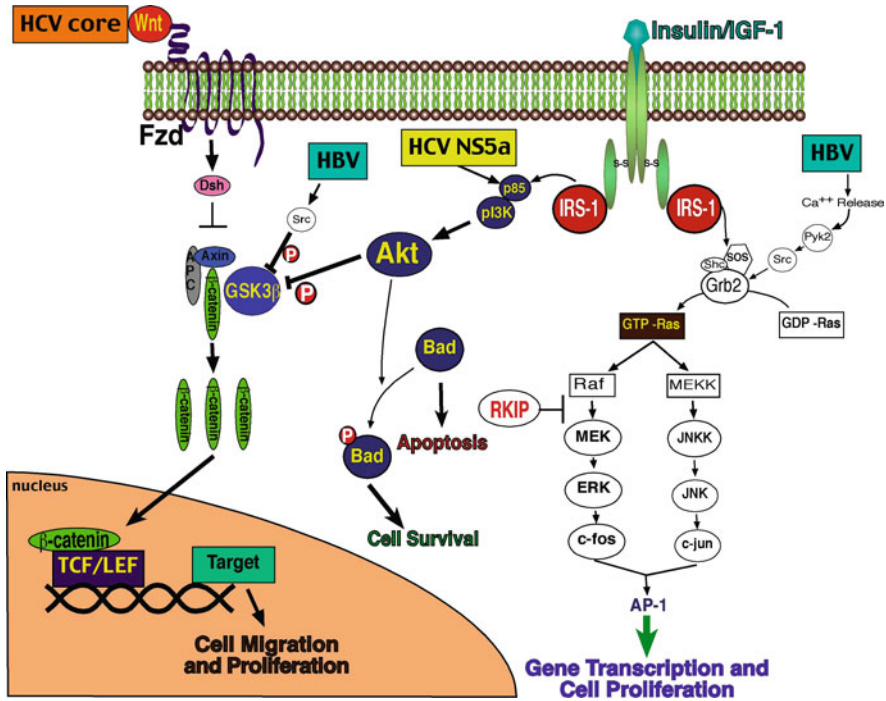


Fig. 6.1 Simplified cartoon of the two major signaling pathways activated during hepatocarcinogenesis with points of interactions between cellular and some hepatitis B and C viral proteins. These interactions lead to cellular behavioral and functional changes important in malignant transformation. Arrows indicate stimulatory pathways and targets of viral proteins, perpendicular lines indicate inhibitory pathways, P indicates phosphorylation. HBx, hepatitis B X protein; HCV core, hepatitis C core protein; HCV NS5a, hepatitis C non-structural protein 5a; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol-3 kinase; FZD, frizzled receptor; GSK-3β, glycogen synthase kinase-3β; APC, adenomatous polyposis coli; ERK, extracellular signal-regulated kinase; JNK, jun N-terminal kinase; AP-1, activator protein-1; RKIP, raf kinase inhibitor protein

2 MAPK/ERK Signaling Pathway

The insulin/IGF-I/IRS-1/MAPK cascade plays an important role in regulating liver regeneration following 2/3 hepatectomy, and in embryonic development (Branda and Wands 2006; Khamzina et al. 2003; Sasaki et al. 1993). In situations of unrestrained growth, constitutive activation of the insulin/IGF-I/IRS-1/MAPK cascade due to enhanced IRS-1 expression has been identified in the majority of HCC (Khamzina et al. 2003). Indeed, IRS-1 over-expression is associated with activation of the ERK/MAPK cascade resulting in increased HCC tumor size (Ito et al. 1996; Tanaka et al. 1996, 1997). The IRS-1 protein is the main substrate for the insulin/IGF-I receptor, and emits downstream signals through its interaction with SH-2 domain containing molecules. The role of insulin/IGF-I/IRS-1 signaling in human HCC is illustrated by studies demonstrating that IRS-1 over-expression, and/or activation of one or more of the components of this signaling pathway,

occurs in the majority of tumors (Wands and Moradpour 2006). Furthermore, inhibition of insulin/IGF-I/IRS-1-mediated signaling by a dominant-negative IRS-1 mutant protein reversed the malignant phenotype of human HCC cells (Tanaka and Wands 1996). Other investigators have emphasized that the MAPK pathway was constitutively activated as well (Alexia et al. 2004b; Ito et al. 1998; Tsuboi et al. 2004).

The MAPK signaling pathways activated in over 90% of HCCs (Wands and Moradpour 2006) are highly conserved and involved in cell growth, differentiation, survival, and invasion (Nottage et al. 2003; O'Neill and Kolch 2004). Although previous studies revealed this pathway can be activated by over-expression of upstream components such as IRS-1 both in human tumor and in a transgenic mouse model (Tanaka et al. 1997), other possible mechanisms may act on various pathway components to promote or accelerate the signal transduction cascade and in particular, loss of inhibitory proteins that regulate, for example, Ras (Calvisi et al. 2006) and Raf. Raf kinase inhibitory protein (RKIP) was initially identified as an inhibitor of the MAPK signaling pathway (Keller et al. 2004; Odabaei et al. 2004; Schuierer et al. 2004). The RKIP is a conserved cytosolic protein with wide tissue expression that does not share significant homology with other kinase inhibitors (Banfield et al. 1998; Serre et al. 2001). There is evidence that RKIP directly interacts and disrupts the Raf-1/MEK interaction thereby preventing MEK activation and other downstream components of the signaling cascade (Yeung et al. 2000, 1999). In this context, over-expression of RKIP suppresses MAPK signaling while downregulation has the reverse effect.

One investigation evaluated expression of RKIP by immunohistochemical staining in human HCC tissues. It was observed that 82.3% (14/17) of the matched peritumoral tissue expressed RKIP as demonstrated by high staining intensity of hepatocytes; in contrast, only two of the 17 tumors (11.8%) showed any immunoreactivity for the RKIP protein ($p < 0.001$). In addition, there appeared to be a general association between the expression level and degree of cellular differentiation (Lee et al. 2006); thus, high concentrations were found in more differentiated cell lines.

Low RKIP levels correlated with ERK/MAPK pathway activation. It is known that activated ERK translocates to the nucleus regulating gene expression through the phosphorylation of transcriptional factors. Therefore, the effect of RKIP on nuclear phospho-ERK (p-ERK) accumulation has been established. In this regard, IGF-1 stimulation of HCC cells resulted in accumulation of p-ERK in the cytoplasm, which was abolished by restoration of RKIP as shown in Fig. 6.2a.

Fig. 6.2 Restoration of RKIP levels inhibited phospho-ERK nuclear accumulation in FOCUS cells. **(a)** RKIP or vector-transfected FOCUS cells were stimulated with (+) or without (–) IGF-1. Cytosolic and nuclear fractions were immunoblotted for RKIP, phospho-ERK (pERK), and total ERK expression; actin and histone-H1 served as cytosolic and nuclear loading controls, respectively. **(b)** Control and RKIP-transfected FOCUS cells were immunostained with RKIP (red) and phospho-ERK (green), and the nucleus was counterstained with 4',6-diamidino-2 phenylindole (blue). The bottom panel shows the merged images of phospho-ERK and 4',6-diamidino-2-phenylindole staining indicating nuclear colocalization. The arrows indicate the nuclear p-ERK

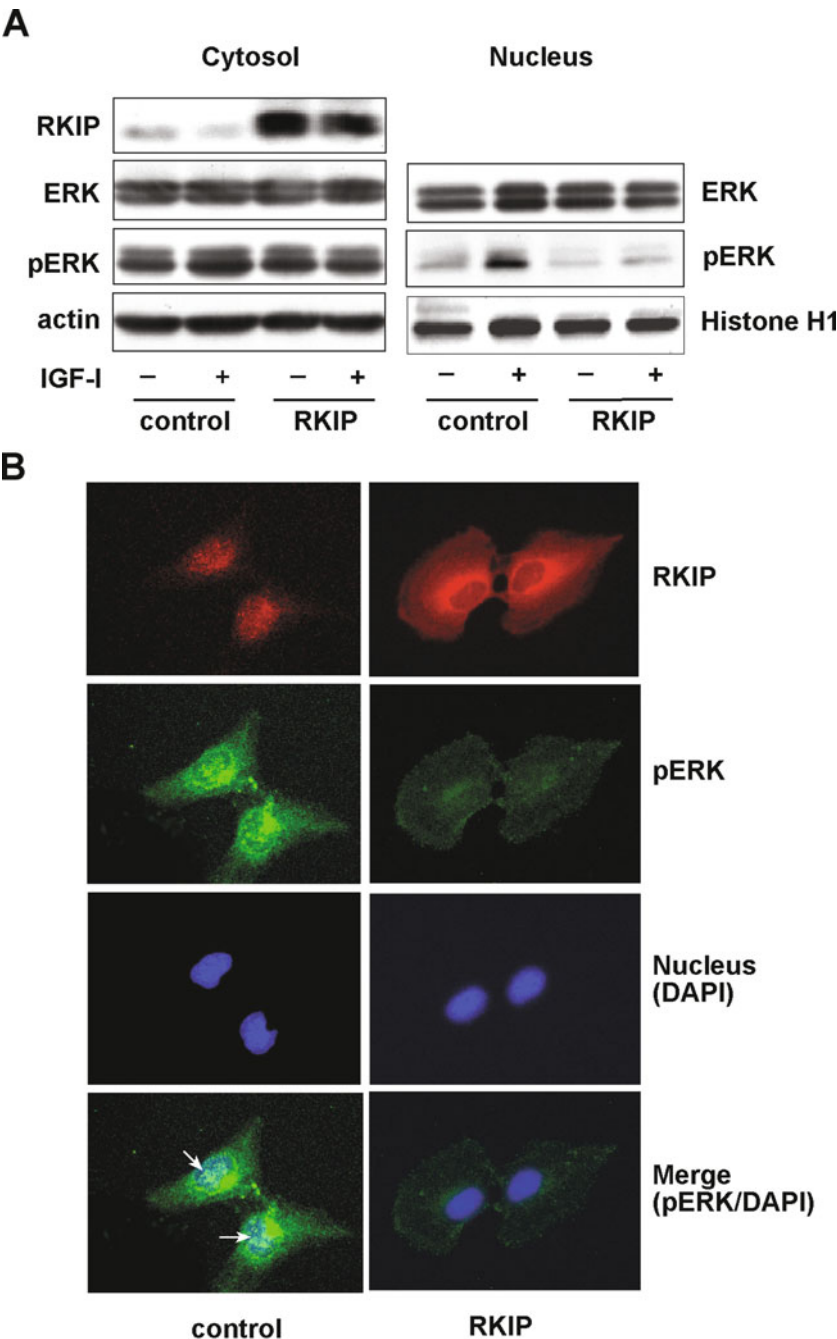


Fig. 6.2 (continued)

Double labeled immunofluorescent staining with anti-RKIP, and anti-phospho-ERK antibodies of FOCUS HCC cells transiently transfected with either RKIP or empty vector plasmid as controls were performed. Strong cytoplasmic staining of RKIP in RKIP-transfected compared to control cells was observed and p-ERK levels were strikingly reduced by RKIP over-expression as shown in Fig. 6.2b. Taken together, such observations suggest that restoration of RKIP blocks IGF-1 induced activation of the ERK/MAPK pathway in HCC cells, leading to a reduced nuclear phospho-ERK accumulation. Finally, functional analysis was accessed since activation of the ERK/MAPK pathway leads to cell proliferation, migration, and inhibition of apoptosis. In this regard ectopic expression of RKIP significantly decreased HCC cell migration rates compared to controls (Fig. 6.3c) with further confirmation by a wound-healing assay (Fig. 6.4). The findings are consistent with the idea that

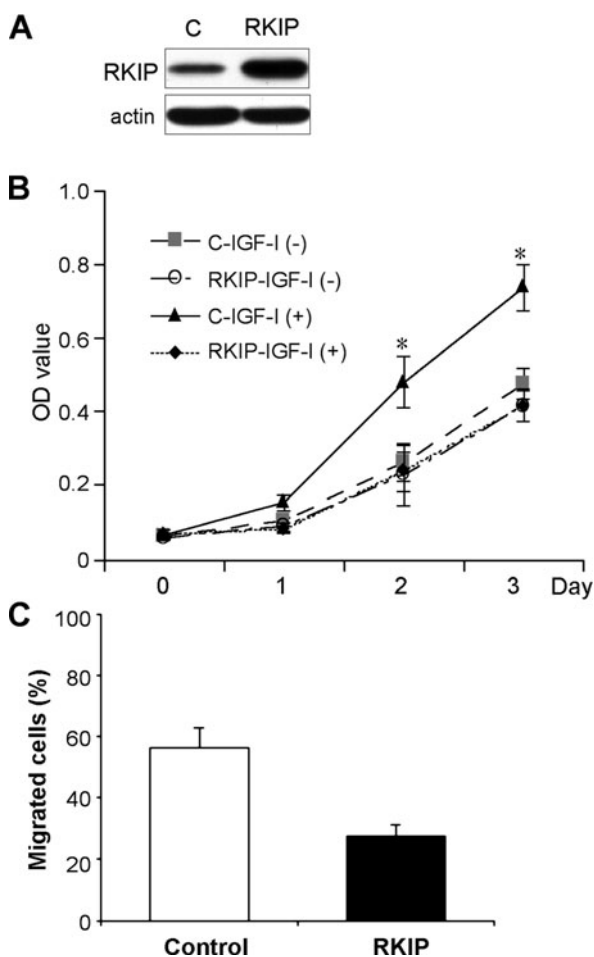
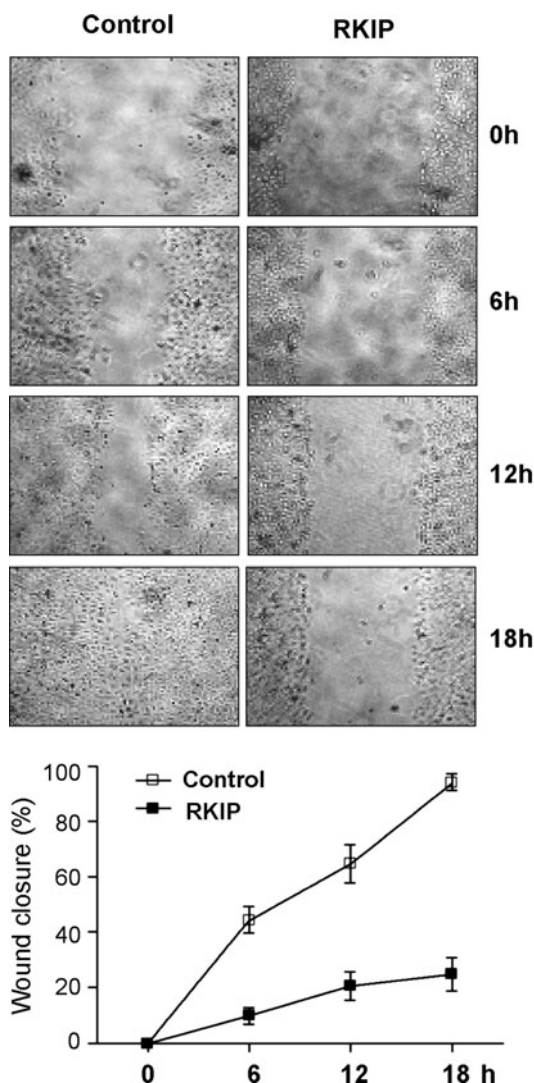


Fig. 6.3 Restoration of RKIP reduced cell proliferation and migration in FOCUS cells. FOCUS cells were transfected stably with either empty vector (control) or RKIP expression plasmid. (a) Demonstration of RKIP protein expression by Western blot analysis. (b) RKIP expression inhibits FOCUS cell proliferation. Results are expressed as the mean $\pm$ SE of triplicate assays. IGF-1 stimulation increased cell proliferation, (c) RKIP expression inhibits FOCUS cell migration

Fig. 6.4 RKIP inhibited FOCUS cell proliferation and motility. A wound was created in confluent monolayers and the percentage of wound closure was shown via the area of the wound that remained open at each time and plotted below. Note that etopic RKIP expression in FOCUS cells strikingly reduced cell migration



RKIP over-expression antagonized IGF-1 activation of the ERK/MAPK cascade with resultant downstream biological consequences being reduced proliferation and migration of HCC cells.

In summary, downregulation of RKIP expression in HCC tumors contributes to constitutive activation of the ERK/MAPK pathway and promotes proliferation and migration of HCC cells. More important, IGF-1 activation of the ERK/MAPK pathway can be blocked by restoration of RKIP levels probably contributing to HCC cell differentiation. Thus, RKIP provides an attractive molecular target to regulate HCC proliferation and differentiation, and it is of considerable interest that

Sorafenib, a drug with Raf kinase inhibitor activity, has been shown to prolong survival of patients with HCC (Llovet et al. 2008). It will be interesting to correlate the treatment response to the level of RKIP expression in future investigations since it is likely that those tumors responding most favorably to Sorafenib are those individuals that lost all RKIP expression.

3 The WNT/ β -Catenin Signaling Pathway

Constitutive activation of the Wnt/ β -catenin signaling cascade also appears to be of major importance during hepatic oncogenesis. Wnt proteins are cysteine-rich secreted glycoproteins serving as extracellular signaling molecules, which play significant roles in normal and pathologic developmental processes (Logan and Nusse 2004). The Wnt family of proteins consists of 350–380 amino-acid molecules, which are the Frizzled (FZD) ligands for FZD receptors. There are 19 known highly conserved human Wnt ligands involved in the generation of cell polarity, embryonic patterning, and cell fate determination. Wnt/ β -catenin signaling appears important in hepatocyte proliferation during early embryonic liver development (Micsenyi et al. 2004; Monga et al. 2003) and participates in liver regeneration after partial hepatectomy (Monga et al. 2003; Sodhi et al. 2005). Wnt ligands bind to seven transmembrane FZD receptor proteins (Fig. 6.1). There are ten known FZD receptors in humans and all contain a highly conserved cystein-rich domain (CRD) constituting the ligand-binding region for Wnt proteins. The C-terminal region is essential for receptor signaling (Kuhl et al. 2000; Sheldahl et al. 1999). These specific interactions depend, in part, on the relative affinities of a particular FZD receptor for the Wnt ligands (Du et al. 1995; Hsieh 2004). Constitutive activation of Wnt signaling contributes to the etiology of several human cancers and also may participate in tumor progression and metastasis (Giles et al. 2003). There are multiple pathways by which Wnt ligands transduce signals with the best characterized being the *canonical* Wnt pathway (Macdonald et al. 2007). In this signaling cascade, Wnt ligand (Wnt 1, 2, 3, and 8) binds to a FZD cell surface receptor. The low-density lipoprotein-related (LRP) co-receptor protein inactivates the β -catenin destruction complex resulting in the stabilization of β -catenin in the cytoplasm followed by translocation to the nucleus where β -catenin binds to TCF/LEF transcription factors to activate Wnt target genes such as *c-myc*, cyclin D1, and members of the WISP family (Tanaka et al. 2003). The activated transcriptional program directs cell proliferation and survival, and modifies cell fate. In the absence of Wnt stimulation, β -catenin is phosphorylated at specific serine and threonine residues rendering phospho- β -catenin prone to ubiquitination and proteasomal degradation. The Wnt ligands contribute to activation of the canonical pathway by stabilization of β -catenin (Macdonald et al. 2007).

Non-canonical Wnt signaling may be activated by other ligands that include Wnt 4, 5a, and 11, and are defined as Wnt-initiated signaling independent of β -catenin transcriptional function (Macdonald et al. 2007; Semenov et al. 2007).

Although non-canonical Wnt pathways are diverse and less well-characterized, they are important in polarized cell movement and organ morphogenesis through activation of cytoskeletal pathways, for example, GTPases RhoA and Rac1, intracellular calcium-dependent kinases-protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII), TGF- β activated kinase 1 (TAK1), nemo-like kinase (NLK), and Jun N-terminal kinase (JNK) (Kohn and Moon 2005; Semenov et al. 2007; Veeman et al. 2003).

Evidence mounts that alterations of the canonical Wnt/FZD signaling pathway are a common early event in the molecular pathogenesis of HCC. Nuclear and/or cytoplasmic accumulation of β -catenin is generally accepted as the hallmark of activated canonical Wnt/FZD signaling, and this phenomenon is demonstrated in tumor tissues by immunohistochemical staining. Indeed, in 33–67% of HCCs, nuclear and/or cellular accumulation of β -catenin has been described and is associated with the clinical and pathological features of the disease (Iozzo et al. 1995; Lejeune et al. 1995; Weeraratna et al. 2002). Furthermore, these studies suggest that tumors with β -catenin accumulation are linked to a dismal prognosis due to a poorly differentiated morphology (Devereux et al. 2001), high proliferative activity (Hsu et al. 2000), and vascular invasion (Devereux et al. 2001; Hsu et al. 2000; Weeraratna et al. 2002). It is well-recognized that β -catenin mutations in human HCC range from 8 to 34% (de La Coste et al. 1998; Iwao et al. 1998; Miyoshi et al. 1998; Polakis 1999; Satoh et al. 2000; Sparks et al. 1998; Weeraratna et al. 2002) and are the most common genetic abnormality involving the Wnt/FZD signaling pathway. Huang et al. found β -catenin mutations in 41% of HCCs associated with HCV infection (Huang et al. 1999). In contrast, mutations in the Axin/conductin gene are relatively rare in HCC (5–10% reported) (Satoh et al. 2000; Taniguchi et al. 2002) while APC mutations have not been described (Colnot et al. 2004; Giles et al. 2003; Huang et al. 1999). It is interesting to note, however, that a significant proportion of HCCs with β -catenin protein accumulation do not demonstrate mutations of pathway components such as β -catenin or Axin (Wilson et al. 1988). These observations suggest that other upstream elements may be involved in the upregulation of the canonical Wnt/FZD signal cascade during hepatocarcinogenesis. Over-expression of the FZD receptors in HCC appears to be an important factor as a pathogenic component. For example, FZD7 was markedly upregulated both in HCC transgenic mouse models, and in human tumors. Recently a study evaluated four different HCC transgenic mouse models produced by over-expression of *c-myc* or SV40-TAG alone, or dual expression of IRS-1/*c-myc* or HBx/*c-myc*. The FZD7 gene was the only FZD mRNA among the nine species studied that was upregulated in all four models of hepatocarcinogenesis as measured by real-time RT-PCR and confirmed by Western blot analysis (Merle et al. 2005).

In another study, 90% of HBV-related human HCC tumors ($N = 30$) overexpressed FZD7 compared to adjacent non-tumorous tissue. Normal liver revealed low FZD7 mRNA expression. However, the T and pT regions displayed highly significant FZD7 over-expression ($p < 0.0001$) as shown in Fig. 6.5a. These findings were confirmed by Western blot analysis using a polyclonal antibody prepared to a unique 25 mer FZD7 peptide sequence located in the CRD of the Wnt ligand-binding

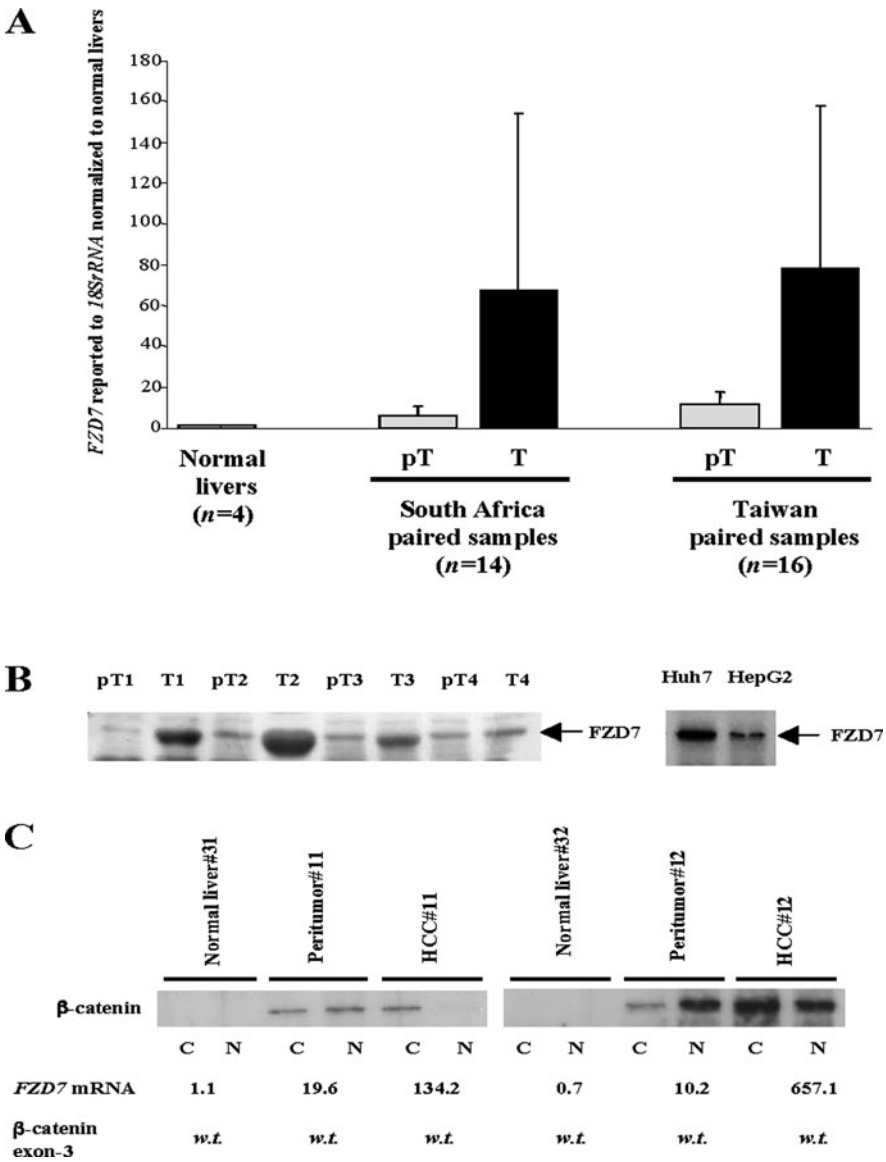


Fig. 6.5 (a) Quantitative real-time RT-PCR assessment of FZD7 mRNA levels in human HCC tumors (T) and the corresponding peritumorous liver parenchyma (pT), derived from Taiwan and South Africa. (b) Western blot analysis of FZD7 receptor protein expression in HCC tumors (T) and the corresponding pT, as well as in Huh7 and HepG2 human hepatoma cell lines. (c) Western blot analysis of β-catenin protein accumulation in cytosolic (C) or nuclear (N) enriched fractions from two HCC tumors and their corresponding peritumoral areas compared to two normal liver samples. Both peritumoral area and tumor overexpressed FZD7 mRNA as shown by the RT-PCR values listed below the Western blots and expressed as relative abundance of FZD7 mRNA. Each tumor and peritumor region had a wild-type β-catenin exon-3 as assessed by PCR and sequencing

region as revealed in Fig. 6.5b. Further support for its role in oncogenesis was provided by cell fractionation experiments in tumors containing wild-type β -catenin (as determined by sequencing of exon-3). It is apparent that in the context of increased FZD7 mRNA expression there was accumulation of β -catenin in both cytoplasm and nucleus compared to normal liver by Western blot analysis. It is important to note that there was increased expression of FZD7 mRNA in the peritumoral region as compared to normal liver suggesting that gene activation was an early event in the pathogenesis of these HBV-related tumors. Indeed, high levels of FZD7 mRNA expression as defined by a value above the cut-off (mean of normal liver $\pm$ 3 SD) was observed in 90% of tumors and 77% of peritumoral regions as compared with normal adult liver controls. Taken together, FZD7 is highly over-expressed and functionally active during HCC tumor development (Merle et al. 2004) and is an observed early abnormality perhaps partially responsible for the β -catenin accumulation in human HCC tumors without β -catenin, AXIN, or APC mutations. Further support for this concept was provided by animal models of tumor development described below. These findings strongly support the hypothesis that activation of FZD pathway is an early event during the pathogenesis of HCC.

Interestingly, in the presence of high-level FZD7 expression, HCC tissues containing only the wild-type β -catenin gene demonstrated nuclear accumulation. These observations suggest that upregulation of FZD7 alone was sufficient to activate the canonical Wnt/FZD7 signaling pathway; mutations in the β -catenin gene or other components of its destruction complex are not necessary as has been shown in squamous cell esophageal tumors (Tanaka et al. 1998). The molecular mechanisms by which FZD7 is activated during hepatocarcinogenesis have not been fully elucidated. A central question is which ligand(s) are responsible for initiation of Wnt signaling through the FZD7 receptor?

4 Wnt3 is a Ligand for FZD7 and Activates the β -Catenin Signaling Cascade in HCC

Little information exists on which Wnt ligand(s) are involved in FZD7 binding resulting in activation of the Wnt/ β -catenin pathway during HCC tumor development. Wnt mRNAs expression has been determined in HCC cells using RT-PCR; Wnt3, Wnt5A, Wnt6, and Wnt11 were the only ligands expressed among the 19 Wnt family members (Kim et al. 2008). It was of interest that Wnt3 was detected in all four HCC cell lines tested (FOCUS, HepG2, Hep3B, and Huh7) notable because Wnt3 induces cellular transformation through activation of the canonical Wnt/ β -catenin signaling cascade (Shimizu et al. 1997; Wong et al. 1994). It was determined if Wnt3 could activate β -catenin signaling through FZD7 in HCC cells and the physical and functional relationship between Wnt3 and FZD7 was explored. Wnt3 mRNA expression levels were determined by real-time RT-PCR in a cohort of

17 pair(s) of human HBV-related HCC (T) and corresponding peritumoral tissues (pT), and 14 normal liver samples. Thirteen of 17 (76%) HCC tumor, and 9 of 17 (53%) peritumoral tissues had increased Wnt3 mRNA expression above that found in normal liver. Again, both HCC and peritumoral tissue revealed increased FZD7 mRNA expression in 11 of 17 (65%) paired samples compared with normal liver. More important, 11 of 17 (65%) showed increased expression of FZD7 mRNA in HCC tumors compared with peritumoral tissues ($p = 0.031$). Thus, in combination, 15 of 17 (88%) had upregulation of either Wnt3 and/or FZD7 in tumor tissue. Only 4 of 17 (24%) of HCC tumors were mutations in exon-3 of the β -catenin gene detected. Using specific monoclonal antibodies (mAbs), it was observed that both Wnt3 and FZD7 proteins were present in tumor cells. Peritumoral dysplastic areas, however, revealed reduced expression, normal liver and other cell types (biliary epithelial cells, etc.) demonstrated no detectable expression. Such results are consistent with the hypothesis that Wnt3 may stimulate FZD7 via autocrine or paracrine mechanisms(s) in HBV-related HCC.

Indeed, the expression level of five β -catenin targets genes as evaluated in these (T) and pT tissues as well (Kim et al. 2008). The five genes examined included c-Myc and cyclin D1, and three recently identified β -catenin target genes over-expressed in human HCC: GS (Cadoret et al. 2002), GPR 49 (Yamamoto et al. 2003), Tbx3 (Renard et al. 2007). It was found that GS, Tbx3, and c-Myc were expressed at significantly higher levels in tumors with both mutated and WT β -catenin than in peritumoral tissues (Kim et al. 2008). Evidence was provided that Wnt3 over-expression activated the β -catenin pathway in HCC cell lines. In this regard, it has been determined if exogenous expression of Wnt3 could activate the canonical pathway in HCC cells. Stable transfection with the Wnt3-Myc construct in FOCUS HCC cells (FOCUS-Wnt3) resulted in marked increases in both mRNA and protein levels compared to vector-transfected controls (FOCUS-C) shown in Fig. 6.6. Expression levels of downstream targets such as cyclin D1, GS, and c-Myc proteins were also increased. In Panel B, control (FOCUS-C) or FOCUS-Wnt3 cells were double immunostained with anti-Myc tag (red color) and anti- β -catenin (green color) mAbs with the nucleus being counterstained with Dapi (blue color). This experiment revealed nuclear localization of β -catenin. The canonical β -catenin pathway was activated as shown in Panel C where the TCF transcriptional activity was increased approximately threefold in FOCUS-Wnt3 cells compared with controls ($*p < 0.01$). Finally, additional evidence for functional pathway activation was provided in Panel D since Wnt3 expression increased the FOCUS cell proliferation rate. Thus, Wnt3 is the first ligand to be identified as a binding partner for FZD7 in human HCC (Kim et al. 2008). However, there is no information on how the Wnt3/FZD7 complex activates the LRP6 co-receptor, but this presumably occurs through phosphorylation of motifs located in the C-terminus; it is also unknown how FZD7 interacts with disheveled (Dvl) following binding to Wnt3. Characterization of these early events in the canonical signaling cascade will be important areas for future research. In summary, these findings lead to the general conclusion that Wnt signaling is increased in HCC due to (1) mutations that stabilize β -catenin and render it resistant to proteasomal degradation (de La Coste et al. 1998; Prange et al.

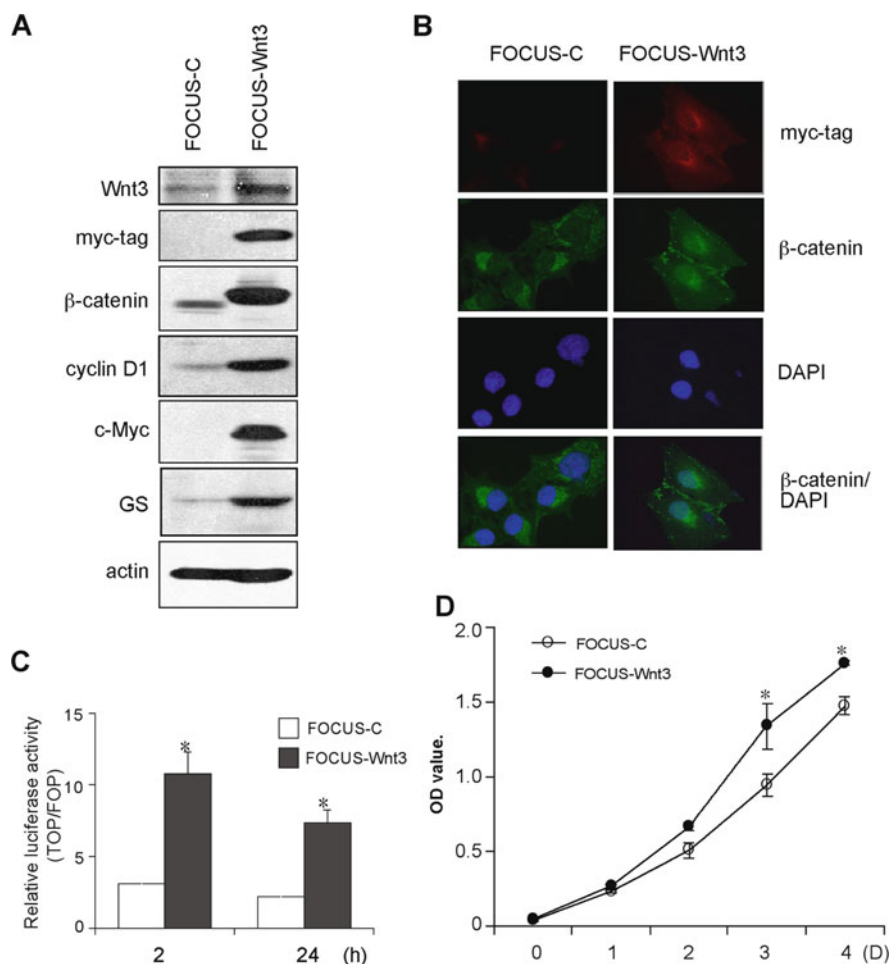


Fig. 6.6 Over-expression of Wnt3 activated Wnt/b-catenin signaling in FOCUS HCC cells. (a) Western blot analysis in FOCUS-Wnt3 or FOCUS-C cells. Wnt3 was overexpressed in FOCUS-Wnt3 as demonstrated by anti-Wnt3 and anti-myc tag mAbs. Expression levels of Cyclin D1, GS, and c-Myc proteins were also increased. Note that the anti-c-Myc mAb does not recognize the myc-tag, but rather the c-Myc protein. Actin was used as a loading control. (b) Control (FOCUS-C) or FOCUS-Wnt3 cells were double-immunostained with anti-myc tag (red color) and anti-β-catenin (green color) Abs; the nucleus was counterstained with the DAPI (blue color). The bottom panel reveals the merged images indicating nuclear localization of β-catenin. (c) The TCF transcriptional activity was increased by threefold in FOCUS-Wnt3 cells compared with control (* $p < 0.01$). (d) Wnt3 expression increased the FOCUS cell proliferation rate. The results are expressed as the mean $\pm$ SE of triplicate assays. * $p < 0.05$ vs. control

2003); (2) upregulation of stimulatory components, such as FZD7 or Wnt3 (Merle et al. 2004; Tanaka et al. 1998) or factors such as PIN-1 that reduce β-catenin interaction with APC (Pang et al. 2004); or (3) mutations in structural components of the complex, for example, Axins (Satoh et al. 2000).

5 Expression Analysis of Wnt/FZD/ β -Catenin Signaling Components in HCC Tumors of Different Etiology

Studies on Wnt/FZD/ β -catenin signaling have been performed principally with HBV-related tumors. Therefore, it would be of interest to extend these investigations and present a comprehensive analysis of HCC due to other etiologies, for example, chronic HBV ($n = 18$) or HCV ($n = 20$) infection as defined by positivity for HBsAg or anti-HCV antibodies in serum, respectively. Others were presumed as non-viral or non-B/non-C (NBNC) related tumors ($n = 24$). The expression patterns of all ten known FZD receptors, and their extracellular soluble regulators (19 Wnt ligands and 4 sFRP inhibitors) as assessed by qRT-PCR in 62 HCC (T) of different etiology, along with their matched pT areas. The expression of these signaling molecules in ten normal livers was assessed for comparison to the dysregulation pattern that may be present in T and pT tissues. In addition, PCR and denaturing high performance liquid chromatography (DHPLC analysis) were employed to test for the most common mutated genes found thus far in HCC namely TP53 and β -catenin (Bengochea et al. 2008).

Increased or decreased expression in HCC was defined as values >3 SD above or below the mean found in normal liver for FZD, LRP, Wnt, and sFRP genes. In this context, FZD3 was increased (41% T, 23% pT), FZD6 (31% T, 8% pT), and FZD7 (33% T, 10% pT). In contrast none of the samples showed any significant up- or downregulation of LRP genes in T or pT tissue when compared to normal. With respect to the soluble extracellular regulators of the FZD membrane receptors, Wnt3, Wnt4, and Wnt5A were strikingly upregulated in comparison to normal (range 3–60-fold): Wnt3 (39% T, 25% pT), Wnt4 (20% T, 16% pT), Wnt5A (25% T, 7% pT). Among the potential inhibitors of FZD two sFRP genes were found to be downregulated: sFRP1 (53% T, 21% pT), and sFRP5 (28% T, 12% pT). In regard to the relationship to etiologic factors of HCC, only FZD7 showed a higher rate of upregulation in HBV vs. non-HBV-related HCC (69 % vs. 23%) Chi-squared test $p = 0.035$. However, Wnt3, Wnt4, Wnt5A, FZD3/6, and sFRP1/5 were equally dysregulated statistically between HBV, HCV, and NBNC-related HCC. These results demonstrated that upregulation of potential activators (FZD3, FZD6, FZD7, Wnt3, Wnt4, Wnt5A) or repression of inhibitors (sFRP1, sFRP5) were aberrantly regulated in 68% pT and 95%T. More striking was the observation that such events accumulated with progression from non-cirrhotic liver to HCC (Bengochea et al. 2008). Correlations were performed between Wnt/FZD/sFRP expression to the mutation status of β -catenin and TP53 genes in the 62 HCC tumors. The β -catenin gene was found mutated mainly in HCV-related HCC (HBV 17%, HCV 40%, NBNC 21%) whereas TP 53 mutations did not correlate with etiologic factors (HBV 33%, HCV 30%, NBNC 13%). Finally, there was no correlation between these mutations and a specific Wnt/FZD/sFRP expression pattern in HCC. This investigation provided a comprehensive analysis of Wnt/FZD signaling elements and revealed that dysregulation may be one of the most common early events described thus far during hepatocarcinogenesis independent of mutations in the TP-53 and β -catenin genes.

6 Constitutive Activation of the Wnt/ β -Catenin and IN/IGF-1/IRS-1/MAPK Signaling Pathway is Necessary and Sufficient to Transform the Mammalian Liver in the Absence of Genetic Mutations

Since the majority of HCCs are related to HBV or HCV chronic infection (Block et al. 2003), it would be of interest to evaluate the potential interactions of viral and cellular proteins to activate signaling cascades associated with HCC (Longato et al. 2009). For example, the X-gene (HBx), the smallest open reading frame in the HBV genome, encodes a 154 amino-acid protein essential for productive HBV infection and replication (Bouchard and Schneider 2004; Cougot et al. 2005; Feitelson and Lee 2007; Keasler et al. 2006; Zhang et al. 2006). HBx has pleiotrophic functions including its broad transcriptional transactivation properties, activation of signal transduction cascades, and interference with a proteosomal, mitochondrial, and DNA repair functions (Zhang et al. 2006). The HBx is often integrated into the cellular genome of HCC (Feitelson and Lee 2007). In addition, HBx was shown to have transforming capabilities in some but not all HBx transgenic mice (Kim et al. 1991; Lee et al. 1990), and to have a role in activating both the Wnt (29, 30) and Ras/MAPK (Benn and Schneider 1994; Klein and Schneider 1997) pathways. Nonetheless, by itself, HBx is generally regarded as weakly oncogenic at best (Bouchard and Schneider 2004; Zhang et al. 2006). In view of the independent evidence that both IN/IGF-1/Ras/MAPK and Wnt/FZD/ β -catenin pathways are upregulated in human HCCs, definitive causal evidence with respect to the molecular pathogenesis of hepatocellular dysplasia, transformation, and/or HCC development in the previously normal liver is lacking. Since over-activation of either pathway alone has proven to be insufficient to cause HCC in vivo (Lee et al. 1990; Tanaka et al. 1997), it has been hypothesized that more than one hit was required for malignant transformation of the liver. The observation that HBx activates both signaling mechanisms, is expressed during active HBV replication, and integrated in human HCCs (Feitelson and Lee 2007), led investigators to design experiments to determine if the combined effects of HBx expression and constitutive activation of the IN/IGF-1/Ras/MAPK pathway would cause hepatocellular dysplasia and HCC. Therefore, a new transgenic mouse model was generated to study potential synergistic effects of HBx and IRS-1 in relationship to IN/IGF-1/Ras/MAPK and Wnt/FZD/ β -catenin signaling and hepatocellular transformation in vivo (Longato et al. 2009).

In this regard, mice expressing the human IRS-1 gene (9) were mated with ATX transgenic mice expressing the HBx gene derived from the adw2 strain. Transgene expression was driven by a liver-specific promoter (Lee et al. 1990; Tanaka et al. 1997). Non-transgenic wild-type (WT) littermates were used as controls. Heterologous ATX mice were crossed with heterologous IRS-1 and the offspring (WT, ATX+, IRS-1+, and ATX+/IRS-1+) were analyzed. For the ATX mice, the human α 1-antitrypsin regulatory region was used to drive the HBx expressing transgene (Lee et al. 1990); the IRS-1 mice employed the albumin promoter (Tanaka

et al. 1997). Four groups of male mice have been studied: wild type (WT), ATX+, IRS-1+, and IRS-1+/ATX+. Upregulation of molecules related to these two-signal transduction cascades were assessed by quantitative real-time RT-PCR.

Co-expression of IRS-1 and HBx caused pre-neoplastic changes in the liver. Histologic sections of WT liver showed the expected ordered and cord-like architecture with well-delineated portal and centrilobular areas, and occasional hepatocytes with micro-steatosis but no evidence of apoptosis, necrosis, or dysplasia (Fig. 6.7a, b). ATX+ mice, which expressed HBx in the liver at levels similar to that found in chronic HBV infection, had minimal histopathologic change relative to WT controls as previously reported (Lee et al. 1990; Madden et al. 2000, 2001). Constitutive IRS-1 over-expression increased the frequency of micro- and macro-steatosis. In addition, small foci of apoptosis or necrosis with Councilman bodies were occasionally detected (Wiedmann et al. 2003). However, 25% of livers from double-transgenic ATX+/IRS-1+ mice exhibited striking nuclear pleomorphism, hyperchromasia, micro- and macro-steatosis, necrosis, apoptosis, and high-grade dysplasia. In addition, dysplastic foci in the ATX+/IRS-1+ livers were nodular; the lesions were histologically indistinguishable from HCC based on hypercellularity, nuclear pleomorphism, mitosis, necrosis, architectural disarray, and infiltrative pattern of growth; $p < 0.0001$ compared to WT controls and single ATX+ and IRS-1+ transgenic lines for high-grade dysplasia plus HCC (Fig. 6.7c–f). Thus far, only the male ATX+/IRS-1+ double-transgenic line has exhibited high-grade dysplasia and about 25% of animals develop HCC. Figure 6.8a depicts gross morphologic features of a representative tumor at 15 months of age; histologic features are in Fig. 6.8b. Loss of RKIP during tumorigenesis may be particularly informative with respect to understanding the enhanced hepatocyte proliferative stimulus in the ATX+/IRS-1+ transgenic mice, as illustrated in Fig. 6.9. No examples of HCC have been observed in over 400 and 250 livers derived from the single IRS-1 and HBx transgenic lines, respectively (Keasler et al. 2006; Mohr et al. 2008). These findings confirm that constitutive expression of HBx or IRS-1 alone is not sufficient to promote hepatocarcinogenesis. However, dual expression causes high-grade dysplasia and HCC.

The effects of IRS-1 and HBx expression on downstream target gene expression have also been evaluated. Previous studies established that constitutive expression of the IRS-1 transgene led to activation of downstream signaling with enhanced binding of tyrosyl phosphorylated IRS-1 to Grb2 and SOS resulting in activation of Ras, Raf, and the MAPK cascade leading or contributing to hepatocyte proliferation (Tanaka et al. 1997); PI3K and MAPK enzymatic assays revealed enhanced activity as well (Tanaka et al. 1997). Moreover, a proliferating cell nuclear antigen (PCNA) was also increased (Tanaka et al. 1997). In addition, aspartyl asparaginyl- β -hydroxylase (AAH) which has a demonstrated role in cell motility and invasion (de la Monte et al. 2006; Ince et al. 2000; Lavaissiere et al. 1996) is also overexpressed in the majority of HCCs (Cantarini et al. 2006; de la Monte et al. 2006). It has been observed that AAH is stimulated by IN/IGF signaling through PI3K/Akt and Erk/MAPK (Cantarini et al. 2006; de la Monte et al. 2006) and upregulated with IRS-1 over-expression in liver. Therefore, AAH mRNA

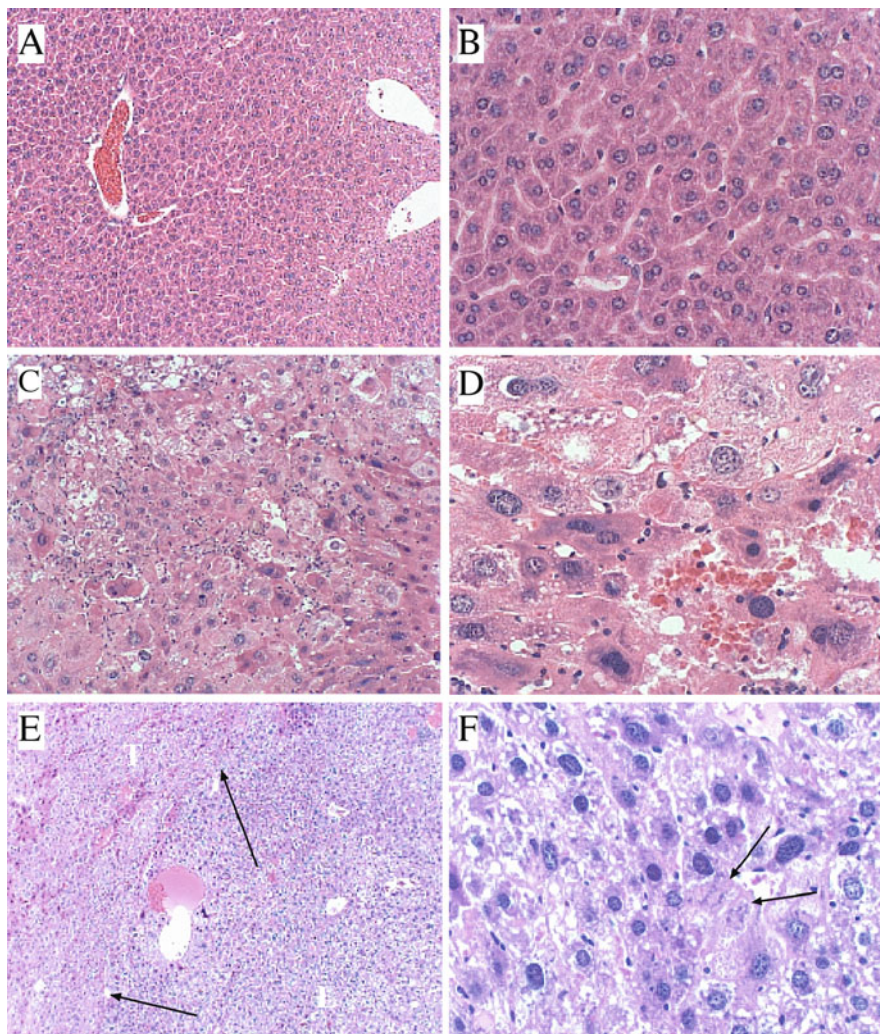


Fig. 6.7 The histopathological features of WT, ATX+, IRS-1+, and ATX+/IRS-1+ livers from mice 15–18 months old were examined. (a, b) Wt control livers exhibited well-organized lobular architecture with minimal inflammation and relatively uniform morphology. (c, d) Livers from ATX+/IRS-/- mice exhibited focal areas of hepatocellular dysplasia juxtaposed to regions with micro-steatosis. Higher magnification images demonstrate hepatocellular pleomorphism, disarray, and nuclear hyperchromasia. (e, f) Dysplastic and HCC foci in ATX+/IRS-1+ livers. Nodular, well-delineated dysplastic focus (*left side*) compresses adjacent liver tissue (*arrows* point to boundaries with liver tissue on *left side*). Note bluer hue of cells within the nodule due to increased cellularity and nuclear hyperchromasia (*right side*). Infiltrating HCC with disorganized growth of cells with nuclear hyperchromasia and atypical mitoses (*arrows*). Original magnifications: A-160x; B,C-320x; D-640x; E-100x; F-240x

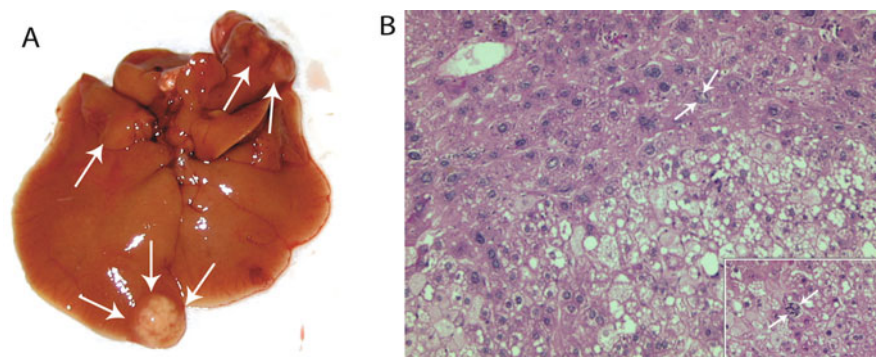


Fig. 6.8 Morphologic and histologic features of HCC in the ATX+/IRS-1+ transgenic mice. (a) Note the gross appearance of small nodular HCC's in the right and left lobes of the liver. (b) The HCC's generally exhibited non-trabecular growth with steatosis and embedded micro-acini, irregularly increased density of nuclei, high nuclear-cytoplasmic ratios (*arrows*), and scattered mitosis. Nodularity with compression of adjacent uninvolved liver parenchyma imparted an encapsulated appearance to the neoplasms

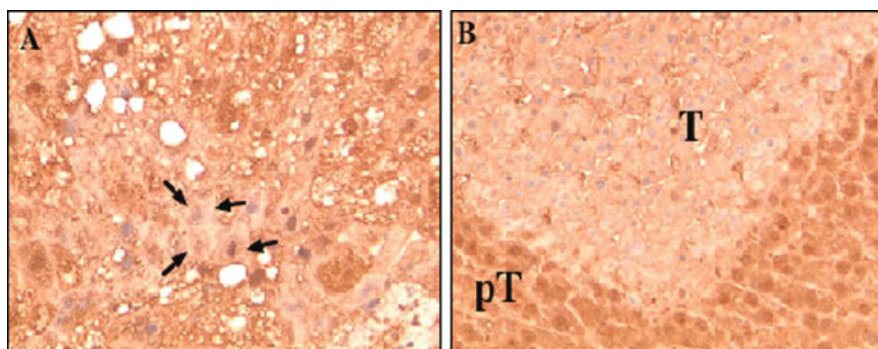


Fig. 6.9 Loss of RKIP expression in dysplastic hepatocyte and HCC of ATX+/IRS-1+ transgenic mice. Here we show loss of RKIP protein expression in dysplastic hepatocytes derived from the liver of an IRS-1/HBx double-transgenic animal at 15 months as depicted in panel (a). Note dysplastic cells with low level RKIP expression by immunohistochemical staining with polyclonal anti-RKIP antibodies (*arrows*). Panel (b) shows a small HCC (T) with near absence of RKIP expression as compared to high-level expression (*brown color*) in normal surrounding hepatocytes (pT) in the liver

levels have been measured to provide another marker of pre-malignant phenotype associated with increased signaling through the IN/IGF-1/IRS-1/MAPK cascade. Increased AAH expression was found only in the ATX+/IRS-1+ double-transgenic livers.

It was then determined if constitutive expression of IRS-1 or HBx enhanced Wnt/FZD signaling. In this regard, Wnt1, Wnt3, FZD3, and FZD7 were evaluated in the transgenic livers since they were highly upregulated in human HCC as demonstrated above. Both Wnt1 and Wnt3 mRNA levels were significantly increased in

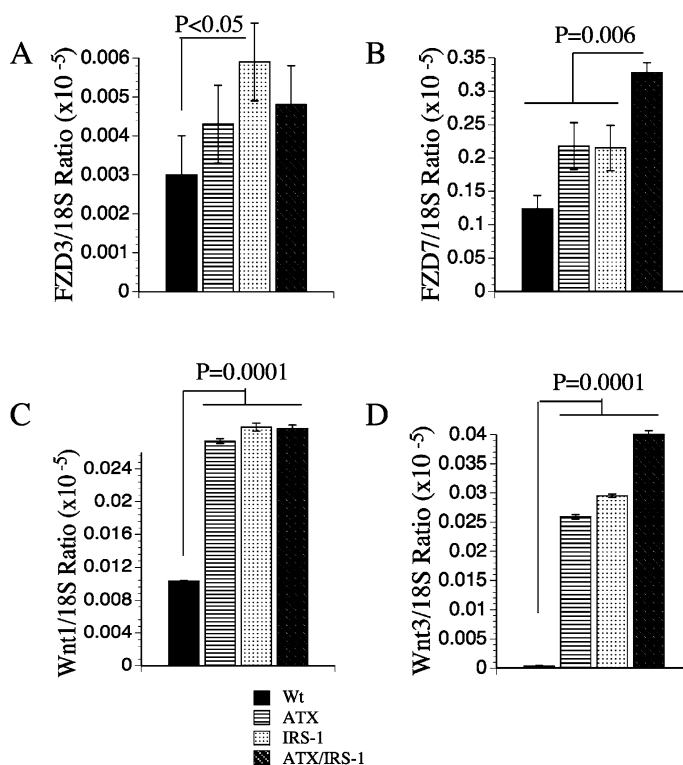
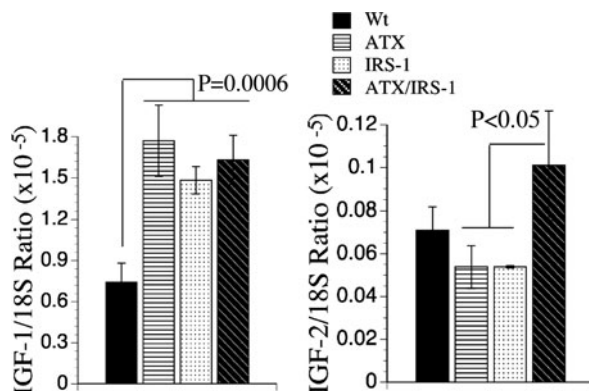


Fig. 6.10 Effects of ATX, IRS-1, and ATX+IRS-1 constitutive expression on mRNA levels of (a) FZD3, (b) FZD7, (c) Wnt1, (d) Wnt3 and Wnt3. Graphs depict the mean $\pm$ S.E.M. levels of gene expression in 8–10 mice per group. Significant intergroup differences are indicated with *p*-values above the bars

the livers of all transgenic lines relative to WT controls (Fig. 6.10c, d) but Wnt3 expression was higher in the ATX+/IRS-1+ mice than the single ATX or IRS-1 transgenic lines ($p<0.01$). In addition, FZD3 was significantly increased in the IRS-1+ livers (Fig. 6.10a), whereas FZD7 was selectively increased in the ATX+/IRS-1+ double-transgenic livers (Fig. 6.10b). Thus, both Wnt3 and FZD7 were highly overexpressed similar to the observations made with human HCC tumors. Finally, sequence analysis revealed a WT TP53 gene in all three transgenic lines; the β -catenin gene was also WT as well. Another common feature of HCC is constitutive activation of pro-growth signaling. Increased IGF-1 gene expression in the liver was demonstrated in both single- and double-transgenic mice. However, the ATX+/IRS-1+ mice were further distinguished by a selective increase in IGF-II mRNA levels as shown in Fig. 6.11. This observation carries significance because IGF-II is the main growth factor that is dysregulated in both human and experimental HCC animal models (Alexia et al. 2004a; Breuhahn et al. 2006).

Fig. 6.11 Expression of insulin like growth factors



Increased levels of cytoplasmic β -catenin immunoreactivity were found in transgenic livers, and much higher levels were observed in the cytoplasm and nuclei of the double transgenic compared to single-transgenic lines by immunohistochemical staining and Western blot analysis. It is noteworthy that HBx and IRS-1 over-expression may both contribute to β -catenin accumulation in hepatocytes. In this setting, the IRS-1 pathway cross-talks with the Wnt-mediated cascade through GSK-3 β (Branda and Wands 2006). Since GSK-3 β is a component of the β -catenin destruction complex, inhibition of its activity by phosphorylation via Akt promotes β -catenin accumulation and translocation to the nucleus where it acts in concert with Tcf/Lef transcription factors to upregulate Wnt responsive genes. Similarly, HBx expression has been shown to inhibit GSK-3 β activity as well allowing β -catenin to accumulate in the cytoplasm of HCC cells (Ding et al. 2005). Also, participating in β -catenin accumulation in hepatocytes of the ATX+/IRS-1+ double-transgenic line is the over-expression of Wnt3 and FZD7, which activate the canonical signaling cascade in HCC (Kim et al. 2008). Taken together, constitutive expression of both IRS-1 and HBx promotes hepatocyte dysplasia and HCC. Activation of IN/IGF/IRS-1/MAPK and Wnt/ β -catenin signaling cascades is necessary and sufficient to transform mammalian hepatocytes. Thus, the double HBx+/IRS-1+ transgenic mouse model replicates many of the cellular and molecular abnormalities found in human HCC.

In summary, aberrant levels of β -catenin accumulation in the cytoplasm or the nucleus leads to inappropriate transcription of various target genes involved in cell proliferation and migration. The abnormal β -catenin accumulation may be due to mutations of β -catenin, APC, and Axin genes, but these are relatively rare in HCC. Thus, regulation of the Wnt pathway can be affected by over-expression of other upstream components such as Wnt ligands and FZD receptors without concomitant genetic alterations. Future studies will be required to identify which specific Wnt ligands and FZD receptors are responsible for activation of the canonical β -catenin pathway in HCC. In addition, given the general importance of this pathway in hepatic oncogenesis it will be essential to assess new TCF/ β -catenin responsive target genes. Because of the intersection of this signaling cascade with GSK-3 β ,

there is an identifiable entry point allowing cross-talk with other signaling systems such as the insulin/IGF-1/IRS-1/MAPK cascade. Finally, there are well-known interactions with HCV and HBV structural and non-structural proteins, which activate both signaling cascades as shown in Fig. 6.1. Therefore, it seems likely that further definition of the canonical Wnt/FZD/ β -catenin and IN/IGF-1/IRS-1/MAPK pathways and their role in hepatocarcinogenesis may reveal new molecular targets for potential therapy of HCC. In this regard, a number of small molecules have been developed and partially characterized that inhibit the TCF/ β -catenin interaction to downregulate canonical Wnt3/FZD7 responsive genes (Katoh and Katoh 2007; Lepourcelet et al. 2004). Two of the most promising are TZ MOO990 and SKF 118–330 which impair β -catenin-dependent activity including reporter gene activation, c-myc or cyclin D1 expression, and cell proliferation. These effects have been observed at 0.64 and 0.8 μ M, respectively (Katoh and Katoh 2007; Lepourcelet et al. 2004). Another compound of interest is FJ9, which disrupts the interaction between FZD7 receptor and the PZD domain of Dvl and subsequently downregulates canonical signaling and suppression of tumor cell growth in vivo (Fujii et al. 2007). It will be interesting to determine if such types of agents that inhibit either canonical Wnt3/FZD7 signaling through alteration in TCF/ β -catenin transcriptional activity or FZD7/Dvl interaction will alter HCC tumor growth rate in the future.

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Chapter 7

Epigenetic Effects of Persistent Hepatitis C Virus Infection and Hepatocellular Carcinoma

David R. McGivern and Stanley M. Lemon

Abstract Chronic hepatitis C virus (HCV) infection is associated with an increased risk for hepatocellular carcinoma (HCC). Following the initial acute phase of HCV infection, a majority of patients develop a chronic infection that may be without symptoms for many years. During this time, the virus infection causes progressive damage to the liver, eventually leading to cirrhosis, liver failure and cancer. The mechanisms underlying the progression of HCV infection to cancer remain poorly defined and the lack of a small animal model for HCV infection has hampered understanding of how HCV infection promotes development of HCC. Unlike other viruses that are associated with cancer, HCV has an RNA genome and an exclusively cytoplasmic lifecycle. Indirect mechanisms including chronic inflammation with associated oxidative stress and the potential for DNA damage are likely to contribute to carcinogenesis. Several lines of evidence suggest that viral factors may also contribute to carcinogenesis. Transgenic mice expressing HCV proteins develop cancer in the absence of inflammation. Several HCV proteins have been shown to directly interact with host proteins that have cell cycle regulatory and tumor suppressor activities. The consequences of these interactions for development of cancer are not clear but may contribute to HCC development by modulating host cell pathways that control cell growth, apoptosis and response to stresses such as oxidative DNA damage.

Keywords Hepatitis C virus · Hepatocellular carcinoma · Cell cycle · Tumor suppressor · Cirrhosis

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1 Introduction

Chronic liver disease caused by the hepatitis C virus (HCV) represents a significant global health burden (Perz et al. 2006). Among human RNA viruses, HCV has a unique propensity to establish persistent infection, which is the outcome of the majority of acute infections, even in immunologically competent adults. As a result, it is estimated that almost 200 million people worldwide are currently infected with HCV. Chronic infection with HCV is associated with hepatic inflammation and progressive fibrosis, and an increased risk for development of hepatocellular carcinoma (HCC). In common with HCCs of diverse etiologies, HCV-associated HCC almost always develops in a background of cirrhosis. Contributing risk factors include alcohol abuse, age older than 50 years, and male gender. In many developed regions of the world, including the United States and Japan, HCV infection has emerged as a leading cause of HCC and led to significant increases in the incidence of this cancer (Abe et al. 1998; Yoshizawa 2002).

2 The HCV Lifecycle

HCV is classified as a unique genus, the Hepaciviruses, within the family *Flaviviridae*. It is highly hepatotropic, replicating primarily if not exclusively within hepatocytes, although some evidence suggests it may also infect lymphoid cells (Lemon et al. 2007). The virus possesses a single-stranded, positive-sense RNA genome of approximately 9.6 kilobases, containing a single large open reading frame, flanked on either side by untranslated regions (UTRs) (Fig. 7.1). For a review of the molecular virology of HCV, see Lemon et al. (2007). The 5' and 3' UTRs are highly structured and contain sequences required for viral genome replication. In addition, the 5' UTR contains an internal ribosome entry site (IRES) that directs translation of the viral polyprotein via a 5' cap-independent process.



Fig. 7.1 Organization of the single-stranded RNA genome of HCV. The genomic RNA is positive (messenger) sense, and approximately 9.7 kilobases in length. Nontranslated RNA segments at each end are shown as lines, and regulate the translation of the polyprotein encoded by the single large open reading frame, and the synthesis of both negative-strand intermediates and new positive-strand progeny molecules. The polyprotein is processed by both cellular and viral proteases, resulting in the production of 10 individual mature virus proteins described in the text. The three structural proteins (core, E1 and E2) that comprise the virus particle along with the genomic RNA are highlighted in blue, while the seven nonstructural proteins required for replication of the genome and production of new virus particles are highlighted in pink. For further details, see Lemon et al. 2007

The viral polyprotein is processed by several host and viral proteases to produce 10 individual viral proteins. The structural proteins, which form the HCV virion, include the core (nucleocapsid) protein and two glycosylated envelope glycoproteins, E1 and E2. Nonstructural proteins involved not only in viral RNA replication, but also in virus assembly and maturation, include the ion channel, p7, the NS2 protease, the NS3 serine protease and RNA helicase, NS4A (a cofactor of the NS3 protease), NS4B, NS5A, and the RNA-dependent RNA polymerase, NS5B. NS4B and NS5A are essential components of the viral replicase complex, but NS5A also interacts with several host cell proteins (as do many other HCV proteins) (Lemon et al. 2007). These interactions are presumed to have evolved because they alter the cellular environment in ways that favor replication of the virus. Viral RNA replication is associated with the formation of a cytoplasmic structure referred to as the membranous web: a virus-induced structure derived from membranes of the perinuclear endoplasmic reticulum and possibly the proximal Golgi. The association of HCV with cancer is unique among viruses with RNA genomes and an exclusively cytoplasmic lifecycle.

Available model systems in which the epigenetic effects of HCV infection can be studied include over-expression systems, RNA replicons (i.e., selectable RNA constructs that replicate autonomously in the cytoplasm of transfected cells, but do not produce virus) (Lohmann et al. 1999), and more recently developed cell culture-infectious virus systems (Wakita et al. 2005; Yi et al. 2006). A severe limitation for these in vitro models is that very few cell types are permissive for HCV replication; most studies have used Huh-7 cells, which are derived from a human hepatocellular carcinoma. In vivo models of HCV infection are also limited. Current understanding of HCV pathogenesis and its association with HCC has been hindered by the lack of a small animal model permissive for HCV infection. Chimpanzees are susceptible to HCV, and although disease is typically milder than what is seen in humans, can develop the full range of pathologic consequences of HCV infection, including HCC (Bukh 2004). However, ethical and financial considerations have severely restricted the use of this model. There is also a chimeric mouse model, in which human hepatocytes are transplanted into *SCID/Alb-uPA* mice (Mercer et al. 2001). The transgene (*Alb-uPA*), under control of the albumin promoter, drives liver-specific expression of the urokinase-type plasminogen activator, which is toxic to the mouse liver cells. In an immunodeficient background, this allows for transplantation and repopulation of the mouse liver with human hepatocytes. The human hepatocytes can be infected with HCV, either by inoculation with virus or by intrahepatic injection of HCV RNA (Mercer et al. 2001; Joyce et al. 2009). This model of HCV infection is useful for studying various aspects of HCV replication, but it is technically challenging and does not allow studies of the interaction of HCV with the immune system, which are critical to pathogenesis. HCV-infected chimeric mice do not develop cancer. An alternative approach has been the development of transgenic mouse lines that express HCV proteins, some of which have a liver cancer phenotype. These transgenic mice have added significantly to our understanding of HCV-associated hepatocellular carcinogenesis, and will be discussed in further detail below.

3 HCV Infection and Hepatic Inflammation, Fibrosis and Cirrhosis

The chronic nature of most HCV infections means that virus-induced liver damage is associated with persistent activation of inflammatory and wound-healing responses. Replication of the RNA genome produces double-stranded RNA (dsRNA) intermediates that are sensed by host proteins, including retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3), leading to activation of interferon regulatory factor 3 (IRF-3) and NF- κ B dependent signaling (Loo et al. 2006; Wang et al. 2009b). Although the virus has evolved redundant mechanisms to disrupt interferon responses, many patients as well as chimpanzees with persistent infection show marked transcriptional up-regulation of interferon-stimulated genes. In addition to the well-studied dsRNA-triggered pathways, some studies have suggested that HCV-induced endoplasmic reticulum stress (Waris et al. 2002) or that HCV-encoded proteins may directly activate NF- κ B signaling (Waris et al. 2003; Sato et al. 2006).

The NF- κ B pathway plays a central role in activation of innate immune and inflammatory responses (reviewed in Sun and Karin (2008)). Elevated levels of cytokines and increased deposition of extracellular matrix proteins lead to fibrotic scarring and ultimately cirrhosis. In the case of HCV infection, the progression to fibrosis and cirrhosis is variable from patient to patient, but most HCC develops in the setting of cirrhosis. Both are late events, typically occurring decades after infection with the virus. Chronic inflammation related to HCV infection drives fibrogenesis. Activation of hepatic stellate cells (HSCs) is known to be important, but the mechanisms by which HCV infection leads to activation of HSCs is unclear. Some studies have suggested a pro-fibrotic role for viral proteins (Bataller et al. 2004; Mazzocca et al. 2005) while others have suggested that immune responses trigger fibrogenesis (Baroni et al. 1999). Regardless of the mechanism, activation of HSCs results in secretion of proteins such as collagen and the formation of scar tissue.

Recent data have demonstrated a patchy distribution of HCV infection within the human liver, with only a small minority of hepatocytes expressing a detectable abundance of viral proteins (Liang et al. 2009). Infected cells appear in clusters, possibly due to cell-to-cell spread of the virus, and not always in close proximity to inflammatory cells. Nonetheless, immune responses against infected cells are considered to result in repeated cycles of hepatocyte destruction and regeneration. This pattern of inflammation, disturbances in the cellular homeostasis of the liver, and increased hepatocellular proliferation provide a favorable environment for the accumulation of mutations.

However, while the link between chronic inflammation and cancer is well-established, several lines of evidence suggest that HCV may have a more direct role in HCC development. In particular, several lineages of HCV transgenic mice develop liver cancer in the absence of hepatic inflammation or fibrosis. Also, *in vitro* studies have demonstrated potentially key interactions between HCV proteins and host proteins that involved in cell-cycle regulation. These studies will be discussed below.

4 Transgenic Mouse Models of HCV-Associated HCC

A considerable number of transgenic mouse lines have been developed with a view to examining the role of HCV protein expression in disease pathogenesis and tumor development in vivo. These mice vary in their propensity to develop cancer depending upon several factors, including the transgene promoter, the genetic background (C57Bl6 transgenics being particularly susceptible), and whether HCV proteins are expressed individually, or together with other HCV proteins. Expression of the core protein has been particularly implicated in carcinogenesis. Two transgenic mouse lines, both in a C57Bl6 background, expressing high levels of core protein developed hepatic steatosis at an early age, and went on to develop adenomas and hepatocellular carcinoma (Moriya et al. 1998). The absence of inflammation in the livers of these mice suggests a direct role for core protein in carcinogenesis, however, the level of protein expression in these transgenic animals probably exceeds that present in most if not all infected human liver tissues. Importantly, the expression of core in other (non-C57Bl6) transgenic lineages has not been associated with liver cancer (Kawamura et al. 1997; Pasquinelli et al. 1997).

Transgenic mice that express a lower abundance of the entire HCV polyprotein may represent a more physiologically relevant model of human disease. FL-N/35 mice express a low abundance of the polyprotein under control of the albumin promoter that is detectable by sensitive immunohistochemical methods (Keasler et al. 2006) and is likely to approximate the level of viral protein expression present in infected human tissues. These mice develop steatosis, and subsequently liver cancer at increased but relatively low rates (Lerat et al. 2002). There was no detectable inflammation or immune recognition of the HCV proteins encoded by the transgene, suggesting a direct role for HCV proteins in carcinogenesis. Cancers occurred exclusively in male animals. The mechanism underlying carcinogenesis in this

Table 7.1 Co-carcinogens that promote development of HCC in HCV transgenic mice

HCV transgene	HCC observed in absence of other agents?	Synergistic effect observed in presence of other agents?
Polyprotein	Yes (Lerat et al. 2002)	Yes: iron overload (Furutani et al. 2006), HBV X protein (Keasler et al. 2006), <i>Helicobacter hepaticus</i> colonization (Fox et al. 2009)
NS5A	Yes (Wang et al. 2009a) No (Majumder et al. 2003)	Yes: alcohol (Machida et al. 2009)
Core	No (Kato et al. 2003)	Yes: carbon tetrachloride-mediated hepatocyte injury(Kato et al. 2003)
Core/E1/E2	No (Kawamura et al. 1997)	Yes: diethylnitrosamine (Kamegaya et al. 2005) ^a

^aHCV structural proteins were referred to as “tumor accelerators,” i.e., an increase in tumor size but not frequency was observed

transgenic lineage is not understood, but an impairment in Fas-mediated apoptosis has been described (Disson et al. 2004). Importantly, a companion transgenic mouse line expressing only the structural proteins of HCV demonstrated only background rates of cancer development, thus implicating indirectly the nonstructural proteins of the virus in carcinogenesis (Lerat et al. 2002). Potentially relevant to this observation, transgenic mice expressing NS5A demonstrates impressive steatosis and some go on to develop cancer (Wang et al. 2009a).

In mice from the FL-N/35 transgenic lineage, the development of liver cancer is promoted by iron overloading, co-expression of the hepatitis B virus X protein, or intestinal colonization with *Helicobacter hepaticus* (Furutani et al. 2006; Keasler et al. 2006; Fox et al. 2009). These and other similar studies are summarized in Table 7.1.

5 HCV Protein Expression and Oxidative Stress

Increased levels of oxidative stress have been linked to HCV infection, and in particular to the expression of HCV core protein, in several experimental systems. Transgenic mice with liver-specific expression of the HCV core protein displayed increased levels of the products of lipid peroxidation (Moriya et al. 2001). Conditional expression of core protein in cultured cells under control of a tetracycline-regulated promoter produces oxidative stress (Okuda et al. 2002). Importantly, core protein is a multifunctional protein, the expression of which has pleiotropic effects on the cell. Some core protein localizes to mitochondria in cultured cells, leading to inhibition of mitochondrial electron transport and increased production of reactive oxygen species (Korenaga et al. 2005). In addition to core, NS5A has been implicated in generation of oxidative stress (Gong et al. 2001). Consistent with these findings, microarray studies comparing cirrhotic liver tissue from HCV-infected patients with non-diseased tissues have demonstrated the up-regulation oxidative stress response genes in the former (Shackel et al. 2002). However, it is not clear how much of this results directly from viral protein expression versus the inflammatory response that it generates. Regardless, increases in oxidative stress have the potential to cause damage to both mitochondrial and nuclear DNA, and to lead to the progressive accumulation of mutations.

6 HCV and the Cell Cycle

Several studies have linked HCV replication to the cell cycle. HCV genome replication is dependent upon the cell cycle in Huh7 cells (Pietschmann et al. 2002; Scholle et al. 2004) and this has been linked to intracellular pools of nucleosides (Nelson and Tang 2006) and cell-cycle dependence of viral protein translation directed by the HCV IRES (Honda et al. 2000a). HCV infection of Huh-7.5 cells (a Huh-7-derived cell line defective in RIG-I function) has been reported to result in cell-cycle arrest

at the G1/S phase transition of the cell cycle and this arrest was associated with apoptosis (Walters et al. 2009). However, recent studies from our laboratory suggest instead an arrest at the G2/M phase of the cell cycle (unpublished data). In normal adult liver, most hepatocytes are quiescent, while in liver samples from patients with chronic hepatitis C, markers for cell-cycle entry have been observed more frequently than in samples of uninfected liver. However, few of the cells from HCV-infected liver that entered the cell cycle were observed to progress past G1 phase suggesting cell-cycle arrest (Marshall et al. 2005).

7 HCV Infection and Cellular Apoptosis

The effect of HCV on apoptosis has been studied using several different experimental approaches, including the over-expression of individual viral proteins, use of replicon-bearing cell lines, cell culture-infectious virus systems, and direct analysis of liver samples from HCV-infected patients. Such studies have suggested both pro- and anti-apoptotic roles for HCV proteins. Over-expression of core or NS5A proteins has been suggested to modulate apoptosis. The role of core is particularly unclear, as some studies suggest that core expression inhibits apoptosis (Ray et al. 1996; Machida et al. 2001; Sacco et al. 2003) while others indicate a pro-apoptotic effect (Zhu et al. 1998; Honda et al. 2000b; Chou et al. 2005). The viral E2 (Lee et al. 2005), NS2 (Erdtmann et al. 2003), NS3 (Tanaka et al. 2006), and NS5A (Gale et al. 1999; Lan et al. 2002) proteins have all been suggested to have an inhibitory effect on apoptotic pathways, while other results indicate that NS3 (Prikhod'ko et al. 2004) and NS4A (Nomura-Takigawa et al. 2006) may promote, or sensitize cells to, apoptosis. It is not clear how these studies should be interpreted, and indeed the biologic relevance of many of these results can be questioned.

More recent cell culture studies using infectious virus have demonstrated that HCV infection causes apoptosis in Huh-7 cells (Deng et al. 2008; Mateu et al. 2008; Walters et al. 2009). Furthermore, in the chimeric *SCID/Alb-uPA* mouse model, apoptosis of the transplanted human hepatocytes has also been observed in response to HCV infection (Joyce et al. 2009).

8 Modulation of Tumor Suppressor Protein Functions by HCV Proteins

HCCs from different patients are genetically diverse and contain a wide variety of different genetic alterations. However, some tumor suppressor and signaling pathways are mutated or altered in a high proportion of HCC tissues. These include the p53, Rb, and Wnt pathways (Edamoto et al. 2003; Suriawinata and Xu 2004). Several HCV proteins are thought to interact with and modulate components of each of these pathways.

8.1 p53 Pathway

Several HCV proteins appear to interact with the p53 tumor suppressor and modulate its activity. Core, NS3 and NS5A have all been implicated using *in vitro* approaches. Numerous studies have implicated NS5A in directly regulating p53 activity. NS5A has been shown to interact with p53, and to sequester it at the nuclear membrane when expressed in the p53^{+/+} HepG2 cell line (Majumder et al. 2001). NS5A also interacts with p53 in the rat hepatoma cell line FAO, and inhibits p53-dependent transcription in the p53^{+/+} cell line HCT116 (Qadri et al. 2002). Expression of NS5A can also promote anchorage-independent growth in NIH3T3 cells (Ghosh et al. 1999). Ectopic expression of p53 in the p53-null Hep3B cell line induces apoptosis, but this effect is abrogated by co-expression of NS5A (Lan et al. 2002).

The core protein also has been reported to interact with p53, and to either enhance (Lu et al. 1999; Otsuka et al. 2000) or repress (Kao et al. 2004) its transcriptional activity. Whether core acts to stimulate or inhibit p53-dependent transcription has been suggested to depend on its level of expression (Kao et al. 2004).

Other studies have suggested a role for NS3 in regulating p53 activity. NS3 has been shown to interact with p53 when both proteins are over-expressed in HeLa cells (Ishido and Hotta 1998). NS3 expression can repress p53-dependent transcription and stimulate proliferation of NIH3T3 cells (Kwun et al. 2001). The N-terminal 180 amino acids of NS3 are sufficient for both binding to p53 and suppression of actinomycin D-induced apoptosis. Point mutations that abolish the NS3 interaction with p53 also abolish the anti-apoptotic activity (Tanaka et al. 2006).

While these observations may well be relevant to the development of liver cancer in some lineages of HCV transgenic mice, as discussed above, an important caveat is that the immortalized cell lines used in many of the studies of HCV and p53 possess defects in the p53 pathway. For example, Huh-7 cells and their derivatives over-express a mutant form of p53 (Bressac et al. 1990). Other cell lines frequently used in the studies described above include NIH3T3, COS-7 or HeLa cells, all of which express viral oncoproteins that directly interact with the p53 protein. In addition, the studies often use over-expression systems in which HCV proteins are expressed to much higher levels than in HCV-infected liver. Therefore, studies describing these potential epigenetic effects of HCV proteins need be interpreted with considerable caution.

In addition to the possible direct regulation of p53 by HCV proteins, there are studies that suggest HCV proteins may interact with proteins that interact with p53 or function in the p53 pathway. For example, the core interacts directly with the RNA helicase DDX3, a candidate tumor suppressor protein that regulates activity of the p21(waf1/cip1) promoter (Chao et al. 2006). This is discussed in greater detail below. In addition, NS5B has been shown to interact with another RNA helicase, DDX5, resulting in its relocalization from the nucleus to the cytoplasm (Goh

et al. 2004). In addition to its RNA helicase activity, DDX5 is a transcriptional co-activator of p53 (Bates et al. 2005), and its relocalization to the cytoplasm is likely to prevent this function of DDX5.

8.2 *Rb Pathway*

The cellular pathways controlled by the retinoblastoma tumor suppressor protein (Rb) are frequently mutated in many types of cancers, including HCC (Edamoto et al. 2003). Several groups have reported effects of HCV proteins on the Rb pathway using different systems. In immortalized rat embryo fibroblasts, for example, the conditional expression of the HCV core protein was shown to down-regulate Rb at the level of transcription (Cho et al. 2001); however, no mechanism was elucidated. Tsukiyama-Kohara and colleagues used a *Cre/loxP* conditional expression system to express the HCV genome in HepG2 cells (Tsukiyama-Kohara et al. 2004). Anchorage-independent growth, increased hyper-phosphorylation of Rb and activation of E2F transcription factors was observed in cells expressing the HCV genome after passaging for 44 days.

Work in our laboratory has revealed a remarkable interaction of the NS5B RNA-dependent RNA polymerase and Rb, and shown that the abundance of Rb is sharply reduced in HCV-infected Huh-7.5 cells (Munakata et al. 2005, 2007). NS5B interacts with Rb through a conserved sequence with homology to the LXCXE Rb-binding motifs of DNA virus oncoproteins (Munakata et al. 2005). This interaction targets Rb for ubiquitin-dependent proteasomal degradation, which appears to be mediated by the E6-associated protein (E6AP) ubiquitin ligase (Munakata et al. 2007). Ectopic expression of wild-type NS5B but not NS5B-containing mutations in the LXCXE-like motif leads to enhanced activity of E2F-responsive promoters, and an increased rate of proliferation in U2OS cells (Munakata et al. 2005). HCV mutants in which the LXCXE motif was altered to ablate the NS5B interaction with Rb failed to down-regulate Rb abundance in infected cells, and demonstrated reduced replication potential in cell culture (McGivern et al. 2009).

8.3 *Wnt Pathway*

Mutations in the Wnt signal transduction pathway are frequently found in HCC (de La Coste et al. 1998; Satoh et al. 2000; Boyault et al. 2007). Specifically, activating mutations are common in the β -catenin gene (de La Coste et al. 1998), as well as mutations in the Wnt pathway that lead to stabilization and accumulation of β -catenin (Satoh et al. 2000). Some studies have suggested that HCV modulates components of the Wnt pathway. NS5A has been shown to activate signaling pathways that stabilize β -catenin (Street et al. 2004, 2005). NS5A also

has been reported to bind to and activate the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), thereby initiating a PI3K signaling cascade leading to activation of the downstream kinase, Akt (Street et al. 2004). A consequence of increased Akt activity is the phosphorylation and inactivation of glycogen synthase kinase-3 β (GSK-3 β), a key component of a multiprotein complex that normally targets β -catenin for proteasomal degradation. Increased stability of β -catenin and increased β -catenin-dependent transcription were both observed in cells expressing NS5A (Street et al. 2005).

9 HCV Interactions with Other Regulatory Host Proteins

9.1 ATM

The ataxia telangiectasia mutated kinase (ATM) is a tumor suppressor protein that detects double-strand DNA breaks and phosphorylates its target proteins to activate signal transduction pathways that lead to initiation of the DNA damage checkpoint. This mechanism leads to an arrest of cell-cycle progression until the DNA damage is effectively repaired. Two groups have suggested that there is an interaction between the NS3/4A protease of HCV and ATM (Ariumi et al. 2008; Lai et al. 2008). Both have demonstrated a partial relocation of ATM from the nucleus to the perinuclear region of the cytoplasm in cells expressing NS3/4A. Furthermore, Ariumi and co-workers (2008) have observed that NS3/4A interacts with both ATM and another DNA damage sensor, checkpoint kinase 2 (Chk2). Knockdown of either ATM or Chk2 resulted in decreased viral RNA replication and reduced virus yields, suggesting that the viral modulation of these pathways promotes HCV replication (Ariumi et al. 2008). The sequestration of ATM and Chk2 by HCV may prevent these proteins from performing their normal functions, potentially leading to an impaired DNA damage response and enhancing the accumulation of mutations. Indeed, expression of NS3/4A was shown to impair responses to DNA damage following ionizing radiation (Lai et al. 2008).

9.2 DDX3

The DEAD box RNA helicase, DDX3, was identified as an interacting partner of the HCV core protein by three different groups (Mamiya and Worman 1999; Owsianka and Patel 1999; You et al. 1999), initially in yeast two-hybrid screens and subsequently by co-immunoprecipitation in mammalian cells expressing the core protein. In uninfected cells, DDX3 has a diffuse, predominantly cytoplasmic distribution, while in HCV-infected cells, DDX3 is found in punctate foci that co-localize with core protein. Several studies indicate that HCV usurps DDX3 as a host factor that facilitates virus replication (Ariumi et al. 2007; Randall et al. 2007). The mechanism

by which this occurs is not understood, but the ability of DDX3 promote HCV replication is independent of its core-binding activity (Angus et al. 2009). DDX3 likely has several different functions in the cell including roles in translation (Shih et al. 2008) and in innate immunity (Schroder et al. 2008). Recently, a number of studies have demonstrated differential regulation of DDX3 in several different tumor types (Chang et al. 2006; Chao et al. 2006; Botlagunta et al. 2008), including in liver cancer, suggesting a role for DDX3 as a novel tumor suppressor. The sequestration of DDX3 by core protein may well have implications for the tumor suppressor function of DDX3.

9.3 Disruption of Innate Immunity as a Route to Carcinogenesis

Persistent antagonism of innate immune responses may be a contributing factor in HCV-associated hepatocellular carcinogenesis. Several components of host cell antiviral-signaling pathways are targeted by HCV as a means of evading innate immune responses. For example, NS5A has been shown to interact with and inhibit the interferon-induced, double-stranded RNA-activated protein kinase R (PKR) (Gale et al. 1997). PKR has well-documented tumor suppressor activity (Meurs et al. 1993) and the disruption of PKR signaling by NS5A is thought to be a mechanism by which HCV could promote cancer (Gale et al. 1999; Gimenez-Barcons et al. 2005). In addition, the NS3/4A protease proteolytically cleaves adaptor proteins that are essential for signaling induced by RIG-I and TLR3, thereby disrupting viral activation of the transcription factor, IRF-3 (Foy et al. 2003; Li et al. 2005a,b), a protein that has been shown to have anti-proliferative properties (Kim et al. 2003).

9.4 Modulation of Signal Transduction Pathways by HCV-Encoded Proteins

Several studies have implicated HCV proteins in the disruption of cellular growth-signaling pathways. The transforming growth factor beta (TGF- β) signaling pathway exerts both an anti-proliferative and pro-apoptotic influence. The core has been suggested to interact with a component of the TGF- β pathway, Smad3, thereby modulating its signaling activity, and reducing the responsiveness of the cell to the tumor suppressive effects of TGF- β (Cheng et al. 2004; Pavio et al. 2005; Battaglia et al. 2009). In addition, NS5A contains several proline-rich motifs, which are known to interact with Src homology 3 (SH3) domains that are found in many proteins involved in signal transduction. NS5A has been suggested to interact via these proline-rich motifs with several SH3-containing proteins, including Grb2 (Tan et al. 1999), the p85 subunit of PI3K (Street et al. 2004), and some members of the Src tyrosine kinase family (Macdonald et al. 2004). NS5A appears to disrupt growth-signaling pathways as a result of its interaction with Grb2 (Tan et al. 1999).

10 Summary

Persistent HCV infection may promote the development of HCC by several mechanisms, some reflecting the inflammatory host response engendered by the intrahepatic expression of viral antigens, and others mediated directly by viral proteins. While it may seem surprising that so many different epigenetic effects of the latter type may be in play given that there are only ten individual viral proteins, multi-functionality is characteristic of the highly evolved proteins of most positive-strand RNA viruses. Moreover, there is evidence that only a minority of the nonstructural protein molecules produced by the virus are actively engaged in viral RNA synthesis. However, the field remains bedeviled by the lack of a good, small animal model of HCV-mediated hepatocellular carcinogenesis, and many of the putative viral-host interactions described above lack rigorous confirmation in infectious virus systems or in an *in vivo* context. Despite this, it seems probable that cancer arises, at least in part, as a result of direct epigenetic effects of viral protein expression, including the modulation of host cell pathways that control cell proliferation, apoptosis, and the response to oxidative DNA damage (McGivern and Lemon 2009).

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Chapter 8

DNA Methylation Status in Chronic Liver Disease and Hepatocellular Carcinoma

Yae Kanai and Eri Arai

Abstract Alterations of DNA methylation are among the most consistent epigenetic changes observed in human cancers. In comparison with normal liver tissue, such alterations occur in a genome-wide manner in non-cancerous liver tissue showing chronic hepatitis or cirrhosis, which are widely considered to be precancerous conditions. DNA methylation alterations at the precancerous stage may rapidly generate more malignant cancers. Multiple tumor-related genes, such as the E-cadherin, HIC-1, p16, p15, TMS1/ASC, TIMP3, MGMT, RASSF1A 1, 14-3-3- σ , and SOCS-1 genes, are silenced by DNA hypermethylation in hepatocellular carcinomas (HCCs). Expression of DNA methyltransferase (DNMT) 1 is significantly higher in non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue. DNMT1 overexpression is also correlated with poorer tumor differentiation, portal vein involvement and intrahepatic metastasis of HCCs, and poorer patient outcome. On the other hand, overexpression of DNMT3b4, an inactive splice variant of DNMT3b, may lead to chromosomal instability through induction of DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis. Genome-wide DNA methylation profiling provides optimal indicators for carcinogenetic risk estimation in patients with chronic liver diseases, and for prognostication in patients with HCCs. With the intention of controlling hepatocarcinogenesis from the chronic liver disease stage, translational epigenetics have come of age.

Keywords Bacterial artificial chromosome array-based methylated CpG island amplification · DNA methylation · DNA methyltransferase · DNMT1 · DNMT3b · Precancerous condition · Prognostication · Risk estimation

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1 Introduction

DNA methylation, a covalent chemical modification resulting in addition of a methyl group at the carbon 5 position of the cytosine ring in CpG dinucleotides, is one of the most consistent epigenetic changes occurring in human cancers (Jones and Baylin 2002; Jones and Baylin 2007; Esteller 2008). DNA methyltransferases (DNMTs) transfer methyl groups from S-adenosylmethionine to cytosines. DNA methylation normally promotes a highly condensed heterochromatin structure associated with deacetylation of histones H3 and H4, loss of histone H3, lysine 4 (H3K4) methylation, and gain of H3K9 and H3K27 methylation. DNA methylation is a stable modification inherited throughout successive cell divisions, and is essential for X-chromosome inactivation, genome imprinting, silencing of transposons and other parasitic elements, and proper expression of genes (Cedar and Bergman 2009; Delcuve et al. 2009).

Human cancer cells show a drastic change in DNA methylation status, i.e., overall DNA hypomethylation and regional DNA hypermethylation. DNA hypomethylation induces chromosomal instability through decondensation of heterochromatin and enhancement of chromosomal recombination during carcinogenesis. DNA hypermethylation of CpG islands around the promoter regions silences tumor-suppressor genes. Translational epigenetics have come of age (Issa and Kantarjian 2009), and empirical analysis of DNA methylation status in clinical tissue samples in connection with the clinicopathological diversity of human cancers is assuming increasing importance for the diagnosis, prevention, and therapy of cancers (Kanai and Hirohashi 2007; Kanai 2008, 2009).

2 DNA Methylation Alterations During Multistage Hepatocarcinogenesis

Although in the 1990s various genetic alterations were revealed using classical analytical techniques such as Southern blotting, especially in hepatocellular carcinomas (HCCs) that were poorly differentiated, large in size, and associated with metastasis, only a few of the molecular events occurring in the earlier stage of hepatocarcinogenesis were known. Since DNA methylation alterations may be correlated with chromosomal instability, we examined DNA methylation status on chromosome 16, which is known to be a hot spot for loss of heterozygosity (LOH) in HCCs, using Southern blotting with a DNA methylation-sensitive restriction enzyme. DNA methylation alterations at multiple loci on chromosome 16, in comparison with normal liver tissue samples obtained from patients without HCCs, were frequently revealed even in samples of non-cancerous liver tissue showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, indicating that DNA methylation alterations are a very early event during multistage

hepatocarcinogenesis. This was one of the earliest reports of DNA methylation alterations at the precancerous stage (Kanai et al. 1996).

Since the molecular weight of DNA fragments digested using a DNA methylation-sensitive restriction enzyme was higher in HCCs than in precancerous conditions, and the intensity of larger-sized bands appeared to be enhanced in the HCCs, the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation may increase progressively as precancerous conditions develop into HCCs. The incidence of DNA hypermethylation on chromosome 16 was significantly correlated with higher histological grade, portal vein involvement, and intrahepatic metastasis of HCCs. The presence of DNA methylation alterations in both precancerous conditions and progressed HCCs suggests that DNA methylation alterations in the precancerous stage may rapidly generate more malignant cancers (Kanai et al. 1996).

The E-cadherin tumor-suppressor gene is located on 16q22.1 near the hot spots for both DNA hypermethylation and LOH in HCCs. E-cadherin acts as a Ca^{2+} -dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells (Hirohashi and Kanai 2003). Significant correlations between reduced expression of E-cadherin and poor prognosis have been reported in patients with cancers. We have demonstrated that the promoter region of the E-cadherin gene shows DNA methylation in human cancer cell lines lacking E-cadherin expression, and that E-cadherin expression is induced after treatment with the DNMT inhibitor 5-azacytidine in such cell lines (Yoshiura et al. 1995). At that time, only two genes, RB and VHL, were known to be tumor-suppressor genes silenced by DNA methylation. On the basis of our data, the E-cadherin gene became chronologically the third example of a tumor-suppressor gene silenced by DNA methylation.

DNA hypermethylation around the promoter region of the E-cadherin gene has been detected even in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis. Heterogeneous E-cadherin expression in such non-cancerous liver tissue, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation (Kanai et al. 1997). In HCCs, we found a significant correlation between DNA hypermethylation around the promoter region and reduced expression of E-cadherin (Kanai et al. 1997). This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in a cohort of clinical tissue samples. DNA hypermethylation around the promoter region may participate in hepatocarcinogenesis through reduction of E-cadherin expression, resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

The HIC (hypermethylated-in-cancer)-1 gene at the D17S5 locus (17q13.3) was the first tumor-suppressor gene to be identified in commonly methylated chromosomal loci in human cancers. We showed that DNA hypermethylation at the D17S5 locus was frequently detectable in non-cancerous liver tissue showing chronic hepatitis or cirrhosis (Kanai et al. 1999). The incidence and degree of DNA methylation at the D17S5 locus increased progressively as precancerous conditions developed

into HCCs. The expression level of HIC-1 mRNA in non-cancerous liver tissue showing chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissue, and was further decreased in HCCs (Kanai et al. 1999).

The list of tumor-related genes whose expression levels are altered due to DNA hypo- or hypermethylation during hepatocarcinogenesis has recently been increasing. Silencing of cell cycle regulators such as p16 (Matsuda et al. 1999) and p15 (Wong et al. 2000), proapoptotic proteins such as TMS1/ASC (Kubo et al. 2004), matrix metalloproteinase inhibitor TIMP3 (Yu et al. 2002) and DNA repair protein MGMT (Matsukura et al. 2003), and multifunctional tumor-suppressor proteins such as RASSF1A (Schagdarsurengin et al. 2003) and 14-3-3- σ (Iwata et al. 2000), due to DNA hypermethylation has been reported in HCCs. DNA methylation of the cytokine mediator gene SOCS-1 (Okochi et al. 2003) has attracted attention because it may activate the JAK/STAT signaling pathway and mediate the molecular linkage between inflammation and hepatocarcinogenesis.

Microdissection techniques and PCR using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissue samples. LOH has been reported even in microdissected specimens from tiny precancerous lesions in several organs. In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, we examined 308 microdissected specimens obtained from lobules, pseudo-lobules, and regenerative nodules in non-cancerous liver tissue from patients with HCCs, and the HCCs themselves, for LOH and microsatellite instability (MSI) using 39 microsatellite markers. In addition, using methylation-specific PCR and combined bisulfite restriction enzyme analysis, we also studied the DNA methylation status of C-type CpG islands of the p16, THBS-1, and human hMLH1 genes, and MINT 1, 2, 12, 25, and 31 clones, which are known to be methylated in a cancer-specific, but not age-dependent manner (Kondo et al. 2000). The low incidence of microsatellite instability in HCCs was compatible with absence of silencing of the hMLH1 gene by DNA hypermethylation during hepatocarcinogenesis (Kondo et al. 1999, 2000). In non-cancerous liver tissue showing chronic hepatitis, LOH for at least one marker was found in 20% of informative microdissected specimens. In non-cancerous liver tissue showing cirrhosis, LOH for at least one marker was found in 15% of informative microdissected specimens. LOH was never detected in normal liver tissue from patients without HCCs or in non-cancerous liver tissue showing no remarkable histological findings from patients with HCCs. Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissue from patients without HCCs, DNA hypermethylation was found on at least one CpG island in 58% of microdissected specimens of non-cancerous liver tissue showing no remarkable histological features obtained from patients with HCCs in which LOH was never detected (Kondo et al. 2000). The incidence of DNA hypermethylation on CpG islands overwhelmed that of LOH at all stages of chronic hepatitis, liver cirrhosis, and HCC. Thus, aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using PCR-LOH analysis and microdissection techniques.

3 Abnormalities of DNMTs During Hepatocarcinogenesis

3.1 Overexpression of DNMT1

With respect to the molecular backgrounds of DNA methylation alterations, we focused on abnormalities of DNMTs during hepatocarcinogenesis. The major DNMT, DNMT1, shows a preference for hemimethylated over unmethylated substrates *in vitro*, and targets replication foci by binding to proliferating cell nuclear antigen (PCNA) (Hermann et al. 2004). Thus, DNMT1 has been recognized as a “maintenance” DNMT that allows copying of the DNA methylation pattern on the parental strand to the newly synthesized daughter DNA strand. Mutational inactivation of the DNMT1 gene that can potentially cause genome-wide alterations of DNA methylation was never detected in HCCs (Kanai et al. 2003).

On the other hand, levels of DNMT1 mRNA expression are significantly higher in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in HCCs (Sun et al. 1997; Saito et al. 2001). The incidence of DNMT1 overexpression in HCCs is significantly correlated with poorer tumor differentiation and portal vein involvement (Saito et al. 2003). Moreover, the recurrence-free and overall survival rates of patients with HCCs showing DNMT1 overexpression are significantly lower than those of patients with HCCs that do not (Saito et al. 2003).

3.2 Splicing Alteration of DNMT3b and DNA Hypomethylation in Pericentromeric Satellite Regions

Dnmt3b is required for DNA methylation of pericentromeric satellite regions in early mouse embryos (Okano et al. 1999). DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. Germline mutations of the DNMT3b gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions (Hansen et al. 1999). In HCCs, DNA hypomethylation of these regions is correlated with copy number alterations on chromosome 1, where satellite regions are rich (Wong et al. 2001).

The major splice variant of DNMT3b in normal liver tissue samples is DNMT3b3, which possesses the conserved catalytic domains. DNMT3b4, on the other hand, lacks the conserved catalytic domains, although it retains the N-terminal domain required for targeting to heterochromatin sites. Samples of normal liver tissue show only a trace level of DNMT3b4 expression. The levels of DNMT3b4 mRNA expression and the ratio of DNMT3b4 mRNA to DNMT3b3 in samples of non-cancerous liver tissue obtained from patients with HCCs, and in HCCs themselves, were significantly correlated with the degree of DNA hypomethylation in

pericentromeric satellite regions (Saito et al. 2002). DNA demethylation on satellite 2 was observed in DNMT3b4-transfected human epithelial 293 cells (Saito et al. 2002). Since DNMT3b4 lacking DNMT activity competes with DNMT3b3 for targeting to pericentromeric satellite regions, DNMT3b4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in such regions.

Furthermore, the growth rate of DNMT3b4 transfectants was approximately double that of mock-transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated. STAT 1, which acts as an effector of interferon signaling, and the genes implicated in interferon signaling, were upregulated in DNMT3b4 transfectants relative to mock-transfectants (Kanai et al. 2004). It had been reported previously that inhibition of DNA methylation in cultured human cancer cells by 5-aza-2'-deoxycytidine induces a set of genes implicated in interferon signaling, primarily via overexpression of STAT1, 2, and 3 (Karpf et al. 1999). In cancer cells, DNMT3b may act to maintain the DNA methylation status of not only pericentromeric satellite regions, but also specific genes, probably in cooperation with DNMT1, and this may explain why inhibition of DNMT3b activity by induction of DNMT3b4 produced a similar result to the general inhibition of DNA methylation obtained with 5-aza-2'-deoxycytidine. Overexpression of DNMT3b4 plays a role in multistage carcinogenesis not only by inducing chromosomal instability, but also by affecting the expression of specific genes.

4 Altered Expression of Methyl-CpG Binding Proteins (MBDs)

MBDs, such as MeCP2, MBD1, MBD2, and MBD3, bind to methylated CpG dinucleotides, and their transcriptional repression domain recruits a transcriptional co-repressor complex containing histone deacetylases (Esteller 2008). Although many researchers have focused on cross-talk between DNA methylation and histone modification, abnormalities of MBDs in human cancers do not seem to have attracted much attention. The expression level of MeCP2 mRNA in HCCs with portal vein involvement is significantly lower than that in HCCs without such involvement, suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCCs (Saito et al. 2001). Reduced expression of MBD2 mRNA has been observed in HCCs, suggesting that this may be associated with a particular step in human carcinogenesis (Saito et al. 2001). Unlike other MBDs recruiting histone deacetylase complexes, MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites. The expression level of MBD4 mRNA in HCCs is significantly lower than that in the corresponding non-cancerous liver tissue and is significantly correlated with poorer tumor differentiation and portal vein involvement (Saito et al. 2001). Reduced MBD4 expression may result in frequent C-T transitions in tumor-suppressor genes.

5 Genome-Wide DNA Methylation Analysis

5.1 DNA Methylation Alterations During Multistage Hepatocarcinogenesis Occur in a Genome-Wide Manner

Recently, we have employed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) (Inazawa et al. 2004) for DNA methylation analysis on a genomic-wide scale. Many researchers in this field use promoter arrays to identify genes that are methylated in cancer cells. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large chromosome regions, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention (Frigola et al. 2006). Therefore, we used a BAC array that may be suitable for overviewing the DNA methylation status of individual large regions among all chromosomes. In fact, using BAMCA, we have shown successfully that particular DNA methylation profiles in the kidney at the precancerous stage are inherited by clear cell renal cell carcinomas developing in individual patients, and that these determine the aggressiveness of the tumors and patient outcome (Arai et al. 2009a). Diagnostic indicators for urothelial carcinomas have also been established using BAMCA (Nishiyama et al. 2009).

In samples of non-cancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hypermethylation (Panel N of Fig. 8.1a) in comparison with normal liver tissue from patients without HCCs (Panel C of Fig. 8.1a). Patients showing DNA hypo- or hypermethylation on more BAC clones in their non-cancerous liver tissue samples frequently developed metachronous or recurrent HCCs after hepatectomy, whereas patients showing DNA hypo- or hypermethylation on fewer BAC clones in their non-cancerous liver tissue samples rarely did so, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to development of more malignant HCCs potentially through the induction of chromosomal instability and silencing of tumor-suppressor genes (Arai et al. 2009b). In HCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, the degree of which was further increased (Panel T of Fig. 8.1a) in comparison with non-cancerous liver tissue obtained from the same patients (Arai et al. 2009b).

There were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation in samples of normal liver tissue from male and female patients without HCCs, and in non-cancerous and cancerous liver tissue from male and female patients with HCCs, respectively. Wilcoxon test identified BAC clones in which DNA methylation status differed significantly between hepatitis B virus (HBV)- and hepatitis C virus (HCV)-positive patients with HCCs in both cancerous

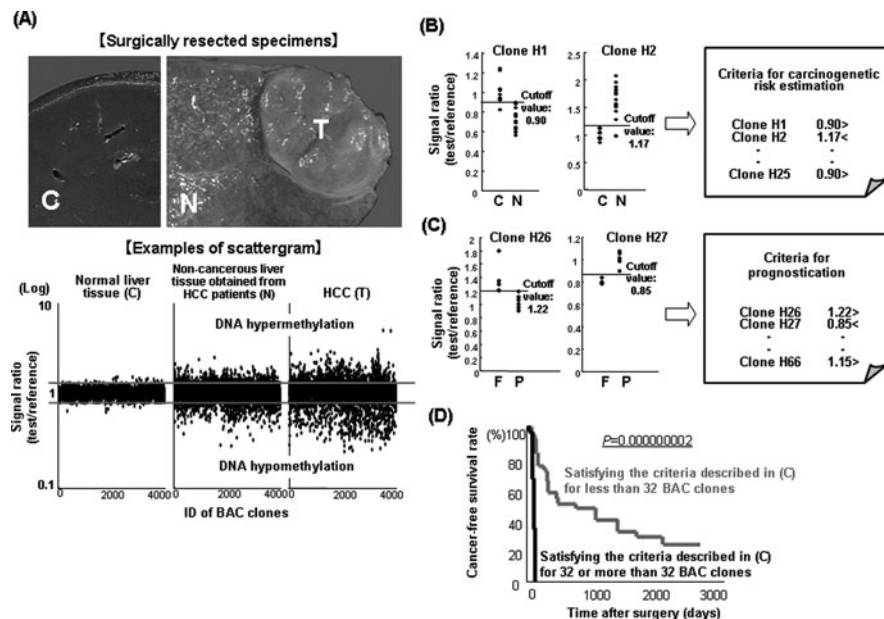


Fig. 8.1 Carcinogenetic risk estimation and prognostication based on genome-wide DNA methylation profiling. (a) Results of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA). In non-cancerous liver tissue obtained from patients with HCCs (N), many BAC clones showed DNA hypo- or hypermethylation in comparison with normal liver tissue from patients without HCCs (C). In HCCs themselves (T), more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation was further increased. (b) Cutoff values for each of the 25 BAC clones selected using a bioinformatics approach were set to discriminate non-cancerous liver tissue obtained from patients with HCCs (N) from normal liver tissue (C), and the criteria for carcinogenetic risk estimation were established using the 25 BAC clones. (c) Cutoff values for each of the 41 BAC clones selected using a bioinformatics approach were set to discriminate the poorer outcome group (P) from the favorable-outcome group (F), and the criteria for prognostication were established using the 41 BAC clones. (d) The cancer-free survival rate of patients with HCCs in the validation cohort satisfying the criteria for 32 or more BAC clones was significantly lower than that of patients with HCCs satisfying the criteria for less than 32 BAC clones

and non-cancerous liver tissue. The DNA methylation status of such BAC clones may reflect the HBV- and HCV-specific molecular mechanisms inducing DNA methylation alterations.

5.2 Carcinogenetic Risk Estimation Based on DNA Methylation Profiles

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at the precancerous stage will become a priority. To reveal the baseline liver histology, microscopic

examination of liver biopsy specimens is performed in patients with HBV or HCV infection prior to interferon therapy. Therefore, carcinogenetic risk estimation using such liver biopsy specimens would be advantageous for close follow-up of patients who are at high-risk of HCC development. Since even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA double strands by covalent bonds, they may be better indicators for risk estimation than mRNA and protein expression profiles, which can be easily affected by the microenvironment of precursor cells.

To estimate the degree of carcinogenetic risk based on DNA methylation profiles, we omitted potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focused on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage, i.e., BAC clones in Groups I, II, III, and IV. Group I: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs was even higher than that of non-cancerous liver tissue obtained from patients with HCCs. Group II: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue, and the average signal ratio of HCCs did not differ from that of non-cancerous liver tissue obtained from patients with HCCs. Group III: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue, and the average signal ratio of HCCs was even lower than that of non-cancerous liver tissue obtained from patients with HCCs. Group IV: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue, and the average signal ratio of HCCs did not differ from that of non-cancerous liver tissue obtained from patients with HCCs. From the BAC clones of Groups I, II, III, and IV, in which the DNA methylation status was inherited by HCCs from non-cancerous liver tissue, the top 25 BAC clones for which DNA methylation status was able to discriminate non-cancerous liver tissue from patients with HCCs in the learning cohort from normal liver tissue with sufficient sensitivity and specificity were identified using a bioinformatics approach (Arai et al. 2009b). By 2D hierarchical clustering analysis using these 25 BAC clones, normal liver tissue and non-cancerous liver tissue obtained from patients with HCCs in the learning cohort were successfully subclassified into different subclasses without any error. We set the cutoff values for each of the 25 BAC clones to discriminate non-cancerous liver tissue obtained from patients with HCCs in the learning cohort from normal liver tissue, and established the criteria for carcinogenetic risk estimation using the 25 BAC clones (Fig. 8.1b). Based on these criteria, both the sensitivity and specificity for diagnosis of non-cancerous liver tissue samples obtained from patients with HCCs in the learning cohort, as being at high-risk of carcinogenesis, were 100% (Arai et al. 2009b). Our criteria enabled diagnosis of additional non-cancerous liver tissue samples obtained from patients with HCCs in the validation cohort as being at high-risk of carcinogenesis with a sensitivity and specificity of 96% (Arai et al. 2009b).

The number of BAC clones satisfying the criteria in non-cancerous liver tissue samples showing chronic hepatitis obtained from patients with HCCs was not significantly different from that in non-cancerous liver tissue samples showing cirrhosis obtained from patients with HCCs. In addition, the average number of BAC clones satisfying the criteria was significantly lower in samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs, than that in non-cancerous liver tissue samples obtained from patients with HCCs. Our criteria not only discriminated non-cancerous liver tissue obtained from patients with HCCs from normal liver tissue, but may also be applicable for classifying liver tissue obtained from patients who are followed up because of HBV or HCV infection, chronic hepatitis, or cirrhosis into that which may generate HCCs and that which will not (Arai et al. 2009b). We intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients.

5.3 Prognostication of Patients with HCCs Based on DNA Methylation Profiles

To establish criteria for prognostication of patients with HCCs, in the learning cohort, HCC samples obtained from patients who had survived more than 4 years after hepatectomy and HCC samples obtained from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test revealed that the signal ratios of 41 BAC clones differed significantly between the two groups (Arai et al. 2009b). By 2D hierarchical clustering analysis using the 41 BAC clones, HCCs in two groups were subclassified into different subclasses without any error. We set cutoff values for each of the 41 BAC clones to discriminate the poor-outcome group in the learning cohort from the favorable-outcome group, and established criteria for prognostication using the 41 BAC clones (Fig. 8.1c). Multivariate analysis revealed that satisfying the criteria for 32 or more BAC clones was a predictor of recurrence, and was independent of parameters that are already known to have prognostic impact, such as histological differentiation, portal vein tumor thrombi, intrahepatic metastasis, and multicentricity (Arai et al. 2009b).

To confirm these criteria, additional HCC samples were analyzed by BAMCA as a validation study. The cancer-free (Fig. 8.1d) and overall survival rates of patients with HCCs satisfying the criteria for 32 or more BAC clones were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones. Such prognostication using liver biopsy specimens obtained before transarterial embolization and radiofrequency ablation may be advantageous even for patients who undergo such therapies. The reliability of such prognostication needs to be validated again prospectively using surgically resected specimens or biopsy specimens.

6 Perspective

DNA methylation alterations associated with DNA methyltransferase abnormalities, such as overexpression of DNMT1 and splicing alterations of DNMT3b, may participate in multistage hepatocarcinogenesis from the precancerous stage to the malignant progression stage. DNA methylation alterations at the precancerous stage may rapidly generate more malignant HCCs. Genome-wide DNA methylation profiling can provide optimal indicators for carcinogenetic risk estimation and prognostication using surgically resected specimens or liver biopsy specimens. Elucidation of the molecular backgrounds of DNA methylation alterations in chronic liver disease may provide clues for epigenetic prevention and therapy of HCCs.

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Part V

Animal Models

Chapter 9

Transgenic and Knockout Mouse Models of Liver Cancer

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Abstract Hepatocellular carcinoma (HCC) is one of the most frequent and deadliest tumors worldwide. Only few patients are amenable to surgery due to the late HCC diagnosis, and alternative treatments do not significantly improve the patient prognosis when tumor is unresectable. Thus, the investigation of HCC biology is required to identify new targets for early diagnosis, chemoprevention, and treatment. To study the molecular events leading to liver malignant transformation and tumor progression, a number of mouse models have been generated. Here, we highlight some of the genetically engineered mouse models that have proved to be valuable tools to study the molecular pathogenesis of human liver cancer. Also, we briefly describe the similarities between human and mouse HCC at the molecular level with emphasis on the advantages and disadvantages of each model. Although additional work is required, the data show that engineered mouse models have provided a significant contribution in our understanding of the pathogenesis of HCC. In particular, the mouse models have allowed the step-by-step analysis of the multiple stages of liver carcinogenesis with the identification of the underlying alterations in signal transduction pathways, cell cycle, and epigenetic and genetic mechanisms involved. Furthermore, the information obtained from these mouse models will help to design new, more specific and effective therapeutic approaches against human HCC.

Keywords Genetically engineered mouse models · Hepatocellular carcinoma · Signal transduction pathways · Oncogenes · Tumor suppressor genes

1 Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm and ranks third among the most lethal cancers worldwide (Feo et al. 2000; Thorgeirsson and Grisham 2002; Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph

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2007). Primary HCC represents 0.5–2% of all cancers in most western countries, but its incidence is extremely high in certain endemic areas of Southeast Asia and Southern Africa due to high frequency of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and food contamination with Aflatoxin B1 (AFB1) (Thorgeirsson and Grisham 2002; Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007). The histopathological and molecular alterations leading to HCC development and progression remain poorly delineated mostly because of the late diagnosis of the disease (Thorgeirsson and Grisham 2002; Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007). Mounting evidence supports the hypothesis that a gradual accumulation of genetic changes occurs in preneoplastic hepatocytes, ultimately leading to malignant transformation (Feo et al. 2000; Thorgeirsson and Grisham 2002; Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007). However, relatively little is known about the stage-specific molecular alterations in hepatocytes during neoplastic development in human liver. Therefore, the development of well-defined animal models of liver cancer, which can reproduce human disease is essential both for the basic studies of tumor biology and the therapeutic purposes. To address this issue, a number of experimental animal models have been generated (Fausto 1999; Calvisi and Thorgeirsson 2005; Feo et al. 2007; Leenders et al. 2008; Heindryckx et al. 2009; Newell et al. 2008; Sánchez and Fabregat 2009; Wu et al. 2009) which can be divided into two broad categories: spontaneous and induced models of HCC (Olive and Tuveson 2006; Frese and Tuveson 2007). The major advantage of spontaneous experimental models is development of HCC under natural circumstances without artificial treatment. Similar to human liver cancer, these models reproduce a gradual accumulation of environmental insults and take into account the diversity in cancer susceptibility. However, the low HCC incidence and great individual variability limit the use of the spontaneous models of human cancer as compared to the induced models of HCC which include treatments with chemical carcinogens, diet manipulation, subcutaneous inoculation of cancer cells into immunocompromised mice, and genome engineering (Olive and Tuveson 2006; Frese and Tuveson 2007; Leenders et al. 2008; Heindryckx et al. 2009; Newell et al. 2008). In this chapter, we focus on genetically engineered mouse model of liver cancer.

Genetically engineered organisms have emerged as one of the mainstays of biomedical research since the 1980s to mimic the pathological and molecular features of human tumors (Olive and Tuveson 2006; Frese and Tuveson 2007). These models enable investigation of the molecular events occurring both in the tumor and tumor microenvironment. Furthermore, engineered animals harboring multiple mutations are highly useful in elucidating the crosstalk between the potential oncogenic pathways *in vivo*. However, there are a number of important limitations in mouse cancer models, such as variations in basic cellular processes as well as in telomere length and telomerase expression. In addition, identical genetic lesions can generate different diseases in mice and in humans (Olive and Tuveson 2006; Frese and Tuveson 2007).

2 Genetically Engineered Mouse Models for the Study of Liver Cancer

2.1 Viral Models of HCC

Epidemiologic evidence indicates that chronic viral hepatitis is the most common etiologic factor in HCC development, accounting for more than 80% cases worldwide (Bruix et al. 2004; El-Serag and Rudolph 2007). Hepatitis B virus (HBV) and C virus (HCV)-related HCC are frequently but not always preceded by liver cirrhosis. It may take more than 20 years for HCC to develop in HBV or HCV infected person suggesting the multi-step nature of viral hepatocarcinogenesis. Therefore, various animal models for investigation of viral hepatitis have been developed, including chronic woodchuck hepatitis virus (WHV) infection associated with inflammation, progressive liver damage, and HCC development (Tennant et al. 2004).

A major problem in generating viral models is a stringent human tropism of both HBV and HCV viruses and thus a requirement for human hepatocytes for induction of virus-associated hepatitis. Therefore, a number of animal models have been developed in which human hepatocytes or liver tissue fragments are transplanted into immunocompromised mice and then infected with HBV or HCV *in vivo* or *ex vivo*. These models have an important role in assessing the value of therapeutic and prophylactic strategies against HBV- and HCV-dependent hepatitis, but they are less useful to study HBV- or HCV-associated HCC than transgenic mice expressing HBV or HCV proteins.

2.2 Hepatitis B Virus

HBV is a DNA virus causing an acute and chronic hepatocyte injury, inflammation, and HCC. In chronic HBV infection viral DNA may integrate into the host cell genome and induce genomic rearrangements, a phenomenon that may activate an adjacent cellular oncogene. In addition, HBV-mediated hepatocyte injury can be amplified by the antiviral cellular immune response and, to a lesser extent, by a direct cell injury. Although most cases of HBV-associated HCC arise on a background of inflammation and fibrosis, some HCC develop in the absence of cirrhosis, most likely due to HBV integration into the host genome, thus promoting transcriptional transactivation of mitogenic factors (Farazi and DePinho 2006; El-Serag and Rudolph 2007).

HBV transgenic mice that express the HBV envelope, pre-core, core proteins, and hepatitis delta antigens do not show histological signs of liver injury (Koike 2002). However, elevated levels of HBV surface antigen (HBsAg) may severely damage hepatocytes via accumulation of the protein in the endoplasmic reticulum,

resulting in hepatocyte enlargement and dysfunction (focal necrosis and secondary inflammation). These changes induce sustained hepatocellular proliferation as well as high oxidative DNA damage, leading to the development of preneoplastic foci and micro-nodular regenerative hyperplasia by 6–10 months of age, and HCCs by 12–20 months of age. At the molecular level, no alterations in canonical oncogenes and tumor suppressor genes were found, except for transcriptional activation of Igf2 (insulin growth factor-2) and Mdr-1a (multidrug resistance-1a) genes. Importantly, exposure of HBsAg transgenic mice to AFB1 or DEN significantly reduced tumor latency. Therefore, it is likely that the strong hepatocellular proliferation characteristic of HBsAg transgenic mice selects for chemically transformed cells by allowing fixation of mutations caused by the DNA adducts. It also predisposes cells for genomic instability by increasing exposure of DNA to reactive oxygen species (ROS) generated by inflammatory liver cells. However, chronic cell injury is not the only mechanism contributing to hepatocarcinogenesis in transgenic mice over-expressing the HBX core protein (Koike 2002). In these mice, pericentral dysplasia and preneoplastic foci over-expressing the HBX protein arise rapidly (between 2 and 4 months of age), whereas HCC occurs only after 12 months. Recent evidence suggests that HBX induces transcriptional activation of various putative protooncogenes, including Pkc, Erk, Stat3, Hif-1 α , NF- κ B, and AP-1. Furthermore, HBX downregulates the expression of various tumor suppressor genes, such as E-cadherin and p21^{WAF1}, and binds to p53, resulting in its inhibition via cytoplasmic sequestration (Koike 2002).

2.3 Hepatitis C Virus

HCV infection is an increasing risk factor for HCC (Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007). The HCV genome is a RNA molecule of approximately 9500 nucleotides, encoding a large glycoprotein that is prone to several post-translational modifications producing final viral proteins and enzymes. A number of HCV proteins, such as core, NS3 and NS5A play an important role in the hepatocarcinogenesis (Lunel-Fabiani 2007). Unlike HBV, the RNA genome of HCV does not integrate into the host chromosomes, suggesting that HCV-driven hepatocarcinogenesis does not involve insertional mutagenesis. Transgenic mouse models expressing different HCV proteins have been generated, including the viral structural proteins core/E1/E2 (Koike 2005). Most of HCV transgenic mice develop liver steatosis with a male predominance in animals older than 3 months of age, accompanied by generation of free radicals, oxidative stress, and lipid peroxidation. HCC develops in approximately 30% HCV transgenic mice by 13–19 months of age, predominantly in males (Koike 2005). These studies suggest that HCV core proteins increase the risk of liver cancer even in the absence of marked inflammatory response to infection. The consistent correlation between steatosis and HCC suggests that steatosis may be an early event during HCV-driven hepatocarcinogenesis.

3 Chronic Liver Injury Models

The deficiency in the protease inhibitor alpha-1-antitrypsin (AAT) is associated with pulmonary emphysema and liver disease. AAT-deficient transgenic mice expressing the transport-impaired Z variant of the human disease accumulate AAT in the endoplasmic reticulum and after prolonged cycles of cell death and compensatory proliferation develop HCC. A similar pathogenesis is likely to account for the progressive cirrhosis and HCC in individuals affected by alpha-1-antitrypsin deficiency (Geller et al. 1994; Fausto 1999).

Transgenic mice with hepatocyte-specific overexpression of urokinase-type plasminogen activator (uPA), a serine protease member of the trypsin family, were originally designed to study the effect of uPA on tumor dissemination (Sandgren et al. 1992). Unexpectedly, overexpression of the uPA transgene caused a series of events similar to those described in other models of toxic liver injury, including hepatocyte necrosis and frequent death of uPA mice. In surviving mice, clonal proliferation of the rare transgene-deficient cells was sufficient to produce HCC (Sandgren et al. 1992).

The deficiency in Mdr-2 gene encoding a P-glycoprotein, involved in transporting phosphatidylcholine into the bile, results in accumulation of high amounts of non-emulsified toxic bile salts in the biliary system thereby leading to progressive damage of the hepatobiliary structures followed by ductular proliferation, development of preneoplastic nodules, and HCC (Mauad et al. 1994; Katzenellenbogen et al. 2006). The Mdr-2-knockout mice represent a useful model to elucidate how chronic inflammation of the biliary system might increase liver cancer risk in humans (Mauad et al. 1994; Katzenellenbogen et al. 2006).

A similar pathogenesis is responsible for liver tumor development in acyl-CoA oxidase (AOX) knockout mice, which develop spontaneous hepatitis and steatosis (Fan et al. 1998; Fausto 1999). In this model, regenerating hepatocytes show increased accumulation of peroxisomes and overexpression of PPAR- α -regulated genes. HCCs develop after the clearance of the inflammatory process. Presumably, the oxidative damage of hepatocytes, combined with the proliferative stimulus induced by PPAR- α , constitutes a key factor contributing to liver tumor development in AOX-deficient mice (Fan et al. 1998; Fausto 1999).

4 Mouse Models Recapitulating Molecular Alterations of Human Hepatocarcinogenesis

A number of genetically engineered mouse models have been generated with the aim to unravel the functional consequences of molecular alterations characteristic of human liver carcinogenesis. Here, we highlight some of these models that have proved to be highly useful for understanding the role of oncogenes and tumors suppressor genes in human HCC.

4.1 Disruption of Cell Cycle Regulation: p53, Rb, E2F1, and SV40 T Antigen

Deregulation of cell cycle-related proteins is one of the major factors contributing to human HCC development and progression (Tannapfel and Wittekind 2002). A key guardian of the cell cycle is the p53 tumor suppressor (Trp53). The Trp53 restrains cell proliferation in response to DNA damage or deregulation of mitogenic oncogenes by its ability to upregulate various cell cycle checkpoints genes, to induce apoptosis, and to promote cellular senescence (Weinberg 1991; Campisi 2003). Mice heterozygous for Trp53 are susceptible to HCC development in the context of liver injury, but only in the absence of intact telomerase (Farazi et al. 2006). Furthermore, Trp53 knockout mice develop larger and more aggressive tumors than wild-type mice upon introduction of mouse polyoma virus middle T antigen (PyMT) driven by an albumin promoter (Lewis et al. 2005). Similarly, disruption of Ink4a/Arf tumor suppressor locus exaggerates phenotype of liver-specific knockout of Trp53 (Chen et al. 2007). In addition, loss of p53 seems to be required for maintaining the liver tumor growth induced by oncogenes such as mutated Ras (Xue et al. 2007). The retinoblastoma (Rb) pathway, often disrupted in human hepatocarcinogenesis (Thorgeirsson and Grisham 2002; Tannapfel and Wittekind 2002; Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007), modulates cell cycle progression by guarding and triggering DNA replication and cell cycle division in late G1 phase. The Rb protein (pRb) binds the members of the E2F family of transcription factors thus impeding the transcription of E2F target genes involved in DNA synthesis and cell cycle progression (Nevins 2001). Interestingly, somatic inactivation of pRb in the murine liver results in E2F target gene deregulation and elevated cell cycle progression during post-natal growth (Mayhew et al. 2007). However, in adult livers, E2F targets are repressed and hepatocytes become quiescent independent of pRb, indicating that other genes may compensate for the pRb loss. On the other hand, mice with liver-specific pRb ablation show increased HCC multiplicity as compared with wild-type mice following exposure to the hepatocarcinogen DEN (Mayhew et al. 2007). Furthermore, tumors arising in pRb-deficient livers were significantly more proliferative and exhibited higher levels of genomic instability. A subsequent comparison of global gene expression patterns showed that the pRb-deficient HCC clustered together with human HCC characterized by poor survival (Mayhew et al. 2007). The significance of the pRb checkpoint was also investigated through generating transgenic mice expressing E2F1 under the control of albumin enhancer/promoter (Conner et al. 2000). Different from the pRb-deficient model, all E2F1 mice spontaneously developed hepatocellular adenomas by 10 months of age with some evolving to HCC (Conner et al. 2000). Importantly, hepatocarcinogenesis occurred in a context of a relatively low genomic instability and proliferation (Calvisi et al. 2004). Thus, disruption of either pRb or E2F1 genes produced different effects on hepatocytes proliferation, genomic stability, and HCC development. Presumably, E2F1 upregulation activates signal transduction pathways, which are not limited to cell proliferation. In accordance with this hypothesis, E2F1 overexpression in the liver has been shown to trigger a strong anti-apoptotic response via upregulation of phosphatidylinositol 3-kinase (PIK3CA)/Akt/mTOR

pathway (Ladu et al. 2008). Notably, PIK3CA/Akt/mTOR cascade is similarly activated in human HCC, with levels of PIK3CA/Akt and mTOR inversely associated with patients' survival (Ladu et al. 2008). Thus, the present findings suggest that E2F1 may function as a critical anti-apoptotic factor both in murine and human liver.

The SV40 (Simian Vacuolating Virus 40) large T antigen (TAg) is an oncoprotein derived from the SV40 polyomavirus. SV40 TAg is able to transform various cell types via interference with expression of the pRb, Trp53, and p105. Disruption of these tumor suppressors initiates G1/S phase transition and consequent replication of both the cell and the viral genome (Ali and DeCaprio 2001). In the liver, expression of the SV40 TAg driven by the albumin enhancer/promoter leads to the development of adenomas and HCC within 3–7 months of age (Sandgren et al. 1989). Similar, a tetracycline-inducible mouse model of SV40 is characterized by high frequency of liver tumor development (Manickan et al. 2001).

4.2 Telomere Dysfunction

In most eukaryotic organisms, telomeres are repetitive sequences of DNA that are located at the termini of linear chromosomes and function to compensate for incomplete semi-conservative DNA replication at the chromosomal ends (Gilson and Gèli 2007). Telomeres are synthesized and maintained by telomerase, part of a ribonucleoprotein complex called TERT (telomerase reverse transcriptases). In most human cells, telomere length is progressively decreasing, and once telomeres become critically short, cell undergoes senescence. Inactivation of Trp53 or pRb contributes to continuous telomere shortening resulting in chromosomal instability and cell death. Mounting evidence suggests that progressive telomere attrition during repeated rounds of hepatocyte proliferation caused by liver injury ultimately results in chromosomal instability, a main feature of human HCC (Plentz et al. 2004; Artandi and DePinho 2000; Artandi et al. 2000).

Different from humans, reduction in the telomere length is not observed in mice, presumably due to the long initial telomere span and active telomerase expression. However, deficiency in telomerase promotes formation of non-reciprocal translocations and cancer development in Trp53 mutant mice (Artandi et al. 2000). A rapid HCC formation in Trp53-mutated telomerase knockout mice points to the synergistic role of telomerase-induced chromosomal instability and attenuated p53 function in liver tumors (Farazi et al. 2006). Consistent with this, Trp53 did not affect the HCC occurrence in the presence of intact telomeres (Farazi et al. 2006).

4.3 Transforming Growth Factor Alpha (TGF- α)

Hepatic overexpression of transforming growth factor alpha (Tgf- α), a potent hepatocyte mitogen structurally and functionally resembling epidermal growth factor (EGF), results in epithelial hyperplasia and slow HCC development (by 13–15 m of age) (Jhappan et al. 1990; Sandgren et al. 1990). The subsequent work has

shown that only male transgenic mice generated on the CD1 genetic background but not females or strains resistant to hepatocarcinogenesis were susceptible to Tgf- α -driven hepatocarcinogenesis (Lee et al. 1992). The strain- and sex-dependence together with a long latency point to a multi-factorial nature of Tgf- α -induced hepatocarcinogenesis and a requirement for additional genetic and epigenetic events.

4.4 *Ras/Mitogen-Activated Protein Kinase (MAPK)*

The Ras pathway regulates numerous signals involved in cell growth, survival, and migration (Barbacid 1990). The best-characterized Ras effector promoting cell cycle progression is the MAPK pathway (Chang and Karin 2001). In this cascade, Ras induction triggers activation of RAF, MAPK kinase kinase (MEK), and extracellular signal-regulated kinase (ERK) proteins, leading to upregulation of c-FOS, c-JUN, c-MYC, ELK1, and ETS targets (Chang and Karin 2001). Consistent with the ubiquitous activation of the Ras/MAPK pathway in human HCC (Calvisi et al. 2006), a number of transgenic mouse models over-expressing the mutant H-Ras gene have been generated with a strikingly different outcome. Expression of the mutant H-Ras transgene controlled by the albumin enhancer/promoter produced either hepatomegaly and lung tumor formation (Sandgren et al. 1989) or resulted in hepatic alterations in male (but not female) transgenic mice leading to HCC development in one-third of male mice by 8 months of age (Wang et al. 2005). Similarly, one transgenic line over-expressing the mutant H-Ras oncogene driven by the L-type pyruvate-kinase gene developed HCC as well as polycystic kidney disease and epididymis hyperplasia (Gilbert et al. 1997). Thus, similar to the findings in Tgf- α transgenic mice, these data indicate that perhaps H-Ras *per se* is a relatively weak liver oncogene, which requires additional mutations and/or epigenetic changes to express its full malignant potential. Indeed, a later study showed that introducing H-Ras mutation alone was unable to confer the autonomous growth properties to the emerging dysplastic hepatocytes. However, the simultaneous mutation in the β -catenin gene (a member of the Wnt signaling cascade) caused a clonal expansion of the altered cells followed by HCC development in 100% of mice (Harada et al. 2004).

4.5 *Epidermal Growth Factor (EGF) and Platelet-Derived Growth Factor (PDGF)*

Epidermal growth factor (EGF) is a potent mitogen for epithelial cells, including hepatocytes, which upon binding to its receptor EGFR, triggers multiple biological events in the cell, including proliferation, resistance to apoptosis, and migration (Hackel et al. 1999). Unlike in other malignancies, the EGF receptor is rarely mutated in HCC but is frequently overexpressed, particularly in the liver tumors

characterized by biological aggressiveness (Calvisi et al. 2007). The overexpression of a secreted form of EGF (IgEGF) in the mouse liver results in rapid HCC development, which is further accelerated when IgEGF transgenic mice are crossed with AAT-myc transgenic mice. Indeed, simultaneous overexpression of IgEGF and AAT-myc resulted in tumor-related death in all double-transgenic mice by 4 months of age (Tonjes et al. 1995).

PDGFs proteins belong to a family of ligands of PDGF receptors involved in a plethora of cellular functions, including proliferation, survival, and cell migration (Heldin et al. 1988). In contrast to PDGF-A and PDGF-B, which are secreted as bioactive dimers after intracellular processing, the PDGF-C precursor polypeptide is secreted intact from the cell and requires extracellular proteolytic cleavage of the receptor interacting domain to produce the active growth factor, PDGF-CC. Inducible overexpression of the PDGF-B ligand in the liver resulted in hepatic stellate cell activation and collagen deposition, but not HCC development (Czochra et al. 2006). On the other hand, PDGF-C transgenic mice expressing the human PDGF-C gene driven by the albumin promoter exhibited fibrosis and steatosis, and 80% developed HCC by 12 months of age (Campbell et al. 2005). These data indicate that PDGF proteins play an important pathogenetic role in the generation of liver fibrosis. Furthermore, the PDGF-C transgenic mouse model represents a unique system for the study of hepatic fibrosis progressing to tumorigenesis.

4.6 Akt/Mammalian Target of Rapamycin (mTOR)

The Akt pathway is a pivotal regulator of a variety of cellular processes, including proliferation, apoptosis, and angiogenesis (Bellacosa et al. 1991; Vivanco and Sawyers 2002). Once activated, the serine/threonine kinase Akt promotes cell survival both by inactivating multiple pro-apoptotic proteins, including Bad, FoxO1, caspase 9, apoptosis signal regulating kinase-1 (Ask1), and stimulating transcription of anti-apoptotic genes. Furthermore, Akt sustains cell growth by either direct phosphorylation of the mammalian target of Rapamycin (mTOR) or indirectly through inactivation of tuberlin, an mTOR inhibitor (Vivanco and Sawyers 2002; Hay 2005). Phosphatase and tensin homolog (PTEN) is a negative regulator of this pathway and its suppression results in Akt activation (Vivanco and Sawyers 2002). Liver-specific deletion of PTEN leads to hepatomegaly and steatohepatitis by 10 weeks and HCC in the majority of male mice by 20 months of age (Horie et al. 2004). Similarly, most of the male mice heterozygous for Lkb1, a gene involved in the negative regulation of the mTOR cascade, develop HCC by 12 months of age as compared with 20% female mice showing a sex difference in HCC susceptibility (Nakau et al. 2002).

4.7 Wnt/ β -Catenin

The canonical Wnt pathway has recently emerged as one of the main signaling cascades involved in human hepatocarcinogenesis (De La Coste et al. 1998). A

pivotal factor in this pathway is the β -catenin gene (Gumbiner 1995; Clevers and Van de Wetering 1997; Polakis 1999). β -catenin is physiologically involved in two major functions: cell–cell adhesion by association with E-cadherin, and transmission of the proliferative signal in the Wntless/Wnt pathway. Protein levels of β -catenin are regulated by phosphorylation at the NH₂-terminal region by GSK-3 β complex consisting of APC protein, axin/conductin, and GSK-3 β . Once phosphorylated, β -catenin is rapidly degraded through the ubiquitin-proteasome pathway (Gumbiner 1995; Clevers and Van de Wetering 1997; Polakis 1999). Mutations of APC or β -catenin itself lead to the elevated free β -catenin, which interacts with transcription factors of the Tcf/lymphoid enhancer factor family upon translocation into the nucleus. β -Catenin/Tcf complex then activates target genes involved in the control of cell growth and apoptosis (Gumbiner 1995; Clevers and Van de Wetering 1997; Polakis 1999).

Wnt signaling is upregulated in a subset of HCC. In HCC, mutations of genes encoding several components of the Wnt pathway have been described, including β -catenin (the most frequently detected), Axin1, and Axin2 (rare). β -Catenin activation has been also frequently observed in the absence of somatic mutations (De La Coste et al. 1998; Laurent-Puig et al. 2001; Farazi and DePinho 2006). Although in humans mutations in the β -catenin gene are thought to be tumorigenic, transgenic mouse models over-expressing either a stable mutant form of β -catenin or a constitutively activated, non-mutated form of β -catenin show only signs of hepatomegaly but do not develop HCC (Cadoret et al. 2001; Harada et al. 2002). Interestingly, although mutations in the APC tumor suppressor are extremely rare in HCC, liver-targeted ablation of APC in mice caused HCC through activation of β -catenin signaling (Colnot et al. 2004). Similar to other transgenic mouse models, it seems that additional genetic or epigenetic changes are required for tumor development in β -catenin transgenic mice. In accordance with this hypothesis, simultaneous co-expression of the mutated β -catenin gene and a mutated H-Ras gene, introduced by adenovirus-mediated Cre expression, resulted in 100% HCC incidence in double-transgenic mice (Harada et al. 2004). The interplay between the growth factor signaling pathways and the Wnt/ β -catenin pathway was further illustrated by the simultaneous overexpression of HGF in a β -catenin knockout background. The proliferative effects of HGF overexpression were mediated by β -catenin stabilization and were abrogated in β -catenin null mice (Apte et al. 2006).

4.8 Janus Kinase-Signal Transducer and Activator of Transcription Factor (Jak/Stat)

The Jak/Stat pathway is activated by a variety of cytokines and growth factors (Bromberg 2001; Kisseleva et al. 2002). Cytokines induce phosphorylation of the Janus tyrosine kinases (Jak1, 2, 3, Tyk2) followed by activation of Stat1, 2, 3, 4, 5, and 6 proteins. Nuclear localization of Stats also results in the activation of Stat target genes, including three families of inhibitory proteins, the protein inhibitors of

activated Stats (PIAS), the SH2-containing phosphatases (SHP), and the suppressors of cytokine signaling (SOCS). Induction of PIAS, SHP, and SOCS proteins following Stat activation represents an efficient negative feedback loop mechanism limiting the magnitude of Stat effects on target cells (Bromberg 2001; Kisseleva et al. 2002). Unrestrained activation of the Jak/Stat pathway due to the suppression of PIAS, SOCS, and SHP inhibitors is a hallmark of human HCC (Calvisi et al. 2006, 2007). In order to further investigate the putative tumor suppressive role of Stat inhibitors, mouse models characterized by SOCS1 and SOCS3 disruption have been generated. Of note, mice heterozygous for SOCS1 (SOCS1^{-/+} mice) develop more severe liver fibrosis than did wild-type littermates when subjected to treatment with dimethylnitrosamine (Yoshida et al. 2004). Furthermore, carcinogen-induced HCC development was significantly enhanced by heterozygous deletion of the SOCS1 gene (Yoshida et al. 2004). These findings suggest that SOCS1 contributes to protection against hepatic injury and fibrosis as well as hepatocarcinogenesis. Similarly, mouse models of SOCS3 hepatocyte-specific knockout (KO) have been developed to directly study the role for SOCS3 during liver regeneration and hepatocarcinogenesis (Ogata et al. 2006; Riehle et al. 2008). SOCS3 KO mice exhibited a marked enhancement of cell proliferation as compared to control mice (Ogata et al. 2006; Riehle et al. 2008). SOCS3 ablation caused a prolonged, Stat3 phosphorylation and increased expression of Stat3 target genes (Ogata et al. 2006; Riehle et al. 2008). In regenerating mouse livers, inactivation of SOCS3 enhanced phosphorylation of the mitogenic extracellular signal-regulated kinase 1/2 (ERK1/2) (Riehle et al. 2008). In addition, SOCS3 KO mice developed HCC at an accelerated rate when subjected to a protocol of chemical-induced carcinogenesis than the corresponding wild-type animals (Riehle et al. 2008). Thus, SOCS3 modulates both physiological and neoplastic proliferative processes in the liver and may act as a tumor suppressor.

4.9 Hippo/Warts

Several studies have linked the Hippo kinase pathway to cancer in mammals (Harvey and Tapon 2007; Zeng and Hong 2008; Bertini et al. 2009). The core components of the *Drosophila* Hippo pathway are well-conserved and have been shown to function as tumor suppressors in mammals. In this pathway, the mammalian sterile 20-like kinases 1 and 2 (MST1 and 2, Hippo homologs) phosphorylate the adapter proteins SAV1 (also called WW45) and MOB1 (a Mats homolog), and the kinases LATS 1 and 2 (Wts homologs). MST1/2 phosphorylation of MOB1 leads to the increased affinity of MOB1 for LATS1 as well as an activity of LATS1. This suggests that MOB1 is an important adapter for LATS activation. Induction of the Hippo cascade triggers the inhibition of growth signaling by the putative oncogenes Yes-associated protein (YAP) and the transcriptional co-activator with PDZ binding motifs (TAZ). To study the role of Hippo in mammals, a transgenic mouse model was generated in which the protooncogene YAP1 could be activated in a

doxycycline-inducible manner. Overexpression of YAP1 for 35 days resulted in a more than fourfold increase in the adult liver size. Subsequent microscopic analysis revealed the presence of the dysplastic hepatocytes with irregular enlarged nuclei, a high nuclear to cytoplasmic ratio, and increased proliferation (Camargo et al. 2007). Similarly, transgenic mice carrying two co-integrated DNA constructs, one with reverse tetracycline transactivator (rtTA) under the control of the liver-specific ApoE promoter, and another with a human YAP cDNA (hYAP) driven by the minimal CMV promoter and a tetracycline (Tet)-response element (TRE), exhibited massive hepatomegaly (Dong et al. 2007). Furthermore, when the ApoE/rtTA-YAP mice were exposed to doxycycline for over 8 weeks, they developed numerous nodules throughout the livers (Dong et al. 2007). The nodules were comprised of proliferative hepatocytes compressing the surrounding non-tumorous liver and displayed many features of the trabecular-type HCC. The transgenic mice eventually succumbed to liver tumors, with a mean survival of 7 weeks (from birth) for mice in which YAP was induced starting at 3 weeks of age, and a mean survival of 14 weeks (from birth) in which YAP was induced starting at 8 weeks of age. Importantly, YAP1 was also identified as an important oncogene for HCC development both in mice and humans via an integrated genomic approach (Zender et al. 2006). The role of Hippo pathway has been recently substantiated by the findings that liver-specific ablation of both Mst1 and Mst2 genes, inhibitors of YAP1-driven proliferation, results in the development of massive HCC with a mean latency of 10 weeks, very similar to what was observed with YAP overexpression (Zhou et al. 2009). Taken together, these results reveal a causal association between deregulation of the Hippo pathway and tumorigenesis.

4.10 Transforming Growth Factor- β (TGF- β)

TGF- β 1 is a multifunctional cytokine that can function as a tumor suppressor or as a *bona fide* oncogene (Massague 2008). The resulting TGF- β 1 effect may depend on the cell context and microenvironment. In hepatocytes, TGF- β 1 is an important suppressor factor via inhibiting proliferation and inducing cell death (Oberhammer et al. 1992; Sánchez et al. 1996). However, TGF- β 1 is often overexpressed in human HCC (Ito et al. 1991). Similarly, liver-specific overexpression of TGF- β 1 increased liver tumor development in transgenic mice (Factor et al. 1997). Spontaneous HCC developed with 59% incidence by 16–18 months of age. Co-expression of TGF- β 1 with c-Myc transgene further accelerated both the rate and malignancy of hepatocarcinogenesis. In particular, the combined activity of two transgenes resulted in the upregulation of the Wnt/ β -catenin pathway (Calvisi et al. 2001). This observation suggests that TGF- β 1 oncogenic potential is mediated at least in part via interaction with the Wnt/ β -catenin cascade, as observed during *Xenopus* embryogenesis (Nishita et al. 2000). Additionally, DEN treatment dramatically enhanced hepatocarcinogenesis in the double-transgenic mice as compared to both parental lines, leading to a bigger tumor size, and higher

tumor frequency and malignancy (Factor et al. 1997). Interestingly, progression from benign to malignant phenotype correlated with an early loss of TGF- β receptor II (TGF- β RII) expression. These findings suggest that TGF- β 1-induced transformation requires the cooperation with additional genetic alterations in order to overcome the inhibitory actions of TGF- β 1 and facilitate HCC development. The importance of disruption of TGF- β 1 pathway in HCC pathogenesis has been further substantiated in T β RII heterozygous knockout mice. These mice did not develop spontaneous HCC, but exhibited a marked increase in HCC occurrence when subjected to chemical-induced hepatocarcinogenesis as compared with wild-type mice (Im et al. 2001).

Intracellular signaling from TGF- β occurs through the SMAD family proteins (SMAD2, SMAD4, and SMAD adaptor), which are believed to be tumor suppressors (Massague 2008). Unexpectedly, SMAD mutant models do not develop HCC. On the other hand, SMAD function is dependent on adaptor proteins, including the embryonic liver fodrin (ELF), a β -spectrin protein. ELF associates with SMAD3, SMAD 4, and the TGF- β receptor complex leading to their nuclear translocation. Of note, ELF heterozygous mice develop steatosis and HCC, presumably due to the cell cycle disruption. Consistent with the dual role of TGF- β 1 in tumorigenesis, microarray analysis identified two subsets of TGF- β -responsive genes, reflecting both the suppressive and oncogenic properties of TGF- β (Coulouarn et al. 2008). Within the TGF- β -positive HCC subgroup, two distinct subsets of tumors were discriminated that preferentially expressed early or late TGF- β responsive genes. In particular, patients with the late TGF- β signature displayed a considerably shorter survival time and increased tumor recurrence compared to those with the early TGF- β signature, implying a pivotal role of TGF- β in determining HCC progression and patient's prognosis (Coulouarn et al. 2008).

4.11 C-Met and Hepatocyte Growth Factor (HGF)

C-Met is a protooncogene possessing a tyrosine-kinase activity. Activation of c-Met by its single ligand HGF induces c-Met kinase catalytic activity which triggers transphosphorylation of the tyrosines Tyr 1234 and Tyr 1235 (Trusolino and Comoglio 2002). These two tyrosine residues engage various signal transducers, thus initiating a wide spectrum of biological activities driven by c-Met, including proliferation, migration, invasion, and morphogenesis (Trusolino and Comoglio 2002). Various alterations (overexpression, amplification, and mutation) of the c-MET protooncogene have been detected in human HCC (Park et al. 1999). Furthermore, the presence of c-Met expression signature in human HCC showed a significant correlation with the increased vascular invasion rate and microvessel density as well as with decreased mean survival time of HCC patients (Kaposi-Novak et al. 2006). However, experimental mouse models of HCC have revealed that the outcome of HGF/c-Met activation could be either stimulation or suppression of hepatocarcinogenesis. Transgenic mice over-expressing HGF under the control of

the metallothionein promoter developed HCC, whereas transgenic mice exhibited hepatomegaly but not dysplasia when HGF expression was driven by the CMV promoter (Sakata et al. 1996; Apte et al. 2006). Furthermore, HGF suppressed HCC development in c-myc transgenic mice, even after the treatment with the tumor promoter phenobarbital (Santoni-Rugiu et al. 1996b). Similarly, co-expression of HGF inhibited tumor formation in Tgf- α transgenic mice (Shiota et al. 1995). On the other hand, hepatic overexpression of the wild-type c-Met trasgene led to HCC development (Wang et al. 2001). Interestingly, these mice often harbored β -catenin gene mutations, suggesting that activation of the Wnt pathway may promote the oncogenic potential of c-Met. In accordance with this hypothesis, co-transfection of human c-Met and mutated β -catenin caused a development of large HCC in mice (Tward et al. 2007). In contrast to the latter findings, loss of c-Met signaling has been recently reported to enhance rather than suppress the early stages of chemical hepatocarcinogenesis (Takami et al. 2007). The authors suggested that increased oxidative stressed caused by c-Met deletion was responsible for acceleration of hepatocarcinogenesis in this model (Takami et al. 2007).

4.12 Nuclear Factor κ B (NF- κ B) and Inflammation-Associated Models

In the vast majority of patients, HCC develop in a context of chronically inflamed liver caused by a variety of viral, toxic, or metabolic factors (Thorgeirsson and Grisham 2002; Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007). Following liver injury, compensatory proliferation is driven by hepatomitogens (e.g., TNF- α , IL-6, and HGF) secreted by the surrounding non-parenchymal cells (Lee et al. 1998; Amaro et al. 1999). Continuous rounds of cell death and compensatory proliferation are thought to induce permanent genomic alterations in hepatocytes, ultimately leading to HCC development (Bruix et al. 2004; Farazi and DePinho 2006; El-Serag and Rudolph 2007). It has been hypothesized that the NF- κ B pathway contributes to HCC development by allowing the survival of altered hepatocytes in the chronically diseased liver (Arsura and Cavin 2005; Naugler et al. 2007; Sun and Karin 2008). The classical NF- κ B is a dimer composed of a p50 (NF κ B1) and p65 (RelA) subunit (Karin et al. 2002). In addition, other Rel-related subunits have been identified, including c-Rel, RelB, and p52 (NF- κ B2). In non-stimulated hepatocytes, NF- κ B is sequestered in the cytoplasm by a family of the specific inhibitory proteins termed inhibitors of κ B (I κ Bs). I κ B- α , a member of this family, has been implicated in the regulation of NF- κ B activity during oncogenic transformation of liver cells (Arsura and Cavin 2005; Naugler et al. 2007; Sun and Karin 2008). In response to viral infection, DNA damage, and pro-inflammatory cytokines, the IKK complex, which is comprised of two catalytic subunits, IKK- α (IKK-1) and IKK- β (IKK-2) and two scaffold components termed IKK- γ and ELKS, promotes NF- κ B activation through phosphorylation-induced ubiquitination of I κ B- α , thereby targeting this molecule for proteolysis. Once activated, the NF- κ B

pathway exerts its multiple effects on hepatocytes (proliferation, survival, etc.). Different studies in genetically modified animals have indicated that IkappaB kinase beta (IKK β) links chronic inflammation with liver carcinogenesis. Mice lacking IKK β only in hepatocytes exhibited a marked increase in HCC occurrence following treatment with DEN (Maeda et al. 2005; Sakurai et al. 2006). This correlated with enhanced reactive oxygen species production, increased c-Jun N-terminal kinase (JNK) activation, and hepatocyte death, which triggered a sustained compensatory proliferation of surviving hepatocytes. On the other hand, decreased hepatocarcinogenesis was detected when IKK- β was depleted both in hepatocytes and Kupffer cells, resulting in the abrogation of the inflammatory processes (Maeda et al. 2005). Similarly, liver-specific knockout of IKK- γ /NEMO (NEMOL-KO) resulted in the spontaneous development of HCC in mice (Luedde et al. 2007). Tumor development was preceded by chronic liver disease resembling human non-alcoholic steatohepatitis (NASH). Spontaneous NASH was characterized by secretion of inflammatory cytokines from Kupffer cells and spontaneous apoptosis of hepatocytes, resulting in compensatory hepatocyte proliferation (Luedde et al. 2007). The pro-tumorigenic role of NF- κ B cascade has been substantiated in another recent mouse model, in which liver-specific expression of either lymphotoxin (LT) α or β cytokines induced liver inflammation and HCC (Haybaeck et al. 2009). Since LT α and β and their receptor (LT β R) are upregulated in HBV- or HCV-induced hepatitis and HCC, these findings causally link the hepatic LT overexpression to hepatitis and HCC (Haybaeck et al. 2009). Importantly, the development of HCC depended on lymphocytes and IKK β expressed by hepatocytes, further underlining the pivotal role of NF- κ B pathway in hepatocarcinogenesis (Haybaeck et al. 2009). Taken together, the present results indicate that the NF- κ B pathway might either promote or suppress hepatocarcinogenesis. Further studies are required to clarify this issue.

A relevant aspect of HCC is the gender difference in HCC incidence. Men are about three to five times more likely to develop HCC than women. To analyze the gender disparity, the interleukin-6 knockout (IL-6 KO) mice have been used (Cressman et al. 1996). IL-6 is a multifunctional cytokine involved in the hepatic response to systemic inflammation, and IL-6 concentrations are increased in patients with viral hepatitis and HCC (Soresi et al. 2006; Ogata et al. 2006). IL-6 is necessary for compensatory regeneration in chemical models of hepatocarcinogenesis and deletion of SOCS3, a negative regulator of IL-6-related cytokines, enhances hepatitis-induced or chemically induced hepatocarcinogenesis (Ogata et al. 2006). Recently, it has been shown that ablation of IL-6 in IL-6 KO mice abolished the gender differences in a model of DEN-induced hepatocarcinogenesis (Naugler et al. 2007). In this model, estrogens inhibited IL-6 promoter activity by decreasing expression of NF- κ B and C/EBP β transcription factors, a process dependent on IKK β and the toll-like receptor adaptor Myd-88. The latter was found to be required for IL-6 induction by necrotic hepatocytes, and Myd-88 knockout (Myd-88 KO) male mice developed fewer and smaller HCCs in response to DEN injury than wild-type male mice (Naugler et al. 2007). The results of this important work provide a potential explanation for the gender differences in the incidence of liver cancer in humans and rodents.

4.13 *c-Myc and Tgf-Alpha*

Co-expression of c-Myc and TGF- α has been frequently detected in human HCC (Feo et al. 2000; Thorgeirsson and Grisham 2002). Hepatic expression of the c-Myc transgene resulted in chronic proliferation and increased incidence of HCC, whereas co-expression of c-Myc and Tgf- α transgenes synergistically enhanced neoplastic development when compared with the parental lines (Murakami et al. 1993; Santoni-Rugiu et al. 1996a, 1998; Calvisi and Thorgeirsson 2005). Subsequent work identified increased ROS production, involving iNos and NADPH oxidase systems, and chromosomal instability as the mechanism underlying accelerated oncogenesis in c-Myc/Tgf- α over-expressing livers (Factor et al. 1998, 2000; Calvisi et al. 2004). Accordingly, vitamin E, a potent free radical scavenging antioxidant, reduced liver dysplasia, prevented malignant transformation, and increased chromosomal and mitochondrial DNA stability in c-myc/Tgf- α transgenic livers (Factor et al. 2000). A similar anti-neoplastic effect was obtained when the double-transgenic mice were treated with aminoguanidine, an iNOS inhibitor, further substantiating the pathogenetic role of the redox imbalance in c-Myc/Tgf- α -driven hepatocarcinogenesis (Calvisi et al. 2008).

Rapid HCC growth was accompanied by inhibition of apoptosis and higher rate of cellular proliferation in c-Myc/Tgf- α mice when compared with c-Myc tumors (Santoni-Rugiu et al. 1998). Accordingly, it has been shown that NF- κ B-induced survival signaling was activated only in preneoplastic and neoplastic lesions from c-Myc/Tgf- α but not c-Myc mice (Arsura and Cavin 2005). C-Myc/Tgf- α HCCs also showed a strong induction of cyclin D1, pRb hyperphosphorylation, and upregulation of E2F1 target genes involved in cell cycle progression, including endogenous c-myc, cyclin A, and Cdc2 (Santoni-Rugiu et al. 1998). Interestingly, β -catenin activation was relatively frequent in c-Myc liver tumors, but very rare in more aggressive c-Myc/Tgf- α HCCs with high genomic instability (Calvisi et al. 2004). These data are consistent with the observation that β -catenin activation occurs in a subset of human HCCs with a relatively stable genome and a more favorable prognosis (Legoix et al. 1999; Hsu et al. 2000; Laurent-Puig et al. 2001). Thus, the c-Myc and c-Myc/Tgf- α transgenic mouse models reveal a remarkable similarity with development of human HCC.

4.14 *C-Myc and E2f1*

Deregulation of c-Myc and/or E2F1 protooncogenes is implicated in the development of numerous rodent and human tumors, including HCC (Murakami et al. 1993; Conner et al. 2000). The importance of c-Myc and E2F1 in carcinogenesis is underscored by their ability to induce both cell proliferation and cell death. Transgenic overexpression of either c-Myc or E2F1 in the liver was sufficient to induce tumor growth, albeit with different latencies, whereas their combined activity significantly increased both the rate of tumor development and malignant conversion (Conner

et al. 2003). Different from c-Myc/Tgf- α transgenic mice, c-Myc/E2F1 driven hepatocarcinogenesis was not associated with genomic instability (Calvisi et al. 2004). Furthermore, despite a strong induction of the Wnt/ β -catenin pathway (Calvisi et al. 2004), co-expression with E2F1 did not increase proliferation triggered by c-Myc overexpression, but instead conferred a strong resistance to c-Myc-induced apoptosis via concomitant induction of PIK3CA/Akt/mTOR and c-Myb/COX-2 survival pathways (Ladu et al. 2008). In human HCC, PIK3CA/Akt/mTOR and c-Myb/COX-2 pathways are similarly activated, with levels of PIK3CA/Akt, mTOR, and c-Myb being inversely associated with patients' survival length (Ladu et al. 2008). Thus, E2F1 may function as a critical anti-apoptotic factor both in human and in mouse liver cancer through its ability to counteract c-Myc-driven apoptosis via activation of PIK3CA/Akt/mTOR and c-Myb/COX-2 pathways. Combined activity of c-Myc and E2F1 also increased expression of genes associated with mitochondrial respiration (Coulouarn et al. 2006). Increased energy production may be an important factor contributing to the rapid tumor growth in c-Myc/E2F1 transgenic mice.

5 Other Models

Although most mouse models of HCC express growth factors and oncogenes under liver-specific promoters, hepatic expression is not an absolute requirement for development of HCC as exemplified by a transgenic model over-expressing fibroblast growth factor 19 (FGF19) in skeletal muscle (Nicholes et al. 2002). In this model, FGF19 induction led to HCC development in mice at 10–12 months of age, with predominance in the females. FGF19 oncogenic potential was associated with its ability to bind the fibroblast growth factor receptor 4 expressed by hepatocytes, thus triggering unrestrained proliferation. Furthermore, nuclear accumulation of β -catenin due to somatic mutations was detected in most HCC, implying activation of the Wnt/ β -catenin signaling pathway as one of the potential driver mechanisms for FGF19-driven hepatocarcinogenesis (Nicholes et al. 2002).

S-adenosylmethionine (SAME) is the major methyl donor and a key metabolite regulating hepatocyte growth, death, and differentiation (Mato and Lu 2007). SAME biosynthesis occurs in all mammalian cells as the first step in methionine catabolism in a reaction catalyzed by methionine adenosyltransferase (MAT). SAME synthesis and utilization occur mainly in the liver via MAT1/III and glycine *N*-methyltransferase (GNMT), the main enzymes responsible for the synthesis and removal of hepatic SAME, respectively (Mato and Lu 2007). Decreased hepatic SAME biosynthesis is a consequence of all forms of chronic liver injury; low levels of SAME are a typical feature of human liver cancer (Cai et al. 1996; Avila et al. 2000). To better understand the role of deregulated methionine metabolism in the generation of liver damage and eventually malignant transformation, the Mat1a (encoding the adult hepatic methionine adenosyltransferase) knockout model has been generated (Lu et al. 2001). Importantly, suppression of Mat1 resulted in

chronic hepatic SAMe deficiency which predisposed to steatohepatitis and HCC (Lu et al. 2001). At the molecular level, liver damage and tumor development seem to be the consequence of increased oxidative stress, upregulation of cytochrome P450 2E1, downregulation of prohibitin 1 (a mitochondrial chaperon protein), and impaired mitochondrial function (Martínez-Chantar et al. 2002). On the other hand, impaired SAMe metabolism, which occurs in patients with mutations of the glycine *N*-methyltransferase (GNMT) gene, can also lead to liver injury (Martinez-Chantar et al. 2008). The role of GNMT disruption in the liver was investigated through the generation of two distinct GNMT knockout (GNMT-KO) mouse models. GNMT KO mice, in which the mouse GNMT exon1 was disrupted, exhibited elevated serum aminotransferase, methionine, and SAMe levels and developed liver steatosis, fibrosis, and HCC (Martinez-Chantar et al. 2008). At the molecular levels, GNMT KO livers displayed activation of Ras and Jak/Stat pathways coincidentally with the suppression of the Ras inhibitors, Ras-association domain family/tumor suppressor (RASSF) 1 and 4, and the Jak/Stat inhibitors SOCS 1-3 and cytokine-inducible SH2-protein. Furthermore, methylation of RASSF1 and SOCS2 promoters and binding of trimethylated lysine 27 in histone 3 to these two genes were increased in HCC from GNMT KO mice, indicating that GNMT loss induced aberrant methylation of DNA and histones resulting in epigenetic modulation of critical carcinogenic pathways. In a second mouse model, the knockout of the 1-5 exons in GNMT gene also resulted in HCC development albeit with different molecular mechanisms (Liu et al. 2007). Differently from the first GNMT KO model, HCCs were more frequently detected in female than male mice. Also, GNMT suppression led to unrestrained activation of the canonical Wnt signaling pathway and global DNA hypomethylation rather than hypermethylation and aberrant expression of DNA methyltransferases 1 and 3b both at the early and late stages of HCC development (Liu et al. 2007). The reasons for the discrepancies between the two GNMT KO models are not clear and require additional investigations. Nevertheless, the data demonstrate that both deficiency and excess of SAMe can cause liver damage and presumably HCC underscoring the importance of maintaining the adequate levels of hepatic SAMe.

6 Evaluating Cooperation of Multiple Oncogenic Events in Liver Cancer by Hydrodynamic Gene Delivery

Hydrodynamic gene delivery was developed using the advances in understanding the structure and properties of blood capillaries (Budker et al. 1998; Liu et al. 1999; Zhang et al. 1999; Suda and Liu 2007). The main reason why parenchymal cells are targeted by hydrodynamic delivery is due to the fact that capillary endothelium and parenchymal cells are closely associated, allowing for the immediate access of DNA to cells once the endothelial barrier is disrupted. Hydrodynamic gene delivery uses a hydrodynamic force produced by a pressurized injection of a large volume

of DNA solution into the blood vessel that permeabilizes the capillary endothelium and generates “pores” in the plasma membrane of the surrounding parenchymal cells, through which DNA may reach the intracellular compartment. With time, the membrane pores close trapping the DNA inside the cell. The most successful application of the hydrodynamic technique is the gene delivery to hepatocytes in rodents (Budker et al. 1998; Liu et al. 1999; Zhang et al. 1999; Suda and Liu 2007). The standard procedure consists of a tail vein injection of physiological solution, equivalent to 8–10% body weight. The injection of such a large volume of DNA solution entering directly into the inferior vena cava stretches myocardial fibers over the optimal length for contraction, induces cardiac congestion, and drives the injected solution into the liver in retrograde.

Elegant experiments using this method in mice provided new insights into hepatocarcinogenesis (Tward et al. 2007; Lee et al. 2005; Patil et al. 2009). Several genetic lesions commonly associated with human liver tumors were reproduced in mice to reconstruct genetic progression to hepatocellular carcinoma and adenoma. Carcinogenesis was initiated with transgenic overexpression of the protooncogene c-Met or by hydrodynamic transfection of c-Met in combination with other genes (Tward et al. 2007). HCC in both instances arose due to the cooperation between c-Met and constitutively active forms of β -catenin. Strikingly, inactivation of c-Met transgene triggered regression of HCC despite the persistence of activated β -catenin. HCC eventually recurred in the absence of c-Met expression, presumably due to the occurrence of the additional genetic or epigenetic events in cooperation with activated β -catenin. Since Cyclin D1 (CCND1) is considered a crucial downstream target of the Wnt/ β -catenin pathway, its importance as a mediator of c-Met- and β -catenin-induced hepatocarcinogenesis was investigated in subsequent experiments (Patil et al. 2009). When CCND1 was co-expressed with c-Met in mice, it cooperated with c-Met to promote HCC development. Of note, tumors induced by CCND1/c-Met had a longer latency period, were formed at a lower frequency, and were histologically more benign compared with those induced by β -catenin/c-Met. In addition, when activated β -catenin and c-Met were co-injected into CCND1-null mice, HCC development was paradoxically accelerated by the absence of CCND1. In similar experiments, hepatocellular adenomas were produced by cooperation between c-Met and defective signaling through the transcription factor HNF1 α (Tward et al. 2007). Furthermore, using the same technical approach, the role of the putative tumor suppressor gene Sprouty 2 (Spry2), frequently inactivated in human HCC (Calvisi et al. 2007), was investigated by expressing dominant negative Spry2 (Spry2Y55F) and activated β -catenin (DeltaN90- β -catenin) in the mouse liver (Lee et al. 2005). In this model, Spry2Y55F cooperated with activated β -catenin to confer a neoplastic phenotype in mice, supporting the hypothesis that Spry2 may be a *bona fide* tumor suppressor in HCC (Lee et al. 2005). Taken together, these findings underline the usefulness of the hydrodynamic gene delivery method to investigate and reproduce in vivo the major oncogenic events characteristic of human HCC as well as to assess the functional consequences of interactions among distinct oncogenic pathways.

7 Comparative and Integrative Functional Genomics of HCC: Mouse–Human Comparison

To better define the similarities and differences at the molecular level between mouse and human hepatocarcinogenesis, genome-wide microarray analysis was performed on seven different mouse models, and the gene expression profiles obtained were compared with those of a large collection of human HCC (Lee et al. 2004). The results showed that the gene expression patterns in c-Myc, E2f1, and c-Myc/E2f1 tumors closely resembled those of human HCC characterized by better (longer) survival, whereas expression profiles of c-Myc/Tgf- α HCC were more similar to those of poor (shorter) survival group of human HCC (Lee et al. 2004). Thus, interspecies comparison of gene expression patterns might be extremely useful to identify the mouse models of liver cancer that most closely mimic the human HCC subtypes.

8 Conclusions

Experimental models of liver cancer have significantly increased our knowledge on human hepatocarcinogenesis. In particular, the generation of a wide range of transgenic and knockout mouse models allowed to investigate the multiple, cumulating genetic, and epigenetic events associated with the sequential steps of malignant transformation, to assess tumor–host interactions, signaling pathways crosstalks, and the gender disparity. The generation of conditional mouse models and use of the hydrodynamic gene delivery technique offered a new opportunity to surmount the major limitations of the animal models, such as the ubiquitous expression of the transgene in hepatocytes (and in the tumor microenvironment) and the presence of genetic alterations during embryogenesis, which might either activate compensatory pathways or be detrimental for mouse survival. Although the generation of a “perfect mouse model” seems unlikely given the molecular and clinical heterogeneity of human HCC, we strongly believe that expansion of mouse models mimicking the different subsets of human HCC is feasible and may represent a powerful tool for understanding HCC pathogenesis and developing novel effective treatments.

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Chapter 10

Mosaic Cancer Mouse Models and Functional Oncogenomics in Hepatocellular Carcinoma

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Abstract Cancer gene discovery based on genomic approaches alone remains a tedious process as genomic instability and heterogeneity of human tumors impede a straightforward cataloging of cancer genes and possible therapeutic targets. Based on the development of new, innovative liver cancer mouse models, a series of recent papers described “Cross species oncogenomic comparison” and “Oncogenomics based in vivo RNAi screening” as powerful algorithms to identify new cancer genes in hepatocellular carcinoma. In the following chapter, we will discuss these functional genomic approaches, which hold the great promise to speed up the process of cancer-gene discovery in HCC and should be considered to complement time-consuming and costly endeavors like the Cancer Genome Project.

Keywords In vivo RNAi screening · Functional oncogenomics · Mosaic liver cancer mouse models

1 Introduction

The completion of the human genome project has stimulated various new technologies to study cancer genomes. For example, high-throughput technologies like expression profiling, array-CGH, and genomic re-sequencing allow for cataloging virtually every altered cancer gene in clinically relevant tumors (Velculescu 2008). Copy number alterations of chromosomal regions can be identified by high-resolution array-based comparative genomic hybridization (CGH), whereas regions of chromosomal amplification mostly harbor oncogenes and deleted regions harbor tumor suppressor genes (Chin and Gray 2008). Also, high-throughput sequencing

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technologies (Wood et al. 2007; Greenman et al. 2007) are available for detection of somatic point mutations in human tumors. Conventional use of these technologies, i.e., cancer gene discovery based on genomic approaches alone, has led to the discovery of several new cancer genes, some of which have already been harnessed as therapeutic targets in clinical routine. However, such approaches are expensive and the pace of cancer gene discovery is slowed down by the fact that in human cancers “causal” genomic events are surrounded by bystander genomic alterations, which occur due to genomic instability but do not contribute to the tumor phenotype. Therefore, it remains a major challenge to filter out causal, tumor-driving alterations from “bystander genomic lesions.” Furthermore, it is difficult to pinpoint relevant tumor suppressors in large deletions, as some genes are haploinsufficient tumor suppressors and loss of even one allele can promote tumorigenesis (also without a corresponding mutation in the remaining wild-type allele).

In conclusion, stringent functional validation of candidate genes identified through conventional genomic/statistical approaches is mandatory before expensive drug development efforts can be justified. In this context, functional validation of cancer genes is often a cumbersome process, and it is not always obvious which assays will reveal the putative oncogenic activity of a previously uncharacterized gene. Cell-culture systems are often used for functional testing, but they do not recapitulate important factors like the tumor microenvironment, and so, do not survey all relevant gene activities. For characterization and functional validation of candidate cancer genes it has become gold standard to generate transgenic and knockout mice, and to study the respective genes in a relevant context in vivo (Van Dyke and Jacks 2002). GEMM also plays an important role in preclinical drug testing, as it is becoming more evident that subcutaneously grown tumors on immunodeficient mice (mostly derived from cultured tumor cell lines) may not predict the treatment responses of human tumors (Sharpless and Depinho 2006; Olive et al. 2009).

This chapter will discuss how the combination of powerful cancer mouse models and integrative oncogenomic approaches can be harnessed to pinpoint and validate new cancer genes in hepatocellular carcinoma. Furthermore we will discuss how genetic screens with short hairpin RNA libraries can be used to identify new tumor suppressor genes. The discussed approaches hold the great promise to significantly speed up the process of cancer gene discovery and validation.

2 Cancer Mouse Models for HCC

Conventional liver cancer mouse models are classical germline transgenic/knockout models or chemically induced hepatocellular carcinomas (Verna et al. 1996a,b; Shachaf et al. 2004; Wang et al. 2001; Harada et al. 2004; Sandgren et al. 1989; Murakami et al. 1993; Deane et al. 2001; Manickan et al. 2001; Jhappan et al. 1990). These models not only have yielded important insights into the molecular mechanisms of liver cancer development, but also have several disadvantages. For example, the expression of transgenes by tissue specific promoters does not target

all epithelial cells within the organ (stem-cell and non-stem-cell compartment) and thus may bias toward a cell of origin different from that occurring in the human disease. Also, expression of an oncogene or deletion of a tumor suppressor gene in all cells within a tissue can create field effects such that all cells display altered gene expression, again, a situation, which does not reflect the situation of spontaneous human hepatocarcinogenesis. Furthermore, it is cost- and time-consuming to engineer germline transgenic and knockout animals and generation of mice harboring complex compound mutant alleles involve complicated intercrossing strategies. Some lesions also result in embryonic lethality or induce developmental compensation in the resulting tissue, such that the consequences of the mutation may not reflect the acute activation or loss that occurs in human HCC. Taken together, a mouse model that allows to bypass most of these limitations would be extremely valuable.

Over the past 10 years, chimeric mouse models based on the genetic manipulation and retransplantation of hematopoietic stem cells have proven as efficient tools to gain a better understanding of mechanisms involved in tumor initiation, progression, and treatment response (Schmitt et al. 2002a,b; Hemann et al. 2005; Pear et al. 1998). Using such models, genetically defined tumors can be generated at a fraction of the time and cost required to produce comparable germline models. Cancers arising in these models are derived from stem and/or progenitor cells and therefore do more accurately reflect the evolution of corresponding human hematopoietic malignancies. Importantly, these models are produced by retransplantation of genetically altered cells into recipient mice, thereby producing genetic mosaics where the developing cancer cell is surrounded by otherwise normal counterparts. However, the probably biggest strength of chimeric/mosaic cancer mouse models is, however, the possibility to rapidly test cooperation between multiple genetic lesions. For example, employing a mosaic cancer mouse model, a candidate gene can be functionally tested in different genetic backgrounds *in vivo* within a few weeks, while the generation of standard germline transgenic mice with subsequent intercrossing would take more than a year. A schematic representation of a mosaic liver cancer mouse model is shown in Fig. 10.1.

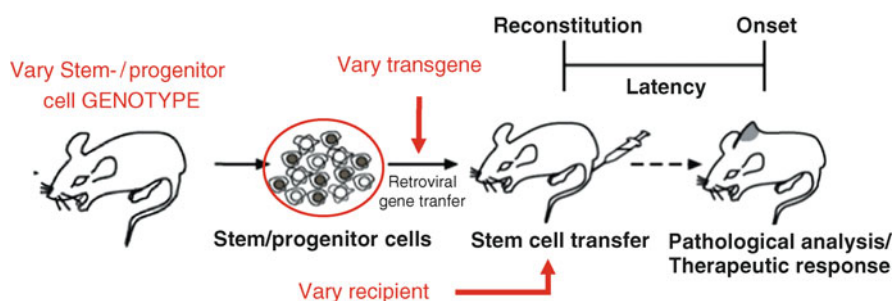


Fig. 10.1 Schematic representation of a progenitor cell-based cancer mouse model

3 Cross-Species Oncogenomic Comparison for Accelerated Cancer Gene Discovery

Genetically defined, cancer mouse models not only represent valuable tools for characterization and functional validation of new cancer genes, but also can be used to identify new cancer genes. Such approaches are based on the rationale that, if tumors in mouse cancer models are initiated based on a limited number of given starting genetic lesions, it should be feasible, using high-resolution genome scanning technologies, to uncover spontaneous, cooperating genetic alterations, which were spontaneously selected for during tumorigenesis. In the following paragraph we will discuss an example for such an approach, where a comparative oncogenomic approach was used to identify and validate two new oncogenes in liver cancer (Zender et al. 2006).

Human HCCs frequently harbor mutations in the p53 tumor suppressor. Therefore, murine HCCs were generated using a progenitor cell-derived mosaic HCC mouse model, where hepatoblasts from p53-deficient embryos were purified and retrovirally transduced with the oncogenes *c-myc*, *Akt*, or *H-RasV12*. Engraftment of these cells into the livers of conditioned recipient mice resulted in liver carcinomas. As discussed above, it was hypothesized that spontaneous genetic alterations may occur during tumorigenesis, which cooperate with given starting lesions. Therefore, a high-resolution array-CGH platform (representational oligonucleotide microarray analysis (ROMA; Lucito et al. 2003)) was used to scan the liver tumors for genome copy number changes that were selected for during liver tumor development. In Akt-induced tumors, no focal genomic alterations (<5 Mb) were found, while tumors derived from H-Ras-transduced hepatoblasts showed selection for a focal amplicon harboring the *c-myc* oncogene and, in another case, of *Rnf19*. While *Rnf19* has not been linked to tumorigenesis, *c-myc* alterations are common in human HCC. Interestingly, when mouse liver carcinomas initiated by the starting genetic lesion *c-myc*; *p53*^{-/-} were analyzed, a recurrent amplicon on mouse chromosome 9 was identified and strikingly the amplified genomic was found to be syntenic with a chromosomal region on human chromosome 11q22, a region frequently found deleted in human tumors (Zender et al. 2006). A cross-species comparison of the minimal region of overlap between amplicon positive mouse and syntenic human tumors limited the number of candidate oncogenes in the region, as, for example, a cluster of matrix metalloproteinases was not included in all 11q22-positive human tumors. Based on the assumption that a driver gene of an amplicon should be consistently overexpressed in amplicon positive tumors, RNA and protein expression levels for all remaining genes that overlapped in the murine 9qA1 and the human 11q22 amplicon were analyzed. Two genes, cIAP1, an inhibitor of apoptosis, and, Yap, a Src-interacting protein, qualified through consistent RNA and protein expression in murine as well as human tumors and were therefore followed-up by functional validation experiments. Combinations of retroviral vectors encoding *c-myc*, cIAP1, and Yap were used to infect p53^{-/-} hepatoblasts and subsequently these cell populations were transplanted on recipient mice to assess tumor growth. cIAP1 overexpression significantly accelerated tumor growth in the “*c-myc*; *p53*^{-/-}”

background, whereas no acceleration was detectable when cIAP1 was co-expressed with either H-Ras or Akt, thus suggesting that the oncogenicity of cIAP1 is context dependent. Yap is a transcriptional co-activator that has been shown to enhance the efficiency of Runx and TEAD/TEF transcription factors. Interestingly, it has been reported to potentiate apoptosis in some contexts. However, co-expression of both Yap and c-myc (unlike H-Ras) in p53-deficient hepatoblasts resulted in significantly accelerated tumor growth, thus validating *Yap* as an oncogene.

It is a central dogma in the oncogenomics field that a genomic amplification is selected for during tumor development because of one “driver gene” which is embedded therein. However, with Yap and cIAP1 two oncogenes in the murine 9qA1 and human 11q22 amplicon were identified and validated. Interestingly, co-expression of Yap and cIAP1 showed a strong synergism between Yap and cIAP1 in accelerating tumorigenesis. The fact that two genes, cIAP1 and Yap, which are embedded in a small genomic amplification, cooperate during tumorigenesis, represents an interesting finding and clearly underlines that a detailed analysis of candidate oncogenes is indispensable.

A big advantage of mosaic cancer mouse models is that they allow for the use of in vivo RNA interference (Zender et al. 2005). Thus, stable RNAi could be used to downregulate cIAP1 and Yap expression in murine tumors harboring the 9qA1 amplicon. Knockdown of both genes led to a significant deceleration of tumor growth, which clearly illustrates the potential of these genes as therapeutic targets and increases confidence for initiating therapeutic development efforts.

In addition to amplified oncogenes, therapeutic development efforts also have to incorporate altered tumor suppressor genes. While amplified oncogenes can be direct targets for small molecule or antibody inhibitors, mutated tumor suppressor genes often pinpoint other targets in the same pathway (e.g., PTEN→PIK3CA or Arf→MDM2), which then can be targeted. While it is obvious to use the above-discussed strategy of “cross species oncogenomic comparison” also for identification of new tumor suppressor genes, in practice this approach is less effective. One reason is that oncogenomic profiles of mouse tumors harbor significantly less focal genomic deletions than amplifications (Zender, Powers and Lowe, unpublished results; Zender et al. 2006; Kim et al. 2006; Maser et al. 2007)). In the next paragraph we will discuss “Oncogenomics based in vivo RNAi screening” as a new strategy to identify new tumor suppressor genes in HCC.

Over the past couple of years, genome wide collections of siRNAs and shRNAs and shRNAmir were made available and have been successfully used for the identification of unbiased and comprehensive collections of new gene targets in different biological systems (e.g., Whitehurst et al. 2007; Berns et al. 2004; Silva et al. 2008). As discussed above, mosaic cancer mouse models allow for the use of in vivo RNA interference technology, thus opening the possibility to perform unbiased RNAi-based functional genomic screens in vivo. Our laboratory recently combined oncogenomic profiling of human tumors with in vivo RNAi screening (Zender et al. 2008). First, a focused shRNAmir library was generated by compiling shRNAs against all genes that were found embedded in focal deletions (<5 Mb) of 98 human HCCs. A total of 362 candidate tumor suppressor

genes were identified, with 301 of them having murine orthologs. All available shRNAs (631) targeting these 301 genes were obtained from the Codex shRNA library (<http://codex.cshl.edu/scripts/newmain.pl>) and these hairpins were screened in the above-described mosaic liver cancer mouse model for their ability to accelerate tumorigenicity of immortalized liver progenitor cells. Owing to the high frequency of p53 mutations and c-myc overexpression in human HCC, the screen was performed in a c-myc; p53^{-/-} background. The integrative approach was highly efficient, resulting in the identification and functional validation of 13 new tumor suppressor genes. The top-scoring candidate tumor suppressor identified in the screen, exportin 4, is a nuclear transporter belonging to the importin- β superfamily and mediates the nuclear export of SMAD3, EIF5A1, and EIF5A2. Loss of XPO4 seems to be protumorigenic by promoting the nuclear accumulation of its key substrates. XPO4 deletions are relatively common in human tumors, thus suggesting that this may be an important mechanism of tumorigenesis. SMAD3 and EIF5A as the major Xpo4 substrates show activities or expression patterns consistent with a role in modulating tumorigenesis. For example, SMAD3 modulates the TGF- β pathway, which is context dependent either anti- or pro-oncogenic. Although it remains to be determined in more detail, to which extent SMAD3 mislocalization contributes to the oncogenic effects of XPO4 loss, it was observed that suppression of XPO4 stimulates TGF- β signaling (Zender et al. 2008), which can promote invasion and metastasis in late stage liver cancer (Teufel et al. 2007).

EIF5A2 has been discussed as a candidate oncogene for many years as its overexpression was found in many human cancers (Clement et al. 2006) and it could be demonstrated that XPO4 loss enhances proliferation through EIF5A2, which is itself oncogenic in mice (Zender et al. 2008). While the precise biochemical mechanism remains to be characterized, the available mouse genetic data strongly suggest a high relevance of XPO4-EIF5A2 signaling circuit for HCC and other tumor types. Looking at all new tumor suppressor genes identified through the oncogenomics-based RNAi screen, it is noteworthy that most of these genes have never been linked to cancer before. Besides Xpo4, examples for other identified and validated genes are an FGF (FGF6), an RNA helicase (DDX20/ GEMIN3), a metabolic enzyme (GLO1), and GJD4 (CX40.1), a gap junction protein. Some of the genes, for example, FSTL5, NRSN2 (C20ORF98), and ZBBX (FLJ23049), are yet completely uncharacterized, however, based on the functional data obtained from the mouse model and based on the notion that all of them were found somatically altered in human cancer, these genes very likely represent important tumor suppressor genes in human HCC. Follow-up studies are necessary to characterize how each of these genes suppresses tumorigenesis, and, given the unexpected nature of each gene, such studies may uncover new pathways or principles relevant to human HCC.

In general, the described results establish the feasibility of integrative cross-species approaches and in vivo RNAi screens and illustrate how combining cancer genomics, RNAi and mosaic mouse models can facilitate the functional annotation of the cancer genome. Such integrative approaches may complement existing large-scale approaches like the human cancer genome project, which have been criticized regarding a potentially poor cost-benefit ratio (Elledge and Hannon 2005).

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Chapter 11

The Zebrafish Model for Liver Carcinogenesis

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Abstract The zebrafish (*Danio rerio*) has been increasingly recognized as a promising animal model for cancer research. Zebrafish tumors can be generated by treatment with chemical carcinogens or by genetic approaches. The liver has been a main target organ for tumorigenesis after carcinogen treatment while many other tissue-specific tumors have been generated by tissue-specific expression of proven oncogenes. We have used both the chemical and transgenic approaches to generate liver tumors. By comparative analyses of transcriptome profiles between human liver tumors and carcinogen-induced zebrafish liver tumors, we have demonstrated a remarkable similarity in the molecular hallmarks during liver tumorigenesis between humans and zebrafish, thus validating the zebrafish model for human cancer studies. Recently, we have also generated stable transgenic zebrafish lines overexpressing the *c-Myc* and *kras*^{V12} in the liver using two different inducible gene expression systems. In both cases, we found that tumors can be reproducibly induced in the liver, and histopathological examination confirmed the production of liver neoplasia including hepatocellular carcinoma. Thus, we have successfully established transgenic zebrafish models for liver cancers and these models will be further characterized in order to understand the molecular and genetic mechanisms of liver carcinogenesis as well as for anti-cancer drug discovery.

Keywords Zebrafish · Hepatocellular carcinoma · Transgenic · Kras · c-Myc · Carcinogen

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1 Zebrafish as an Experimental Model

The zebrafish (*Danio rerio*) has been an excellent experimental model not only in developmental biology, but also in biomedical research. As early as the 1950s, the zebrafish has been used as a developmental and embryological model (Halstead et al. 1955). Several important observations were made in these early studies based on the unique combination of the optical clarity of the embryos/larvae and embryological manipulability. The pioneering works of Streisinger and colleagues in early 1980s on production of pure strains of zebrafish marks the beginning of the zebrafish age (Streisinger et al. 1981). Following this, works on large-scale forward genetic screens have been reported in the 1990s, making the zebrafish a mainstream model system in the understanding of vertebrate development as well as heritable diseases (Driever et al. 1996; Haffter et al. 1996). Now the zebrafish, among a few vertebrate models has its genome sequenced near completion (http://www.sanger.ac.uk/Projects/D_rerio/).

The zebrafish is small in size (3–4 cm long) and readily reproduces all the year round in laboratories with each mating pair to produce more than 200 embryos daily. Zebrafish embryos develop very fast, finishing gastrulation within 10 hours of fertilization and hatching out in 2 days with most organs well-formed. By only about 3 months after fertilization, zebrafish grow to sexual maturity and are capable of reproducing. These features, plus external development and optical clarity of zebrafish embryos, greatly facilitate visual analyses of early developmental processes. Zebrafish embryos are also permeable to many small molecules and thus potentially useful for drug screening. Due to the ease of maintenance and the availability of a large number of embryos, the zebrafish studies are cost-effective and applicable for high-throughput screening. All of these attributes make the zebrafish an excellent experimental model.

Over the last three decades, many experimental tools have been established in zebrafish. In addition to conventional gene expression assays, both forward and reverse genetic approaches have been developed for zebrafish studies. Now saturation mutant screens can be routinely performed after ENU (*N*-ethyl-*N*-nitrosourea) treatment. Other than the standard three-generation screening, smaller scale and quicker screens such as haploid and homozygous diploid screens, which can uncover recessive alleles in a single generation have also been developed (Patton and Zon 2001). Retrovirus-mediated insertional mutagenesis has been used for generation of a large number of genetics mutants with easily identifiable chromosome loci. In reverse genetics, transgenic approaches have been well-developed to express both reporter and function genes in a tissues-specific manner or under an inducible condition (Gong et al. 2001). The efficiency of transgenic technique has been dramatically improved by using transposon systems such as medaka *Tol2* and maize *Ac/Ds* (Kawakami et al. 2004; Emelyanov et al. 2006). Although the gene targeting technology, which has been popularly used in mouse genetics, has not been successful in zebrafish, an alternative approach called TILLING (Targeted Induced Local Lesion IN Genome) has been successfully used in zebrafish to produce targeted gene mutants (Wienholds et al. 2003). For functional analysis of a single gene, other than the transgenic approach, a morpholino antisense oligonucleotide-based knockdown approach has been popularly used to inhibit the translation of a specific

protein in early zebrafish embryonic development (Nasevicius and Ekker 2000). Conversely, embryos can also be microinjected with capped mRNA or plasmids to express specific proteins and variants.

2 Zebrafish Models for Human Diseases

The zebrafish has been increasingly employed to model human genetic disorders caused either by single gene mutations or multiple genetic defects. Many of these zebrafish mutants are phenotypically similar to corresponding human disease states with homologous genes mutated, ranging from hematopoietic, cardiovascular, musculoskeletal, and germ cell chromosomal disorders to behavior and aging-related diseases (North and Zon 2003; Lam and Gong 2009).

The validity of using zebrafish as a model system for human blood disorders has been illustrated by several blood mutants that phenocopy human conditions (North and Zon 2003). The *sauternes* (*sau*) mutant is the first zebrafish model reported for human congenital sideroblastic anemia caused by mutations in δ -aminolevulinate synthase (ALAS2), which is an erythroid-specific enzyme involved in regulating the first step of heme biosynthesis. In other examples, injection of morpholino against *urod* has been shown to lead to uroporphyrinogen decarboxylase deficiency and hepatoerythropoietic porphyria similar to those seen in humans (Nasevicius and Ekker 2000).

In addition, there are many zebrafish heart mutants which display similar phenotypes to human heart disorders, such as cardiac valve defects (*jeekyll*), cardiac hypertrophy (*liebeskummer*), and coarctation of the aorta (*gridlock*) (North and Zon 2003). Other zebrafish mutants include *sapje* for muscle degeneration and decreased motility observed in Duchenne muscular dystrophy, *b4galt7* and jellyfish for cartilage defects in musculoskeletal disorders, *satellite*, *mariner*, and *sputnik* for hearing impairment and deafness. A more completed list of zebrafish disease models of human disorders, including mutants having similar genes mutated in corresponding mammalian or human disease states, is presented in another book chapter in *Human Genetics—Principles and Approaches* (Lam and Gong 2009). With a combination of the phenotype and relevant knowledge of the mutated gene, these zebrafish models can help to understand the molecular basis of these pathologies and screen for potential drug candidates that can suppress the disease phenotypes.

3 Chemical Carcinogen-Induced Tumors in Zebrafish

It has been reported that teleost fish can develop a wide variety of benign and malignant tumors spontaneously in virtually all organs or in response to various water-borne chemical carcinogen exposures, and, as in mammals, tumorigenesis is a phenomenon of advancing age (Hawkins et al. 1985). Swordtails (*Xiphophorus* hybrids) have been shown to develop melanomas as a result of a mutation in a novel receptor tyrosine kinase *Xmrk* (Walter and Kazianis 2001), the medaka fish (*Oryzias latipes*) have been reported to develop hepatocellular carcinomas, melanomas, and lymphosarcomas (Masahito et al. 1989), and liver tumors have

been observed in lungfish (Masahito et al. 1986). In addition, the development of liver tumors in response to 7,12-dimethylbenz[a]anthracene (DMBA), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), or aflatoxin B1 exposures has been investigated in the rainbow trout (*Oncorhynchus mykiss*) (Wolf and Jackson 1963; Hendricks et al. 1980).

Actually, the use of zebrafish for carcinogenicity testing of chemicals and in toxicology long predates their use as a genetic model (Stanton 1965). The zebrafish has been also utilized in carcinogenesis studies to evaluate the risk from environmental hazards such as carcinogens in drinking water (Hawkins et al. 1985). Zebrafish inhabiting natural waters also serves as an important sentinel to indicate carcinogenic risks in the environment (Hawkins et al. 1995). Chemical carcinogenesis was actually the first approach used to induce tumor formation in the zebrafish (Stanton 1965). Carcinogen treatments of zebrafish give robust induction of cancer and are comparatively easy to perform. Water-soluble chemicals can be added directly into the water and lipophilic compounds can be fed or delivered using a carrier solvent. Experimental zebrafish can be exposed for short or extended time periods. A number of chemical compounds that are known to be carcinogenic in mammals have been found to induce tumor formation in the zebrafish (Spitsbergen et al. 2000a, 2000b; Spitsbergen and Kent 2003).

The earlier works of Spitsbergen and colleagues have shown that exposure of zebrafish to structurally diverse carcinogens can induce formation of a significant number of neoplasms, and that the specific types of neoplasms vary depending on the carcinogen used, age of fish at treatment, and genetic strain of the fish (Spitsbergen et al. 2000a, 2000b; Spitsbergen and Kent 2003). DMBA (7,12-dimethylbenz[a]anthracene) induces the broadest tumor spectrum among carcinogens studied extensively in zebrafish, including neoplasms in liver, intestine, pancreas, thyroid, testis, cartilage, blood vessels, muscles, connective and lymphoid tissues, and brain (Spitsbergen et al. 2000a), while MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) provokes neoplasms predominantly in the liver and testis (Spitsbergen et al. 2000b). Not surprisingly, the alkylating mutagen ENU, which introduces DNA point mutations and is commonly used in forward and reverse genetic screens, has also been reported in another study to induce a very high incidence of cutaneous papillomas in Florida wild-type zebrafish within 1 year following ENU treatment (Beckwith et al. 2000). More recently, similar studies treating 2.5-month-old AB line zebrafish with nitrosamine carcinogens showed that *N*-nitrosodiethylamine primarily induces liver and pancreas carcinomas detected grossly at necropsy and confirmed histologically, whereas *N*-nitrosodimethylamine induces only liver tumors (Mizgireuv et al. 2004; Mizgireuv and Revskoy 2006). Some of these carcinogenic treatments have been applied to mutants with a genetic predisposition to cancer but having a low spontaneous tumor incidence and demonstrated an increased susceptibility of mutants as compared with wild-type controls (Shepard et al. 2005, 2007; Haramis et al. 2006). Overall, the liver is a primary target organ and tumors in the liver are the most prominent neoplasms following treatment with most carcinogens in common exposure protocols, regardless of the developmental stage of the zebrafish at exposure (Table 11.1).

Table 11.1 Responsiveness of zebrafish to chemical carcinogens

Agent	Life stage at treatment	Neoplasm frequency	Primary target tissues	Secondary target tissues	Other targets
DMBA	Embryo	15%	Liver	None	Lymphomyeloid, brain, neural crest, skeletal muscle, thyroid
	Fry	65%	Liver, gill, Blood vessels	Intestine	
	Juvenile	28%	Intestine	Gill	
MNNG	Embryo	31%	Liver	Testis	Gill, ultimobranchial gland
	Fry	20%	Testis	Blood vessel	
	Juvenile	1%	None	None	
MAMA	Embryo	61%	Liver	Blood vessel	Heart, eye, brain, nerve sheath, testis, pancreas, fin
	Fry	39%	Liver	Testis	
	Juvenile	24%	Liver	Intestine	
DEN	Embryo	9%	Liver	None	Notochord, ultimobranchial gland, intestine, skin
	Fry	66%	Liver	None	
	Juvenile	1%	None	None	
AFB1	Embryo	8%	Liver	None	Intestine, bone
	Fry	11%	Liver	None	
	Juvenile	16%	Liver	Intestine	

Three treatment methods are commonly used at three different stages: embryo (60 hpf), fry (3 weeks post-fertilization, wpf), and juvenile (2 months post-fertilization, mpf). For all carcinogens, the liver is the major target organs for tumorigenesis. The spontaneous tumor rate is about 1% at 6–14 months old of age

The basic histological appearance of chemically induced tumors in zebrafish cancer has revealed significant similarity to those in human cancer, including increased cell proliferation, atypical nuclear morphology, and low degree of cell differentiation (Spitsbergen et al. 2000a, 2000b). Liver neoplasms in zebrafish as well as in rainbow trout differ from those of humans and other mammals in being more likely to be mixed carcinomas with biliary as well as hepatocellular components, of which such mixed hepatic epithelial neoplasms are rare in mammals (Bailey et al. 1996; Spitsbergen et al. 2000a). Mutant lines of zebrafish such as the Tupfel long fin (TL) line often show pancreatic or intestinal differentiation in more anaplastic biliary neoplasms induced by polycyclic aromatic hydrocarbons, another indication that zebrafish neoplasms are more pluripotent than those of mammals (Spitsbergen, unpublished).

4 Molecular Validation of the Zebrafish Cancer Model by Transcriptome Analysis

Previously, our laboratory has evaluated the molecular conservation between zebrafish and human liver tumors using a DNA microarray approach (Lam et al. 2006; Lam and Gong 2006). In this study, we demonstrated that chemically induced

liver tumors in zebrafish mimic the molecular expression patterns of human liver cancer and possess features that correlate with progressively higher grades of malignancy. The ten zebrafish liver tumors analyzed in this study exhibited a range of histopathological features indicative of neoplastic progression, which allowed a comparison of their gene expression profiles with those of human liver cancer classified according to clinical grade or stage.

A Zebrafish Liver Tumor Differentially Expressed Gene Set (ZLTDEGS) was obtained by statistically comparing gene expression profiles of zebrafish liver tumors with those of normal liver tissues. Ontology annotation of the human homologs using Unigene clusters revealed the deregulation of many genes involved in cell cycle control, apoptosis, DNA replication and repair, and metastasis. In particular, alterations in both the canonical Wnt and Ras signal transduction pathways have been noted, which are all well-characterized aberrancies and molecular hallmarks frequently deregulated in human liver cancer.

By comparing ZLTDEGS with gene lists ranked based on their statistical significance of four different human tumor type datasets (liver, gastric, prostate, and lung) using gene set enrichment analysis (Table 11.2), we have further confirmed that the zebrafish liver tumor signature is indeed correlated with the upper ranking and statistically more significant genes that are deregulated in human liver cancer. In contrast, comparison with other human tumors from the stomach, prostate, and lung does not demonstrate a high degree of similarity. Apart from the general hallmark of liver cancer, we have also found that zebrafish liver tumors possess

Table 11.2 Comparison of zebrafish liver tumors and several human tissue tumors based on microarray data

Human tumor type	Overlapping genes with ZLTDEGS	P _{Binomial}	P _{GSEA}
Liver	128	1.54E-14	7.30E-05
Gastric	55	1.30E-05	1.10E-02
Prostate	9	7.71E-04	8.29E-02
Lung	19	9.30E-06	9.70E-01

The table shows the number of overlapping genes between Zebrafish Liver Tumor Differentially Expressed Gene Set (ZLTDEGS mapped to 1,942 Hs ID – human unigene ID) and differential expressed genes in human liver (1,278 Hs ID), gastric (750 Hs ID), prostate (70 Hs ID), and lung (152 Hs ID) tumors microarray data sets. The Binomial test determines if ZLTDEGS is over-represented in the differentially expressed gene sets in human tumors. Smaller P_{Binomial} value indicates greater statistical significance of the over-representation of ZLTDEGS in human tumors. GSEA (Gene Set Enrichment Analysis) determines if ZLTDEGS correlates with the top differentially expressed genes [ranked based on false discovery rate (FDR) values] in the human tumor datasets. Smaller P_{GSEA} value indicates better correlation between ZLTDEGS and the upper-ranked (i.e., statistically more significant) genes in human tumor datasets. Only the intersection between ZLTDEGS and human liver tumor dataset is highly significant for both the P_{Binomial} and P_{GSEA} values, indicating that ZLTDEGS contains genes that are significantly over-represented and correlated with the top ranking genes in the human liver tumor. Although ZLTDEGS is over-represented with genes in human gastric, prostate, and lung tumor datasets, their correlation with the top ranking genes in these respective datasets were marginally or not significant. The original data is published in Lam et al. (2006)

common molecular features correlating with the morphologic progression of human liver tumors. Zebrafish liver tumors with a high component of anaplastic embryonal cells, which resemble high-grade tumors had expression profiles similar to human high-grade liver tumors. Both zebrafish and human advanced anaplastic tumors have higher expression of genes associated with cell cycle, cytoskeletal organization, metastasis, RNA processing, and protein synthesis. In contrast, the zebrafish tumor that consists primarily of hepatocellular adenoma and well-differentiated hepatocellular carcinoma, which resemble low-grade tumors, had expression profiles that are highly similar to human pre-cancerous nodules and low-grade liver tumors. These low-grade tumors are well-differentiated and therefore retain more of the liver-specific characteristics with higher expression of genes encoding liver-abundant proteins. Moreover, several of the homologs that are conserved in zebrafish and human liver tumors, such as *RAC*, *RHO*, *CDC42*, *HIF1*, *HSP70*, *HSP90*, *PCNA*, *p53*, *BAX*, and *STMN1*, have been previously reported with prognostic, diagnostic, and therapeutic values in humans, hence suggesting that such comparative oncogenomics approaches can help in identifying genes with clinical implications (Lam and Gong 2006). Additionally, we have identified a set of up-regulated genes, which are conserved between zebrafish and humans but are not yet well-characterized in liver cancer. Further study of this gene set may identify genes with potentially novel roles operating in human liver cancer.

Our findings help to establish confidence in the zebrafish as a liver cancer model system, at least in the context of carcinogen-induced liver tumors. Our findings also indicate that the current genomic technologies and tools are sufficiently robust to capture essential common molecular players with clinical implications in liver cancer. Hence, the study provides a framework for comparative genomics between fish and human or other mammalian models that can help to unravel strong common molecular associations underlying a disease phenotype such as liver cancer, whereby molecular conservation emphasizes the fundamental importance of molecular signatures and their potential in predicting clinical tumor behavior.

5 Genetic Approaches to Generate Tumor Models in Zebrafish

Although chemical carcinogenesis can be readily used to produce tumors in zebrafish at low cost, disadvantages of this approach include low incidences, non-specific histological types of tumors, late tumor onset, and that tumor growth under these conditions is spontaneous and heterogeneous in genetic profile and location. To directly assess the functional aspects of specific molecular alterations during tumor development in the zebrafish, several groups have turned to the generation of transgenic zebrafish to relate specific molecular alterations associated with corresponding tumorigenesis in humans.

The first transgenic tumor model in the zebrafish is the creation of a myc-induced T-cell lymphoid leukemia (Langenau et al. 2003). In this study, the zebrafish *rag2* promoter drives the transgenic expression of mouse *c-myc*, the classical dominant oncogene, in lymphoid cells. Zebrafish embryos injected with the oncogene construct at the one-cell stage develop T-cell acute lymphoblastic leukemia in as

early as 30 days post-injection in 6% of the transgenic zebrafish. The tumors first arise in the thymus and rapidly infiltrate multiple organs and tissues, eventually causing animal lethality. In fact, these zebrafish show cancer so rapidly that it precludes efficient maintenance of the zebrafish line with constitutive transgene expression. Subsequently, the researchers improved their transgenic tumor model by rendering conditional *c-myc* expression using the Cre-*loxP* system through a heat-shock promoter-controlled Cre recombinase gene (Langenau et al. 2005a, 2005b; Feng et al. 2007). Additionally, tumor cells transplanted into sub-lethally irradiated recipient zebrafish fully recapitulate the original cancer. Examination of molecular pathways activated in response to *c-myc* overexpression closely resembles the most common and aggressive subclass of human leukemia (Langenau et al. 2005a). In addition, zebrafish leukemia has also been generated by transgenic expression of three human oncogenes that are known to be involved in human leukemia, the first one is human *TEL-AML1* (ETV6-RUNX1) fusion gene (Sabaawy et al. 2006); the second is *NOTCH1*, in which activating mutations have been found in about 60% of leukemia patients (Chen et al. 2007); the third is *MYST3/NCOA2* fusion gene, a fusion of two histone acetyl-transferase genes due to chromosome translocation, leading to acute myeloid leukemia (Zhuravleva et al. 2008). By using these different genes, these studies have demonstrated the likely conserved mechanisms of producing leukemia in both fish and human, and thus established diversified zebrafish leukemia models.

In addition to leukemia, the transgenic approach has also been used successfully to generate several other types of tumors in zebrafish. These include pancreatic neuroendocrine tumors (Yang et al. 2004), melanoma (Patton et al. 2005), exocrine pancreatic cancer (Park et al. 2008), etc. In the study by Yang et al. (2004), human *MYCN* under the control of the zebrafish *myoD* promoter has been expressed in neural tissues and pancreas, as well as in the muscles in transgenic zebrafish and overexpression of *MYCN* results in the formation of pancreatic neuroendocrine tumors which originate from insulin-producing islet cells between 4 and 6 months of age.

In the zebrafish melanoma model, the activated human *BRAF*^{V600E} oncogene, which encodes a mutated serine/threonine kinase, was targeted to express in melanocytes using the melanocyte-specific *mitfa* promoter (Patton et al. 2005). When this transgene was injected into *p53* homozygous mutants, 7% of the zebrafish develop melanoma by 4 months of age, suggesting that the combination of mutated *BRAF* and *p53* makes the zebrafish more susceptible for the pathogenesis of these neoplasms. The melanoma is characterized by dense cellularity, high mitotic index, and local invasion. Overexpression of the *BRAF*^{V600E} oncogene leads to the formation of highly invasive melanomas that display molecular characteristics similar to those of the humans, including mitogen-activated protein kinase (MAPK) activation. In addition, the melanoma cells could be transplanted into irradiated transparent adult *casper* mutant zebrafish, causing tumor formation at a high rate (White et al. 2008). The authors could monitor tumor progression and invasion into distant tissues in the same zebrafish for up to 1 month after transplantation. Although *BRAF* has been previously identified as a melanoma-related oncogene (Shinozaki et al. 2004;

Wellbrock et al. 2004), this observed co-operation between *BRAF* and *p53* in the acceleration of melanoma formation provides a robust method to produce zebrafish melanoma.

Langenau et al. have described the zebrafish model of embryonal rhabdomyosarcoma (ERMS), a pediatric malignancy thought to arise from skeletal muscle (Langenau et al. 2007). Expression of the human activated *kRASG12D* gene under the *rag2* promoter leads to rapid development of ERMS in approximately 50% of mosaic transgenic zebrafish within 3 months of age, as characterized by the presence of multinucleated striated muscle fibers and a series of diagnostic markers. Developing tumors in the skeletal muscle tissues show a high degree of local and distant invasion with tumor cells being present in various distant organs such as the intestine, kidney, and testes (Langenau et al. 2007). Gene expression profiling has been used to confirm that the zebrafish tumors are consistent with those of human ERMS. Remarkably, the molecular oncogenic pathways activated in zebrafish ERMS shows a significant overlap with a subset of ERMS patients. The authors have also selectively labeled differentiated and progenitor tumor cells within the ERMS tumor cell mass and demonstrated that only the undifferentiated early muscle cell progenitors are capable of inducing tumor formation when transplanted into a recipient zebrafish. Interestingly, they are able to identify the cancer stem cells in zebrafish ERMS using this approach.

In another study, oncogenic *KRAS* is fused with GFP and overexpressed in pancreatic progenitor cells in living zebrafish embryos, leading to inhibition of exocrine differentiation in these cells and resulted in the formation of invasive pancreatic cancer in these transgenic zebrafish at 6 months of age (Park et al. 2008). The *KRAS*-induced zebrafish pancreatic cancer shows common histological features with human pancreatic cancer and, similar to the human disease, these tumors were characterized with abnormal activation of Hedgehog signaling (Park et al. 2008).

Other than generation of specific types of tumors in transgenic zebrafish, the transgenic approach has also been reported to produce a broad type of tumors. One report comes from the insertional mutagenesis studies. Amsterdam et al. (2004) have reported that many of the insertional mutants with disruption of ribosomal protein genes become susceptible to tumor formation, primarily with malignant peripheral nerve sheath tumors (MPNSTs). Although association of cancers with ribosomal genes in mammals is rare, the observation in zebrafish indicates the possible roles of ribosomal protein genes as haploidinsufficient tumor suppressors. Another report is from the studies of well-known tumor suppressor gene, *p53*, and some point mutations of this gene have been identified by TILLING (Berghmans et al. 2005). In one of the *p53* mutant lines spontaneous tumors have risen dramatically and that MPNSTs are also predominantly observed. A more recent study has indicated that in the ribosomal protein gene mutants, *p53* is not synthesized apparently due to insufficient ribosomal proteins, thus making the fish be prone to tumors (MacInnes et al. 2008). Similarly, several other tumors have been reported in two other tumor suppressor gene mutant strains: intestinal, hepatic, and pancreatic tumors in *apc* mutant (Haramis et al. 2006) and ocular tumor in *ptenb* mutant (Faucherre et al. 2008). In another study, Le et al. (2007) have employed Cre-loxP

system to activate ubiquitously expressed KRASG12D by heat-shock inducible Cre recombinase expression and they observed multiple types of tumors including ERM, MPNSTs, myeloproliferative disorder, and intestinal hyperplasia. In addition, forward genetic screen by ENU approach can also result in cancer susceptible lines and one such example is *bmyb* mutation which causes approximately twofold increase of tumor rate after carcinogen treatment compared to wild-type fish (Shepard et al. 2005). A recent elegant mutant screen for genomic instability have resulted several genetic strains with dramatic increase of spontaneous tumor rate in a variety of tissues including skin, colon, kidney, liver, pancreas, ovary, testis, and neuronal tissues (Moore et al. 2006).

Thus, the transgenic approach has succeeded to produce both targeted and untargeted tumor types, indicating the power of this technology to develop tumor models in zebrafish. Interestingly, in many of these cases, overexpression of a single oncogene under a tissue-specific promoter is sufficient to lead to histologically and molecularly validated tumors in transgenic zebrafish. These studies further establish the zebrafish as an easily amendable experimental model for cancer research. A particular advantage of using transgenic zebrafish to develop tumor models is the feasibility to express oncogene-GFP fusion genes and observe the progress of GFP-expressed tumor cells in externally developing and transparent zebrafish embryos. Thus, progression and metastasis of tumor cells can be monitored in real time and these GFP tumors can also be easily isolated to culture *in vitro* and transplanted to propagate *in vivo*. Furthermore, GFP-labeled endothelium transgenic line, *Tg(fli1:egfp)*, is available in zebrafish and it provides a powerful tool to image in real time the relationship of tumorigenesis and angiogenesis (Stoletov et al. 2007). Now the technical tools are clearly available in zebrafish to address a role of a particular gene in tumorigenesis.

6 Generation of Transgenic Zebrafish Liver Cancer Models

Although liver tumors have been the predominant type of tumors induced by different chemical carcinogens, the mechanism of tumorigenesis in these carcinogen-induced tumors could vary, be unpredictable and unfeasible for multi-generation investigation. So far, several transgenic tumor models in blood cells, pancreas, skin, and muscle have been reported in zebrafish, no liver tumor model have been reported. Moreover, most of the transgenic systems employs the constitutive transgenic expression of oncogenes in specific tissues and cells and thus it is difficult or impossible to maintain the transgenic lines. We are interested in development of transgenic zebrafish models for liver cancers to investigate molecular mechanisms of liver carcinogenesis as well as for potential future drug screens. Here we report our efforts on tests of two well-established oncogenes, *c-Myc* and *kras*, in development of liver cancer model in zebrafish by using two different inducible systems. Our preliminary data indicate that overexpression of either oncogene causes liver neoplasia and eventually carcinoma.

6.1 Overexpression of *c-Myc* oncogene in Zebrafish liver

The proto-oncogene *c-Myc* is a transcription factor which functions as a heterodimeric complex with the Myc associated factor X (MAX) to transcriptionally regulate hundreds of target genes, which are involved in many diverse programs including cell cycle, cell growth, protein translation, cell adhesion, metabolism, apoptosis, angiogenesis, and DNA repair (Oster et al. 2002). Although *c-Myc* has long been known to be one of the most frequently deregulated oncogenes in human cancer, the exact mechanisms leading to its aberrant activation remain undetermined. Increased expression of *c-Myc* has been detected both in experimentally induced hepatocellular carcinoma in rodents and in primary human liver tumors, confirming its involvement in liver tumorigenesis (Buendia 2000).

Many efforts have been previously made to investigate the roles of *c-Myc* in the initiation of tumorigenesis and the maintenance of different neoplastic phenotypes using mice as a model system (e.g., Jain et al. 2002; Shachaf et al. 2004). Besides promoting cell proliferation as frequently reported, *c-Myc* is also shown to induce apoptosis in the mouse endocrine pancreas, suggesting that *c-Myc* might have distinct functions in different tissues (Pelengaris et al. 2002). In addition to overexpression of *c-Myc* in conditional transgenic systems, other studies have also shown that targeted inactivation of *c-Myc* can act as a potential therapy for neoplasia (Soucek et al. 2008). Moreover, the *c-Myc* transcription signature is strongly associated with malignant conversion of pre-neoplastic lesions and thus may be used for diagnosis of early cancer (Kaposi-Novak et al. 2009). Transgenic model systems can therefore provide a platform to develop and evaluate new therapies targeting *c-Myc* in human cancers.

Our laboratory has established a transgenic zebrafish model by conditionally overexpressing mouse *c-Myc* in the zebrafish liver using the Tet-On system, in which a reverse tet transactivator (rtTA) was specifically expressed in the liver under the control of the liver-specific *lfabp* promoter (Her et al. 2003). When bound to the Tet response element (TRE) in the presence of doxycycline rtTA activates *c-Myc* gene expression (Fig. 11.1a). After 14 days of doxycycline treatment starting from 21 dpf, all of the transgenic zebrafish (100%) showed enlarged liver (Fig. 11.1c), whereas all similarly treated non-transgenic fish showed normal phenotype (Fig. 11.1b) and normal liver histology (Fig. 11.1d). Histological analysis of the transgenic fish suggested that the activation of *c-Myc* caused both liver hyperplasia (Fig. 11.1e) and hepatocellular adenoma (Fig. 11.1f). It seems that there is mainly hyperplasia of liver at the early stage, followed by development of hepatocellular adenoma when *c-Myc* was overexpressed for a longer time. Our findings have also shown that withdrawal of doxycycline could attenuate *c-Myc* expression and led to a recovery of the enlarged liver phenotype after several weeks (data not shown). These results demonstrated the reversibility of zebrafish liver tumors by reduction of *c-Myc* expression and showed that our *c-Myc* transgenic zebrafish could act as a useful *in vivo* model for liver cancer initiation and progression studies.

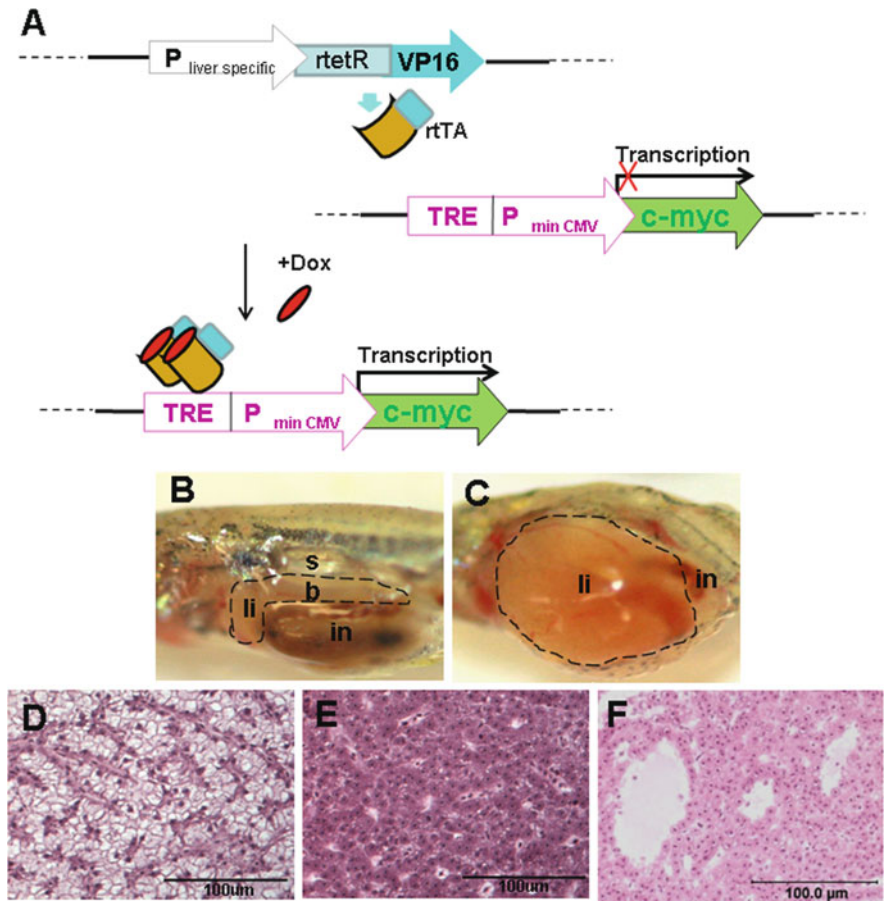


Fig. 11.1 Tet-On *c-Myc* transgenic zebrafish. (a) Schematic view of the Tet-On system to express mouse *c-Myc* under the liver-specific zebrafish *lfabp* promoter. The reverse tetracycline transactivator (rtTA), a fusion of reverse tetracycline repressor (rtetR) and VP16 activation domain, is expressed under control of the *lfabp* promoter. In the absence of inducer doxycycline (Dox), rtTA is unable to bind to the tetracycline responsive element (TRE) to activate the downstream *c-Myc*; while in the presence of Dox, rtTA binds to the TRE located in another transgenic construct and drives the *c-Myc* expression. (b) Gross morphology of normal liver (li) (surrounded by dashed line) and its surrounding intestine (in) and swimbladder (sb) in the 45 dpf (24 days post-treatment) Dox-treated non-transgenic sibling control zebrafish. (c) 45 dpf (24 dpt) transgenic zebrafish over-expressing mouse *c-Myc* showing massive liver tumor (region surrounded by dashed line). (d) A hematoxylin/eosin-stained paraffin section of a normal liver from a Dox-treated non-transgenic sibling control zebrafish (e and f) Histological observation of H&E-stained paraffin sections showing hepatocyte hyperplasia (e) and hepatocellular adenoma (f)

6.2 Overexpression of *kras*^{V12} Oncogene in Zebrafish Liver

RAS genes have long been at the leading edge of signal transduction and molecular oncology. Aberrant *RAS* signaling is common in human tumors and mutated *RAS*

has been implicated in approximately 30% of all human cancers (Schubbert et al. 2007). *KRAS* point mutations that result in constitutive activation most commonly occur in codons 12, 13, and 61, with the oncogenic mutant G12V (*KRAS*^{V12}) being more ubiquitously detected in most types of human tumors (Downward 2003). Approximately 7% of human liver cancers are attributed to activating mutation of this gene (Schubbert et al. 2007).

To test the potential of the constitutively active *kras*^{V12} to produce liver tumors in zebrafish, we developed a mifepristone-inducible Cre-loxP system to conditionally express the *EGFP-kras*^{V12} fusion gene in the liver. In this system, three distinct transgenic lines were generated: the first harbors a liver-specific *lfabp* promoter that drives the expression of a mifepristone-dependent LexPR chimeric activator gene (Emelyanov and Parinov 2006) consisting of bacterial LexA binding domain, ligand binding domain of human progesterone receptor, and human p65 activation domain; the second carries a *cre* recombinase gene under the control of a LexA operator – minimal promoter sequence that provides the binding site for the LexPR activator; and the third harbors a *lfabp:loxP-mCherry-loxP-EGFP-kras*^{V12} in which expression of *EGFP-kras*^{V12} oncogene in the liver can be induced after Cre-mediated excision of mCherry gene. In triple transgenic fish after several rounds of crosses, *EGFP-kras*^{V12} expression is induced only when mifepristone (RU-486) is added and the activation is first by binding of mifepristone to liver-specifically expressed LexPR activator, which in turn binds to the LexA operon to activate the transcription of *cre* gene that causes the excision of loxP-flanked sequence to lead to the transcription of the *EGFP-kras*^{V12} (Fig. 11.2a). In the absence of mifepristone, LexPR activator is not functional and thus neither Cre protein nor *EGFP-kras*^{V12} protein is produced (data not shown).

In the triple transgenic zebrafish, following mifepristone induction at 2 dpf (day post-fertilization), enlarged liver growth could be observed as early as 7 dpf (Fig. 11.2b) compared to normal liver morphology (Fig. 11.1c). By 85 dpf, 29% of induced transgenic fish, showed enlarged abdomen due to over-growth of the liver (Fig. 11.2e, f). Histological analysis confirmed the development of mild to moderate cystic degeneration in hepatocellular tissues and mixed hepatocellular carcinoma. An example of hepatocellular carcinoma is shown in Fig. 11.2h. We also observed an up-trend of hepatocellular carcinoma from adenoma when fish are aged.

We employed a cDNA microarray approach to analyze the gene expression profiles of liver tumor formation induced by the expression of the *EGFP-kras*^{V12} in transgenic zebrafish. Several *ras* oncogene family members and genes involved in the MAPK signaling pathway are shown to be significantly up-regulated. Preliminary results also indicated that most of the pathways involved in tumorigenesis, namely disruption of cell cycle regulation, deregulation of apoptotic pathways, loss of genomic integrity, and angiogenesis have been involved. This study of gene expression profile on a global scale may help to investigate molecular changes and elucidate mechanisms in *kras*^{V12}-induced liver tumor formation and may highlight signature pathways leading to oncogenesis.

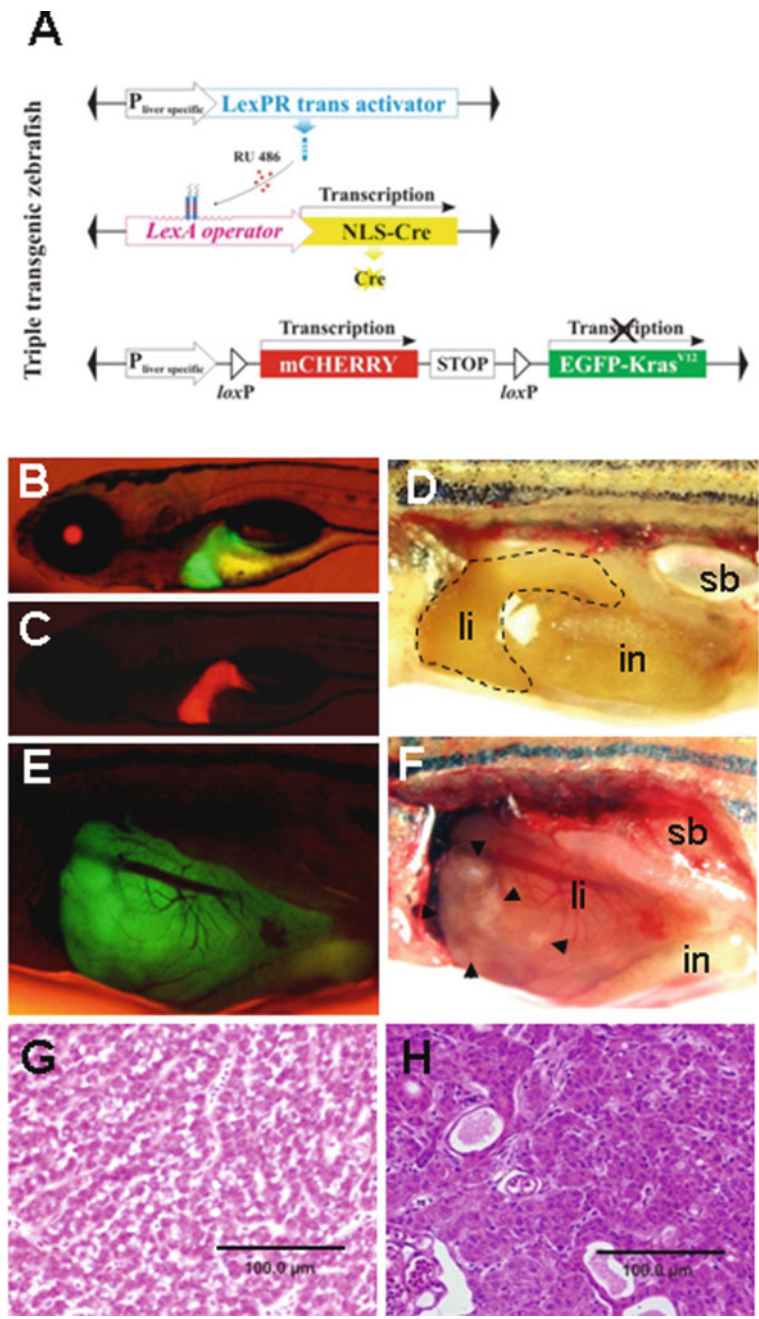


Fig. 11.2 Mifepristone-induced *EGFP-kras^{V12}* transgenic zebrafish. (a) Schematic view of mifepristone-inducible Cre-loxP transgenic system. In this system, three different transgenic lines are developed: (i) Activator line, LexPR activator gene under the liver-specific *lfabp* promoter;

6.3 Comparison of *c-Myc* and *kras*^{V12} Transgenic Zebrafish in Production of Liver Tumors

Both *kras*^{V12} and *c-Myc* have been used to produce tumors in transgenic zebrafish, but they are targeted to different tissues for different tumors. While *c-Myc* has been used to produce mainly leukemia (Langenau et al. 2003, 2005a), *kras*^{V12} has been demonstrated to be potent for production of a variety of tumors including ERM, myeloproliferative disorder, intestinal hyperplasia, MPNSTs, and malignant pancreas tumors. (Langenau et al. 2007; Le et al. 2007; Park et al. 2008). Thus, a direct comparison of the two oncogenes in producing the same tissue tumors is not available. In our studies, both *c-Myc* and *kras*^{V12} are expressed under the same liver-specific *lfabp* promoter to produce liver tumors and these two types of transgenic lines provide an opportunity to compare their potency and effects.

From our preliminary observations, we found that *c-myc* overexpression in the Tet-On system induced homogenous liver abnormalities in all of the transgenic fish. Hepatocyte hyperplasia (early stage) or hepatocellular adenoma (late stage) was observed in 100% of the transgenic fish expressing *c-myc*, and the uniform liver lesions occupied almost in the whole liver without the existence of normal liver tissue. The fish expressing *c-myc* can survive well for at least 3 months without obvious abnormalities other than enlarged liver and small body size. However, in our *kras*^{V12} transgenic fish, focal liver lesions were commonly observed inside the normal liver tissues and liver neoplasia only occupied partial liver tissue instead of the whole organ. These *kras*^{V12} transgenic fish developed malignant liver neoplasia from 2 months after oncogene induction and the existence of liver tumor caused higher mortality in transgenic fish. Therefore, it seems that mutant *kras*^{V12} oncogene is more potent than *c-myc* so that the transgenic fish expressing mutant *kras*^{V12}

Fig. 11.2 (continued) (ii) Cre line, Cre gene (containing nuclear localization signal) under the control *lexA* operator – minimal promoter sequence, and (iii) Effector line, *lfabp* promoter-driven *EGFP-kras*^{V12} fusion gene interrupted by *loxP*-flanked mCherry gene. In triple transgenic fish containing all three constructs obtained via cross breeding of the single transgenic lines, in the presence of inducer RU486 (mifepristone), LexPR activator produced in the liver is capable of binding to *LexA* operator to activate *Cre* expression, which in turn causes *loxP* recombination and excision the mCherry gene, which otherwise prevents expression of the downstream *EGFP-kras*^{V12}. Thus, in induction causes *EGFP-kras*^{V12} expression in the liver of the triple transgenic fish. (b) Green fluorescence image of enlarged liver in mifepristone-induced triple transgenic fry at 7 dpf. Mifepristone treatment was performed from 2 to 4 dpf. (c) Red fluorescent image from the *Tg(lfabp:RFP)* transgenic fry at 7 dpf (Korzh et al. 2008) showing normal liver morphology. (d) Gross morphology of normal liver in a dissected 3-month-old wild-type adult. The liver organ (li) is outlined and surrounded by the intestine (in) and swimbladder (sb). (e and f). Fluorescent and brightfield images of a 3-month-old triple transgenic zebrafish after mifepristone induction. The fish displays massive liver tumor (region surrounded by arrowheads) with EGFP fluorescence. The induction was performed at 30 dpf for 2 days. (g) A hematoxylin/eosin-stained paraffin section of a normal liver from a wild-type fish. (h) A section of an example of hepatocellular carcinoma derived from the mifepristone-induced triple transgenic fish

developed malignant liver tumor at earlier stage. This result is consistent with the previous report by Podsypanina et al. (2008), who have shown that mutant *kras*^{V12} can cause more severe tumor than *c-myc*.

However, the difference in the effects of these oncogenes are possibly caused at least in part due to the use of two different inducible systems. In the Tet-On *c-Myc* transgenic lines, *c-Myc* expression is inducer-dependent and the level of expression is also dosage-dependent; this feature resulted in uniform expression of *c-Myc* in the liver and caused homogenous change of hepatocytes. In contrast, the mifepristone-inducible *kras*^{V12} transgenic lines express oncogene constitutively after inducer-induction because of the Cre-loxP system and the level of *kras*^{V12} expression is relatively high. In most transgenic fish, the Cre-mediated excision does not occur in 100% hepatocytes, thus resulting in formation of tumor foci, which could more mimic the real carcinogenesis situation in human.

Experimentally, the mifepristone-induced Cre-loxP transgenic system requires only a short pulse treatment of inducer while the Tet-On system needs the continued presence of inducer (doxycycline) to maintain the transgene expression, which is laborious and inconvenient. On the other hand, withdrawal of doxycycline can attenuate *c-Myc* expression in the Tet-On system and leads to the reversal of the zebrafish liver tumor phenotype through extinction of *c-Myc* transgene expression. This phenotypic reversal cannot be observed in the mifepristone-inducible *kras*^{V12} transgenic lines. The Tet-On *c-Myc* transgenic lines is a simplex transgenic system with co-integrated activator and effector constructs while the mifepristone-inducible *kras*^{V12} lines start with three different transgenic lines, though the triple transgenic fish can be theoretically maintained by breeding. In addition, in our *kras*^{V12} transgenic lines, GFP-*kras*^{V12} fusion protein is expressed and the GFP fluorescence provides a convenient marker to monitor liver and tumor growth as well as potential metastasis.

7 Prospects

In the past few years, the zebrafish has shown great promise as an animal model system to study different types of cancers. So far, tremendous experimental tools have been established in zebrafish. With the genome resources completely available, genome wide and systematic investigation becomes possible to fully explore the potential of the new animal model in cancer research. In our studies, we have established two inducible systems to demonstrate the feasibility in zebrafish to induce liver carcinogenesis by overexpression of a single oncogene. Thus, the zebrafish may be developed into a rapid, economical, and high-throughput system to systematically test potential and proved oncogenes in liver carcinogenesis from both human and fish.

One area of research that is lagging in zebrafish is investigation of the sequential evolution of mutations in various genes, such as oncogenes, tumor suppressor genes, and other regulatory genes during the carcinogenesis process from initiation through promotion and progression. The issue of mutations in specific genes is not yet systematically addressed in the zebrafish and requires further investigation. In

rainbow trout, it appears that an early genetic change shared by liver neoplasms induced by several carcinogens is an activating mutation in the *kras* oncogene (Bailey et al. 1996). Unlike humans, in trout and medaka liver neoplasia, inactivating mutations in the tumor suppressor gene *p53* have not been detected, at least in tissues sampled relatively early in the disease (Bailey et al. 1996; Krause et al. 1997). To limit the cost of carcinogenesis studies, most fish in such studies have not been maintained for their entire lifespan. To better validate the zebrafish model for liver tumors, more studies on initiation, promotion and progression, alterations in sequence, copy number, and methylation patterns need to be conducted in the full range of oncogenes, tumor suppressor genes, and other regulatory genes influencing pathogenesis of fish liver neoplasia.

Genetic and chemical modifier screens are two major attractions of using the zebrafish model for cancer studies. The genetic modifier screen is to use a forward genetic approach to generate mutants that either enhance or suppress cancer phenotype while chemical screens to use small chemicals to inhibit carcinogenesis for new cancer drug discovery. Zebrafish embryos have already been demonstrated to be suitable for high-throughput chemical screen as a large number of embryos or fry can be generated, arrayed into multi-well plates, and continuously exposed to small volumes of different chemical compounds or mixtures of compounds within a relatively short timeframe (Peterson et al. 2000). This platform will greatly facilitate the screening process, which may lead to the identification of drugs that target pathways influencing carcinogenesis. The first attempt of such screen for chemicals to inhibit tumors has been reported in zebrafish and some compounds acting as suppressors of mitosis have been identified (Stern et al. 2005). Now with many specific types of tumor transgenic lines available, chemical screen can be focused on different tumor types, providing opportunities for discovery of new drugs for specific cancers.

Transcriptome analysis by DNA microarray technology has been a useful tool in understanding of molecular mechanisms of disease processes through multiple bioinformatic tools such as Gene Ontology and Ingenuity Pathway Analyses. Comparative transcriptome has been used as a means of comparing the molecular features of tumors among vertebrate models and humans (Lee et al. 2004; Sweet-Cordero et al. 2005; Lam et al. 2006). As published microarray expression data in cancer model animals and humans continue to grow, comparative functional oncogenomics approaches will be increasingly useful and important for comparing, validating, and identifying expression signatures that are strongly associated with a cancer phenotype. Moreover, the transcriptome analyses also provide a valuable means to investigate genomic response to chemical/drug treatments. A recent deep sequencing strategy using second-generation sequencers will revolutionize transcriptome analysis. The ease of technical handling procedures and the increasing number of reads at reduced costs provide an affordable alternative for higher resolution studies of differential gene expression analysis as compared to current microarray platforms ('t Hoen et al. 2008). Furthermore, the deep sequencing technology also provides new opportunities to search for mutations, deletion, amplification, and methylation in the genome of cancer cells.

Recent work has revealed the existence of microRNAs as short non-coding RNAs that regulate the expression of thousands of gene targets involved in a variety of important biological processes and are therefore likely to be master switches in many signaling pathways (Bartel 2004). MicroRNAs have recently been proposed to contribute to oncogenesis, as their regulatory roles make them strong candidate oncogenes and tumor suppressors. Indeed, many examples of microRNA deregulation have been reported in tumors, and microRNA profiling has been exploited to identify microRNAs that might target genes involved in cancer or represent effectors of activated oncogenic pathways (e.g., Lu et al. 2005; Esquela-Kerscher and Slack 2006). Simultaneous profiling of mRNA and microRNA expressions may thus be a useful strategy to identify functional microRNA target genes during oncogenesis.

Another interesting zebrafish application is tumor transplantation. In particular, the transparent zebrafish strain, *casper*, is available and has been used for transplantation of tumor cells as well as stem cells (White et al. 2008). With this transparent strain, GFP-labeled transplanted cells can be easily traced and re-isolated for new round of transplantation. Previously, Mizqireuv and Revskoy (2006) have performed serial transplantation of zebrafish tumors in homozygous diploid clonal lines and they are able to maintain numerous tumor lines through transplantation as many as 25 passages by the time of publication. The zebrafish also has the potential in xenotransplantation to examine human/mouse tumor cell biology, an approach has long been used by cancer biologists to investigate underlying mechanisms that drive cancer progression in vertebrates. Reports have shown that cancer cells derived from various human tissues can be successfully introduced into young zebrafish embryos and juvenile 30-day-old zebrafish (Lee et al. 2005; Stoletov et al. 2007). Recent findings with human glioblastoma cells xenotransplanted into zebrafish embryos have yielded important insights likely to improve treatment protocols for this tumor, which currently has grave prognosis (Geiger et al. 2008). The ability to successfully transplant cancer cells in the zebrafish might provide a means to develop promising preclinical drugs and determine their mechanisms of action.

While there is a large supply of human cancer cell lines, there are no zebrafish cancer cell lines permanently established in culture. As a result, significant effort needs to be put into developing zebrafish cancer cell lines harboring known oncogenes relevant to those in human cancer. This will further enhance functional cell-based and genetic-profiling studies. The zebrafish cancer cell lines will also be useful to determine if therapeutic strategies with anti-cancer drugs directed against human oncoproteins could impact zebrafish cancer in a similar manner. Given the attributes of the zebrafish, this vertebrate can be expected to provide new avenues for significant discoveries and to unravel novel insights into tumor biology and cancer drug development to improve cancer treatment in humans in the near future.

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Part VI
Global Gene Expression Profiling
of Human Liver Cancer

Chapter 12

Integrative and Functional Genomics of HCC

Cédric Coulouarn and Snorri S. Thorgeirsson

Abstract In this chapter, we review the impact of functional and integrative genomics on hepatocellular carcinoma (HCC) research over the last decade. We focus on how genome-wide and high-throughput technologies have been used successfully to classify HCC at a molecular level and became important tools to refine the diagnosis and prognosis predictions of HCC. We also describe how cross-species comparative oncogenomic emerged as a powerful strategy to improve HCC prognostication, as well as to highlight evolutionally conserved regulatory modules or pathways that may be critical in hepatocarcinogenesis. In order to achieve a complete understanding of the molecular mechanisms involved in the disease, the field is now shifting toward integrative genomics, which now combines multi-parametric data reflecting alterations at genomic, genetic and epigenetic levels. Undoubtedly, functional and integrative genomics promise to yield unprecedented biological insights into the pathogenesis of HCC and will ultimately converge toward a personalized medicine that will improve diagnosis, treatment and prevention of liver cancer.

Keywords Hepatocellular carcinoma · Gene expression profiling · Comparative-integrative functional genomics · System biology

Hepatocellular carcinoma (HCC) is one of the most common visceral neoplasms worldwide, with more than 550,000 new cases, which are diagnosed each year. With a mean survival of 6 months from the time of diagnosis and 600,000 deaths annually, HCC ranks among the deadliest forms of human malignancies worldwide (Parkin et al. 2005). Although being highly prevalent in Southeast Asia and sub-Saharan Africa, mostly due to the high rates of chronic viral hepatitis, the incidence of HCC has increased in the United States and Western Europe over the past 25 years. Since the incidence and mortality are expected to double over the next 10–20 years, HCC has become a major public health problem.

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Hepatocarcinogenesis in humans is a slow process that may take more than 30 years after a chronic hepatitis is first diagnosed (Thorgeirsson and Grisham 2002). During this long process, the accumulation of irreversible structural alterations in chromosomes and genes may eventually lead to the emergence and the expansion of monoclonal populations of transformed hepatocytes and the development of HCC. Accordingly, like other forms of human cancer, liver carcinogenesis is associated with important genomic changes, including genomic instability, aberrant methylation, and profound alterations in gene expression (Thorgeirsson and Grisham 2002). Over the last decade, the constant improvement microarray-based technologies have provided a great opportunity to assess the complexity of cellular and molecular events associated with HCC onset and progression. By monitoring the expression of virtually all-coding and non-coding genes, gene expression profiling of HCC tumors provided unbiased snapshots of cancer-associated gene alterations. Similarly, the application of high-throughput approaches provided new insights into the global epigenetic modifications and DNA copy number changes, which occur in HCC. Gene expression profiling in the context of HCC led to important discoveries. As example, microarray-based technologies opened a new window toward a molecular classification of tumors and several gene expression profiling studies identified new homogeneous subtypes of HCC with clinical relevance (Lee and Thorgeirsson 2004). By identifying cancer-associated genes and gene networks reflecting the dysregulation of specific signaling pathways, gene expression profiling emerged as a useful approach for diagnosis/prognosis prediction. Although the initial applications of large-scale gene expression profiling, commonly referred to functional genomics, were mainly focused on the identification of gene expression signatures characteristic for clinical parameters or tumor features, the field progressively shifted toward an integration of data generated from different “omic” platforms. By apprehending simultaneously the global changes of gene expression profiles, DNA copy number, and epigenetic modifications in the context of HCC, this combined approach, or integrative system genomics, is expected to improve our understanding of molecular mechanisms associated with liver cancer and help the identification of new therapeutic targets.

1 Functional Genomics of HCC: Microarray-Based Technologies, and Gene Expression Profiling

Numerous techniques are commonly used to measure the gene expression but the introduction of microarrays-based technologies revolutionized the study of complex biological systems such as cancer. The main advance offered by the high-throughput technologies compared to the other methods such as Northern Blot or Quantitative-Real-Time PCR is the opportunity to investigate simultaneously at the RNA level the qualitative and quantitative changes in the expression of virtually all genes in a given sample. The high resolution and sensibility of microarrays provide a global picture of the transcriptome that reflects the status of cells, tissues, or organs in particular

physiological or pathological conditions. Thus, when applied to different subsets of samples (such as well-defined experimental conditions or clinical samples) the so-called gene expression profiling strategy allows identifying gene expression signatures, which eventually reflect a specific phenotype, a biological process or a disease condition. Not surprisingly, the application of microarray-based technologies has been extensively applied in the field of cancer biology and has significantly enhanced our understanding of molecular basis of cancer, including HCC.

Since the initial report by Lau et al. (2000), studies of gene expression profiling in the context of HCC has exponentially flourished over the last decade and have greatly improved our knowledge of the molecular pathogenesis of hepatocarcinogenesis. As a complement to the original research reports, review articles have constantly updated and summarized the knowledge on hepatocarcinogenesis derived from microarray studies (Aravalli et al. 2008; Kim and Wang 2003; Lee and Thorgeirsson 2004; Lee and Thorgeirsson 2006; Lemmer et al. 2006; Suriawinata and Xu 2004; Teufel et al. 2007; Thorgeirsson et al. 2006b; Zhang and Ji 2005). By comparing the gene expression profiles of HCC nodules versus surrounding non-tumor tissues, several early studies identified specific subsets of genes, specifically up- or down-regulated in HCC. By using a cDNA microarrays allowing to access the expression of about 17,400 human genes, Chen et al. described a systematic characterization of gene expression patterns in human liver cancers (Chen et al. 2002). By including several tumor nodules from the same patients the authors elegantly described specific gene expression patterns from clonally independent tumor nodules in the same patient. Functional analysis of genes differentially expressed in HCC versus matched surrounding non-tumor tissues highlighted numerous functional modules altered in HCC. As example, early report by Okabe et al. (2001) indicated that activation of the MAPK pathway is a common feature of hepatocarcinogenesis. Increased expression of proliferation-associated genes also represents a common feature of cancer cells. Conversely, the majority of the down-regulated genes encoded hepatocyte-specific gene products and detoxification enzymes, reflecting a loss of differentiation in cancer cells (Okabe et al. 2001). DNA microarrays have also been successfully applied to identify specific gene expression signatures that correlate with clinically relevant parameters in HCC. This functional genomics approach led to a new classification of HCC at a molecular level (Boyault et al. 2007; Thorgeirsson et al. 2006b). By facilitating the discovery of tumor-specific and clinical feature-related molecular markers, high-throughput technologies became important tools for HCC diagnosis, prediction of prognosis, and response to treatment.

1.1 Molecular Classification of Liver Cancer

Somehow contrasting with other forms of human cancers, HCC exhibits a widespread clinical and pathological heterogeneity, including multiple etiological factors and degree of differentiation, as well as variable proliferation rate, metastasis

propensity, and sensitivity to chemotherapeutic agents. Even though clinical prognostic models have been developed, such as the Okuda staging system, the Cancer of the Liver Italian Program (CLIP), or the Barcelona Clinic Liver Cancer (BCLC), no general consensus has emerged for a reliable staging of patients with HCC. The actual staging systems of HCC are mainly based on clinical and pathological variables, which reflect the degree of alterations of liver functions or histological status of the tumors. Despite considerable efforts on rationalization, integrating single, or multiple clinical and pathological variables, the diagnosis of HCC as well as classification of HCC remains unreliable to accurately predict parameters such as patient survival, recurrence or response to therapy. As example, the prognostic factors which largely rely on the differentiation status of tumors and the severity of liver function defects do not take in account the underlying molecular alterations which may be responsible for the tumor heterogeneity and probably determinant for its progression. In this context, the molecular classification of HCC based on gene expression signatures represents a rational complement to the current staging systems. Accordingly, extensive studies have been performed over the last 10 years using high-throughput approach to identify gene expression signatures specific for several clinical and pathological parameters, including risk factors, differentiation (Yu et al. 2007), survival (Lee et al. 2004a), and recurrence (Iizuka et al. 2003).

1.2 Etiology-Associated Gene Expression Signatures

The majority of HCC cases developed on underlying chronic liver diseases. A variety of risk factors have been associated with HCC, including exposure to hepatitis viruses (mainly HBV, HCV), abusive alcohol intake, nonalcoholic fatty liver disease, aflatoxin B1, obesity, diabetes, or hemochromatosis. Microarray-based gene expression profiling has been extensively applied to investigate the pathogenetic mechanisms associated with chronic liver diseases. Although, most of the studies reported differential gene expression in the context of viral infections, gene signatures associated with nonalcoholic fatty liver disease, alcoholic disease, autoimmune hepatitis, and primary biliary cirrhosis have been also reported (Cheung et al. 2008; Rubio et al. 2007; Honda et al. 2005; Younossi et al. 2005; Derambure et al. 2008; Shackel et al. 2001).

In the context of the viral infections, microarrays studies clearly indicated that the molecular mechanisms responsible for the pathogenesis of HCC differ between the HBV and HCV viral infections (Honda et al. 2001). By using of a genome-wide cDNA microarray containing 23,040 genes, Okabe et al. (2001) established the gene expression profiles of 20 primary HCC, including 10 cases derived from HCV-infected livers and 10 cases positive for the hepatitis B surface antigen. A supervised approach identified a gene signature correlated with the infection status and type of hepatitis virus. The signature included key molecules for activating chemotherapeutic drugs or detoxifying xenobiotic carcinogens. Similarly, Iizuka et al. investigated the gene expression patterns of 45 HCC samples (14 HBV and 31 HCV) using high-density oligonucleotide microarrays (Iizuka et al. 2002). A

supervised learning method identified 83 genes differentially expressed between HBV- and HCV-infected HCC. Functional analysis indicated that HBV-related HCC are characterized by a higher expression of imprinted genes, genes relating to signal transduction, transcription, and metastasis, whereas HCV-related HCC exhibit a higher expression of detoxification and immune response-related genes (Iizuka et al. 2002). Honda et al. showed that specific subsets of genes associated notably with apoptosis, cell cycle, cell–cell interaction, cytokines, transcription factors, and stress response are prominent networks able to classify tissue according to HBV and HCV. In hepatitis B tissue lesions, genes involved in inflammation were predominant, whereas in hepatitis C, expression of anti-inflammatory response genes was relatively dominant (Honda et al. 2001).

1.3 Gene Signatures for Recurrence and Metastasis

The establishment of adapted treatments for HCC has been challenged by the clinical and molecular heterogeneity of HCC. When possible, curative treatments include tumor resection, mainly for non-cirrhotic patients, percutaneous ablation, and hepatic transplantation. However, even after curative resection, 80% of patients develop intrahepatic recurrence and the 5 years survival is between 49 and 74% (Llovet et al. 2003). Array-based gene expression profiling has been applied to better understand the molecular mechanisms involved in HCC recurrence and to predict cancer patients with metastatic potential. Such prediction models for HCC metastasis are expected to impact clinical practice since an appropriate therapeutic regime can be applied to patients with high-risk of recurrence (Budhu et al. 2005b). Accordingly, specific gene expression signatures in tumor and surrounding non-tumor tissues have been identified, and prediction models have been developed to predict vascular invasion, early intrahepatic recurrence, and metastasis (Iizuka et al. 2008; Budhu et al. 2005a; Iizuka et al. 2008).

Vascular invasion represents a poor prognosis factor associated with early post-operative recurrence in HCC. Using a supervised learning algorithm, Ho et al. established an expression signature associated with the presence or absence of vascular invasion in HCC (Ho et al. 2006). This molecular signature consisting of 14 discriminative genes for vascular invasion exhibited a predicting value for early recurrence after HCC resection. A 57-gene signature discriminative for vascular invasion and cirrhosis was similarly reported to predict recurrent disease at diagnosis, with 84% accuracy (Wang et al. 2007). Recently, in an effort to identify candidate therapeutic agents capable of targeting the invasive phenotype in HCC, Braconi et al. used a 73-gene signature that correlated with vascular invasion (Chen et al. 2002) to screen a reference database of 453 genomic profiles associated with 164 bioactive small molecules. Using a connectivity map algorithm (Lamb et al. 2006) resveratrol and 17-allylamino-geldanamycin were identified as attractive therapeutic candidates since both of these agents were able to reduce HCC cell invasion at noncytotoxic concentrations (Braconi et al. 2009).

Kurukawa et al. used a PCR-based array to analyze the expression of 3,072 genes in resected HCC specimens from 100 patients (Kurokawa et al. 2004). By using a random permutation approach the authors first identified a subset of 92 informative genes with a predicting value for early intrahepatic recurrence in a testing group consisting of 60 HCC cases. From these 92 genes signature, the top 20 ranked genes with the highest prediction accuracy correctly predicted the early intrahepatic recurrence for 29 of 40 HCC cases within the validation group, and the odds ratio was 6.8 (95% CI 1.7–27.5, $P = 0.010$). The 2-year recurrence rates in the patients with the good signature and those with the poor signature were 29.4 and 73.9%, respectively. Multivariate Cox analysis revealed that molecular signature was an independent indicator for recurrence (hazard ratio 3.82, 95%CI 1.44–10.10, $P = 0.007$). By using a higher-density microarray representing about 6000 genes, Iizuka et al. (2003) investigated mRNA expression profiles in tissue specimens from 33 patients with HCC. This training set was used in a supervised learning manner to construct a predictive system, consisting of 12 genes, with the Fisher linear classifier. The predictive performance of the system was then evaluated on a blinded set of samples from 27 newly enrolled patients. Early intrahepatic recurrence within 1 year after curative surgery occurred in 12 (36%) and eight (30%) patients in the training and blinded sets, respectively. This approach correctly predicted early intrahepatic recurrence or non-recurrence in 25 (93%) of 27 samples in the blinded set and had a positive predictive value of 88% and a negative predictive value of 95%. Recently, Yoshioka et al. analyzed the expression profiles of 139 cases of HCC using pan-genomic microarrays. By similarly dividing a cohort of patients who had undergone curative resection of HCC into a training and a testing set, the authors established a molecular prediction system characteristic for early intrahepatic recurrence. Multivariate Cox regression analysis indicated that the clinical value of molecular prediction system was an independent prognostic factor (Yoshioka et al. 2009). Woo et al. examined the gene expression profile of 65 HBV-associated HCC using Affymetrix chips to generate a genetic classifier that could identify the patients with a high-risk of early recurrence following curative resection (Woo et al. 2008). A 628 gene features signature was identified by a univariate Cox proportional hazard model as a genetic classifier that could classify HCC patients into high-risk and low-risk subtypes of early recurrence. CD24 was identified as a putative biomarker for classifying low- and high-risk groups of early recurrence. The robustness and consistency of predictability was validated when our gene expression signature was applied to a completely independent patient cohort (Lee et al. 2006a). Genetic network analysis suggested that SP1 and peroxisome proliferator-activated receptor-alpha might have regulatory roles for the early recurrence of HCC.

Ye et al. (2003) explored the global associated with HCC progression and metastasis by determining the gene expression profiles of 67 primary and metastatic HCC samples from 40 patients. Using a supervised machine-learning algorithm, the authors generated a molecular signature consisting of 153 genes that permitted to discriminate metastatic HCC patients and identified genes that were relevant to metastasis and patient survival. The authors further reported that the gene expression

signature of primary HCC with accompanying metastasis was very similar to that of their corresponding metastases (Ye et al. 2003). This finding supports the hypothesis that genes favoring metastatic progression are initiated in the primary tumors. Furthermore, osteopontin, which was identified as a lead gene in the signature, was identified as both a diagnostic marker and a potential therapeutic target for metastatic HCC. Osteopontin was over-expressed in metastatic HCC and an osteopontin-specific antibody effectively blocked HCC cell invasion in vitro and inhibited pulmonary metastasis of HCC cells in nude mice. To determine whether metastatic ability is influenced by liver parenchyma, the same group conducted a gene expression profiling of non-cancerous liver parenchyma tissue from HCC patients with or without intrahepatic portal vein metastasis (Budhu et al. 2006). By using cDNA microarrays the authors identified a 17-cytokine gene expression signature in non-cancerous hepatic tissue from patients with metastatic hepatocellular carcinoma (HCC) which exhibits an accurate predicting value for HCC metastasis and recurrence. A global Th1/Th2-like cytokine shift in the venous metastasis-associated liver microenvironment coincides with elevated expression of macrophage colony-stimulating factor (CSF1). These results suggested that a pre-dominant humoral cytokine profile occurs in the metastatic liver milieu and that a shift toward anti-inflammatory/immune-suppressive responses may promote HCC metastases.

1.4 From Supervised to Unsupervised Approach: A Prediction Model for Survival

Numerous microarrays studies identified gene expression signatures that correlate with known clinical parameters or tumor features by using a supervised approach. In contrast, by adopting an unsupervised strategy based on the expression of genes differentially expressed in HCC, we have uncovered new subclasses of HCC (Lee et al. 2004a). The expression profiles of 91 clinically well-documented cases of HCC, and 60 matched non-tumor surrounding tissues were first characterized using a cDNA microarray platform covering 21,329 genes. An unsupervised clustering based on the expression of the more variable genes (4,187 genes in total) identified two homogeneous groups of HCC. Interestingly, by comparing several clinical data, we discovered that the two groups of HCC were highly correlated with the length of patient survival (Lee et al. 2004a). These results were next validated by randomly dividing the HCC samples into a training set and a testing set. The training set was used to build a series of classifiers genes that estimate the probability that a particular HCC belongs to each group of HCC. By using five different prediction algorithms HCC samples were successfully classified based on patient survival. By using a univariate Cox regression model, a gene signature highly correlated with patient survival was identified. Importantly, this survival-associated gene signature was validated in three independent datasets which were generated by other investigators and including HCC cases from various etiologies (Chen et al. 2002; Iizuka

et al. 2003; Ye et al. 2003). Knowledge-based annotation of the survival genes highlighted a functional module associated with cell proliferation as the best predictor of an unfavorable outcome of the disease. Expression of typical cell proliferation markers such as PNCA and cell cycle regulators such as CDK4, CCNB1, CCNA2, and CKS2 was greater in HCC with a poor prognosis. Interestingly, functional analysis of survival genes also pointed to a module of genes involved in ubiquitination and sumoylation, which expression was higher in the subgroup of tumors characterized by a poor prognosis. Therefore, deregulated components in ubiquitin-mediated protein degradation may provide attractive therapeutic targets for novel HCC treatment modalities. This study demonstrated that unsupervised analysis of global variations in HCC transcriptome at the time of diagnosis is able to identify distinct subgroups of HCC patients with variable prognoses.

1.5 Gene Expression Profiling of Non-Coding Genes

Lately, the molecular profiling of HCC has been extent to the expression of non-coding genes, including microRNAs (miRNA). MiRNAs belong to an abundant family of endogenous, short, and non-coding RNAs, which are believed to be key post-transcriptional regulators of gene activity. MiRNAs play important roles in the control of many biological processes, such as development, differentiation, proliferation, and apoptosis (Ambros 2004). Dysregulation of miRNA expression has been widely reported in cancer, including HCC. Aberrant expression of several miRNAs has been reported in HCC and miRNA signatures associated with clinical and histological features of tumors have been identified (Gramantieri et al. 2008). Early report by Murakami et al. (2006) provided one the first comprehensive analysis of miRNA expression patterns in HCC. The authors notably identified a subset of miRNAs, which could predict HCC from matched surrounding non-tumor tissues with an accuracy of 97.8%. By profiling the expression of 250 miRNAs in a series of 46 malignant and benign hepatocellular tumors, Ladeiro et al. identified specific miRNA signatures that could discriminate benign hepatocellular tumors and malignant hepatocellular tumors. Specific miRNA expression patterns were also associated with several subtypes of hepatocellular tumors according to specific risk factors and oncogene and tumor suppressor gene mutations (Ladeiro et al. 2008). By profiling the miRNA expression in a clinically well-defined cohort of 131 patients with HCC, Budhu et al. identified a unique 20-miRNA metastasis signature that discriminated HCC with venous metastases from metastasis-free HCC. As demonstrated by a survival risk prediction analysis the metastasis-related miRNA signature could predict survival and recurrence of HCC in patients with multinodular or solitary tumors, including those with early-stage disease (Budhu et al. 2008). As illustrated by these studies, miRNA profiling may represent a rational complement to the profiling of coding genes for the molecular classification of HCC. MiRNAs may constitute new markers for diagnosis and prognosis of HCC, as well as attractive new therapeutic targets.

2 Comparative Oncogenomics: Cross-species Comparison of Gene Expression Data

The molecular characterization of human HCC derived from the aforementioned microarray studies emphasized the complexity and the heterogeneity of liver cancer, which is associated with the deregulation of numerous genes. In fact, the functional analysis of genes in which expression is altered in HCC suggested that almost all cancer-associated pathways are altered during the course of HCC development (Aravalli et al. 2008). Therefore, the gene expression pattern for each individual HCC sample represents a snapshot that reflects the multiple events that have eventually gradually arose throughout the natural history of the disease. For obvious reasons, this complexity makes more challenging the identification of the underlying molecular mechanisms involved in HCC initiation and progression. In this context, cross-species comparative oncogenomics has emerged as an alternative strategy to reduce this apparent complexity (Lee and Thorgeirsson 2006). Cross-species comparative functional genomics is based on the integration and the subsequent comparison of gene expression profiles derived from different experimental models, including those established in different species. By taking advantage of experimentally well-defined animal models for HCC, this strategy aims to dissect the role of specific pathways or genes in HCC, in an effort to better understand the molecular mechanisms that govern the development of HCC in human (Lee et al. 2005; Thorgeirsson et al. 2006a). By applying a cross-comparison of multiple gene expression data sets from human HCC and the rich database of experimental models for HCC, evolutionally conserved gene expression patterns during HCC progression may be identified.

2.1 The Neutral Theory of Evolution as a Basis for Comparative Oncogenomics

Cross-species comparative functional genomics is based on the neutral theory of molecular evolution, which was introduced in the late 1960s (Kimura 1968; King and Jukes 1969). The theory states that the vast majority of evolutionary changes, which occur at the molecular level, are caused by random drift of selectively neutral mutants. By aligning genome sequences from multiple evolutionarily related species, conserved and divergent sequences have been identified. Conserved sequences, which obviously tend to evolve at a slower rate, may represent functionally important regulatory elements. Thus, if the gene regulatory elements are conserved among species, it is rational to speculate that the gene expression signatures reflecting similar phenotypes in the species would also be conserved. This theory forms the basis for the attempts to cross-compare the gene expression profiles derived from human and mice. Cross-species comparative oncogenomics is a multi-step process, which first includes the generation of gene expression profiles from independent experimental conditions. Since the expression profiles are generated

from different species, the comparison is based on the expression of orthologous genes, which is standardized in each individual datasets.

In the context of hepatocarcinogenesis, cross-species oncogenomics has been notably successfully applied (i) to identify several mouse models that closely mimic human hepatocarcinogenesis (Lee et al. 2004b), (ii) to improve the molecular prognostication of liver cancer by using gene expression signatures specific for rat hepatoblasts (Lee et al. 2006b) or growth factor signaling pathways (Coulouarn et al. 2008; Kaposi-Novak et al. 2006), and (iii) to highlight the predictive value of oncogenic pathways in the early stages of the disease in human (Kaposi-Novak et al. 2009).

2.2 Identification of Best-fit Mouse Models to Study Human HCC

Mouse models for liver cancer have been extensively used as a paradigm to investigate several aspects of human hepatocarcinogenesis. Mouse models not only provided opportunities to functionally test and validate new candidate cancer genes, but also opened a new window to understand the molecular mechanisms underlying the pathogenesis of HCC, and in fine to model human cancer. Widely used in vivo experimental models for HCC include the following: (i) xenograft models, in which HCC cell lines or tumor tissue fragments are implanted in recipient mice, (ii) genetically engineered mice (transgenic and knockout), and (iii) chemically induced models, in which mice are challenged with hepatocarcinogens such as diethylnitrosamine, ciprofibrate, or phenobarbital (Leenders et al. 2008; Newell et al. 2008). Despite the undeniable advantages provided by mouse models in analyzing aspects of the disease that could not easily appraised in human tissues, such as the possibility to characterize early stages of the disease, it remains uncertain if mouse models accurately reproduce the broad spectra of pathological and genetic changes observed in human hepatocarcinogenesis (Rangarajan and Weinberg 2003). In addition, none of the currently available mouse models meet all criteria of the ideal animal model of human cancer, which include biologic, genetic, etiologic, and therapeutic criteria (Hann and Balmain 2001). Thus, it is important to determine which models are the most appropriate for human HCC. In this context, our laboratory applied a cross-species comparative oncogenomic approach to identify the best-fit mouse models for human HCC (Lee et al. 2004b). The direct comparison of the mouse and human HCC transcriptomes was achieved by combining the gene expression patterns of 68 HCC tumors derived from seven different mouse models with 91 human HCC. These mouse models included two chemically induced HCC (ciprofibrate and diethylnitrosamine), four transgenic (targeted overexpression of E2f1, c-Myc, c-Myc/E2f1, and c-Myc/Tgfa in the liver), and one knockout (Acox1^{-/-}). By applying a hierarchical clustering analysis based on the expression of the orthologous genes present in both mouse and human datasets, several clusters were identified. In the combined datasets, the gene expression patterns

of HCC derived from c-Myc, E2f1, and c-Myc/E2f1 transgenic mice were most similar to a subset of human HCC which exhibit a good survival, whereas the gene expressions patterns of HCC developed in c-Myc/Tgfa transgenic mice or induced by diethylnitrosamine recapitulate those of human HCC with a poor prognosis. In contrast, the gene expression patterns of HCC derived from Acox1-/- mice and induced by ciprofibrate were least similar to those observed in human HCC. Since the development of HCC in these two models is driven by peroxisome proliferation in the liver, these results suggest that this molecular pathway does not occur frequently in humans. Interestingly, the similarity between human HCC and HCC derived from the mouse models was not only restricted to the gene expression patterns. Actually, co-clustered human and mouse HCC also exhibit common phenotypic characteristics. As example, the human tumors with increased proliferation, decreased apoptosis, and poor prognosis paired with the mouse models with the same characteristics. Human tumors with the worse prognosis clustered with tumors developed in c-Myc/Tgfa transgenic mice, which are known to exhibit a poor prognosis phenotype, including a short latency and a high incidence of HCC development, a high genomic instability, and a high mortality (Calvisi and Thorgeirsson 2005). By successfully identifying the mouse models, which closely mimic subgroups of human HCC with specific characteristics, this initial study validated the concept of cross-species oncogenomics. By establishing a molecular relationship between the mouse models and subclasses of human HCC, this approach provides an opportunity to orientate the therapeutic strategies and to evaluate the potential of new drug candidates that would benefit to predefined subsets of human HCC.

2.3 A Novel HCC Subtype with Progenitor Cell Origin

From the various gene expression studies that have been conducted in HCC, it has become evident that signaling pathways characteristics of fetal liver development, and which are normally silenced in adult hepatocytes, may be activated in cancer cells. Supporting the notion that the transcriptional reprogramming induced in HCC could mimic that of the developing liver, several oncofetal markers, such as alphafetoprotein (AFP) or glypican-3 (GPC3), have been shown to exhibit a similar expression pattern in fetal liver and HCC as compared to adult liver (Coulouarn et al. 2005; Tellgren et al. 2003; Xu et al. 2001). Given the growing appreciation that signaling pathways that control liver development may be partially activated in human HCC, we addressed whether the molecular classification of human HCC into homogeneous groups could be improved by using a gene expression signature derived from hepatic progenitor cells (Lee et al. 2006b). As an extension of the cross-species oncogenomic approach, we integrated the gene expression profiles derived from rat hepatoblasts with those of human HCC. Hierarchical cluster analysis of the integrated dataset identified a novel subtype of human HCC, which tightly co-clustered with rat fetal hepatoblasts. This new subtype accounted for approximately

20% of HCC patients enrolled in the study and was associated with an extremely poor prognosis. Gene network analysis indicated that the gene expression program that distinguished this subtype from other types of HCC included markers of hepatic oval cells, suggesting that this new HCC subtype may arise from hepatic progenitor cells. This study emphasized the power of the cross-species oncogenomic strategy in discovering new subtypes of human HCC which would not have been identified by conventional approaches.

2.4 Prognostication of Human HCC using Gene Signatures Specific for Signaling Pathways

Growing evidence suggests that hepatocarcinogenesis involved the activation or the suppression of one or several canonical signaling pathways in which alteration may result in the acquisition of a malignant phenotype. Particularly, gene expression profiling in human HCC pointed to the dysregulation of pleiotropic growth factors, receptors, and their downstream signaling pathway components as a central protumorigenic principle in human hepatocarcinogenesis (Breuhahn et al. 2006). Based on this observation and contrasting with our previous unsupervised strategies, we hypothesize that a supervised approach driven by the hypothesis that gene expression signatures specific for the hepatocyte growth factor (HGF) and transforming growth factor-beta (TGF- β) signaling pathways may help to identify clinically relevant subgroups of patients with liver cancer.

HGF is a pleiotropic cytokine that controls many fundamental aspects of cell biology, including cell proliferation, adhesion, migration, differentiation, and modification of the cellular microenvironment (Furge et al. 2000). HGF exerts its effects by activating a tyrosine kinase-signaling cascade after binding to the proto-oncogenic c-MET receptor. Aberrant activation of c-MET is frequently associated with tumor progression and metastasis (Birchmeier et al. 2003). A robust gene expression signature for the HGF/c-MET signaling pathway was achieved under well-controlled experimental conditions. HGF-sensitive genes were identified by profiling the transcriptional responses to HGF of mouse primary hepatocytes isolated from wild-type mice or mice in which the c-Met receptor has been conditionally silenced in hepatocytes (c-Met^{-/-}). The gene signature included genes whose expression was sensitive to the HGF treatment in WT but not in KO hepatocytes. This approach allowed a stringent detection of sensitive genes in a well-controlled experimental environment that closely mimicked the in vivo effects of HGF. To assess the importance of the HGF/c-MET gene expression signature, a cross-species comparative functional genomic approach was applied using 242 human HCC and 7 metastatic liver lesions. Hierarchical cluster analysis of the combined human and mouse dataset, based on the expression of the orthologous HGF/c-MET target genes revealed that a subset of human HCC and all the liver metastasis shared the mouse HGF/c-MET signature. Moreover, the presence of the HGF/c-MET signature in human HCC significantly correlated with increased

vascular invasion rate as well as with increased microvessel density. The subset of patients which exhibited the HGF/c-MET signature had also significantly shortened overall mean survival time compared to other patients. Five different prediction algorithms were used to further assess the significance of c-met regulated expression profiles in determining the disease outcome. Predicted activation of HGF/c-MET signaling showed significant association with poor prognosis and metastases with all algorithms. In addition, functional analysis of genes with the highest predictive value among the classifier genes pointed to genes involved in the cell adhesion (INTGAV, INTB1), motility (CAP1, ARPC1B, NCK2), and proliferation (CKS2, KPNB1, MAPK3) as key contributors to c-met driven tumor progression. As illustrated by this study, cross-species oncogenomics using gene expression signatures derived from genetically well-defined conditions constitutes an attractive strategy for defining both the function of oncogenic pathways and the clinically relevant subgroups of human cancers.

As a paradigm, we subsequently explored whether a gene expression signature characteristic for the TGF- β pathway would also possess a predictive usefulness for HCC and other cancers. Like the HGF/c-MET signaling pathway, genetic alterations in TGF- β signaling have been reported in cancer but the implication of TGF- β in carcinogenesis is complex since TGF- β may exhibit both tumor suppressive and oncogenic properties (Massague 2000, 2008). TGF- β acts as a tumor suppressor at the early stages of tumor development by inhibiting proliferation and inducing apoptosis, but TGF- β also possesses oncogenic potential, which contributes to tumor progression at later stages. Notably, as a potent stimulator of epithelial-mesenchymal transitions (EMT), TGF- β promotes tumor cell invasiveness and metastasis (Thiery and Sleeman 2006). To profile human HCC with regard to the TGF- β signaling pathway, we first established a specific TGF- β gene expression signature under genetically well-controlled conditions. Similar to the previous study, TGF- β dependency of the gene signature was achieved using hepatocytes isolated from WT mice and mice deficient for the TGF- β type II receptor (Tgfr-/-). By performing a time-course gene expression profiling on primary hepatocytes cultured in presence of TGF- β , we identified functionally diverse subsets of TGF- β -responsive genes, reflecting both the suppressive and oncogenic properties of TGF- β . The mouse TGF- β gene signature was then integrated with the gene expression data from 139 cases of human HCC. Cluster analysis based on the expression of orthologous genes present in the mouse and human datasets demonstrated that the temporal TGF- β gene expression signature successfully discriminated significant subtypes of HCC. Importantly, two distinct subsets of tumors that preferentially expressed either early or late TGF- β -responsive genes were identified. The comparison of clinical variables revealed that the subtypes of HCC defined by early and late TGF- β signatures showed significant differences in survival and recurrence. Notably, patients with the late TGF- β signature had a considerably shortened mean survival time and increased tumor recurrence compared to the patients with the early TGF- β signature.

As illustrated by these two studies, the general application of the cross-species comparative functional genomic approach not only provided mechanistic information regarding the underlying oncogenic processes in HCC, but also improved

the molecular classification of human HCC. The characterization of pathway-specific gene expression signatures using well-defined experimental condition or animal models represents a rational strategy to discover novel intracellular regulatory mechanisms with prognosis significance in human HCC. This strategy may be highly relevant to orientate anticancer therapies since the molecular pathways dysregulated in specific subsets of HCC may be targeted using small molecule inhibitors.

2.5 Prediction Model for Early HCC

Hepatocarcinogenesis is a multistage process in which precursor lesions progress into early HCC by sequential accumulation of genetic and epigenetic alterations (Thorgeirsson and Grisham 2002). In an attempt to obtain a comprehensive picture of the molecular alterations associated with the early steps of hepatocarcinogenesis in human, Kaposi-Novak et al. (2009) performed a gene expression profiling of 49 nodular liver lesions ranging from regenerative and dysplastic nodules to early HCC. The expression patterns of genes differentially expressed between the consecutive stages of early human hepatocarcinogenesis, a signature of 460 genes including early markers of neoplastic progression was identified. Gene connectivity network applied to the genes differentially expressed between dysplastic nodules and early HCC outlined an extensive regulatory network centered on MYC oncogene. Activation of the MYC-regulated expression signature during the malignant transformation of preneoplastic liver lesions was further validated by using a comparative genomics approach. By analyzing the liver transcriptome fluctuations at different stages of tumor development in E2f1, c-Myc, and c-Myc/E2f1 mouse transgenic model for HCC, we previously identified oncogene-specific gene expression signatures at an early dysplastic stage of hepatocarcinogenesis (Coulouarn et al. 2006). Nonparametric gene set enrichment analysis (GSEA) validated the presence of the liver-specific MYC expression signature during malignant conversion of human preneoplastic lesions. Furthermore, from the Myc-driven gene signature, the authors identified a set of 36 classifier genes, which could predict the early HCC with 75–85% accuracy. This study not only emphasizes the value of the comparative genomics approach to generate prediction models that could significantly facilitate early diagnosis of liver cancer, but also highlights its relevance to identify potential targets for therapeutic purposes.

In summary, cross-species oncogenomics provides a practical framework for a systematic analysis of gene signatures specific for growth factor and/or oncogenic signaling pathways to derive predictive models for HCC. It would be therefore important in the future to generate and integrate gene expression signatures from various experimental conditions with the gene expression patterns from human HCC. This strategy may provide an accurate molecular classification system embracing the full spectra of preneoplastic and neoplastic liver lesions, and may contribute to the identification of novel diagnostic and therapeutic targets in HCC.

3 Toward an Integrative Systems Genomics

It has become clear from genome-scale studies that liver cancer does not result from a single factor but rather results from the accumulation of multiple abnormalities, which occur at several molecular levels, including DNA, RNA, and protein level. The possibility to access quantitative and qualitative changes of DNA, RNA, and protein molecules at a genome scale has provoked a shift in biological sciences away from the classical reductionism to system biology. Integrative systems genomics refers to a strategy, which consists in merging data derived from various “omic” studies, including as example gene expression profiles, DNA copy number, or methylation profiles. By using multi-parametric measurements it may be possible to improve current diagnostic and therapeutic approaches and enable a predictive and preventive personalized medicine (Hood et al. 2004).

3.1 Chromosomal Alterations and Epigenetic Abnormalities in HCC

Array-based technology for comparative genomic hybridization (CGH) analysis has rapidly advanced in the past decade, resulting in the development of numerous array platforms that are being applied in human cancer research, including HCC. Genome-scale analysis of DNA copy number by CGH has allowed unbiased detection of structural DNA alterations associated with HCC onset and progression. Poon et al. proposed a tumor progression model for human HCC based on bioinformatic analysis of genomic data (Poon et al. 2006). In this study, the authors used CGH to analyze genome-wide chromosomal aberrations of 158 HBV-associated HCC. Based on statistically significant CGH events, a self-organizing algorithm was used to construct an evolutionary tree that could infer patient subgroups with different degrees of tumor progression. Gains of 1q21-23 and 8q22-24 were identified as genomic events associated with the early development of HCC. Gain of 3q22-24, however, was identified as one of the late genomic events found to be associated with tumor recurrence and poor overall patient survival. Moinzadeh et al. performed a meta-analysis of chromosome alterations associated with human hepatocarcinogenesis by compiling CGH data derived from human HCC ($n=785$) and premalignant dysplastic nodules ($n = 30$). The most prominent amplifications of genomic material were present in 1q, 8q, 6p, and 17q, while losses were most prevalent in 8p, 16q, 4q, 17p, and 13q (Moinzadeh et al. 2005). Specific chromosomal imbalances were correlated with etiology and histological grade. Importantly, dysplastic nodules exhibited frequent amplifications in 1q and 8q, while deletions occurred in 8p, 17p, 5p, 13q, 14q, and 16q. The authors reported that gain of 1q appear to be rather an early event in the pathogenesis of HCC that may predispose to further chromosomal abnormalities. This study illustrates well the power of CGH analysis to identify the cause and functional significance of genomic alterations in human HCC.

To discover aberrantly methylated genes in HCC, Fukai et al. performed a gene expression profiling induced in six human hepatoma cell lines following a treatment with the 5-aza-2'-deoxycytidine demethylating agent. Among the 50 genes identified, hepatocyte growth factor activator inhibitor 2/placental bikunin (HAI-2/PB) was reported to be hypermethylated in >80% human HCC tumors. HAI-2/PB promoter hypermethylation was associated with a reduced expression of the gene in HCC tumors (Fukai et al. 2003). HAI-2/PB may act as a negative regulator of hepatocyte growth factor (HGF) signaling and also as an inhibitor of plasmin, which is thought to play a crucial role in the invasion of HCC. Thus, HAI-2/PB might have a suppressive role in the progression of HCC through the regulation of HGF signaling. By analyzing the global levels of DNA methylation as well as the methylation status of 105 putative tumor suppressor genes Calvisi et al. found that the extent of genome-wide hypomethylation and CpG hypermethylation correlates with biological features and clinical outcome of HCC patients (Calvisi et al. 2007). Activation of Ras and downstream Ras effectors (ERK, AKT, and RAL) was notably reported as a consequence of epigenetic silencing of inhibitors of the Ras pathway in all HCC. Selective inactivation of SPRY1 and -2, DAB2, and SOCS4 and -5 genes and inhibitors of angiogenesis (BNIP3, BNIP3L, IGFBP3, and EGLN2) were associated with poor prognosis. Importantly, several epigenetically silenced putative tumor suppressor genes found in HCC were also inactivated in the non-tumorous liver. These results assign both therapeutic and chemopreventive significance to methylation patterns in human HCC and open the possibility of using molecular targets to effectively inhibit HCC development and progression. Recently, Arai et al. performed a bacterial artificial chromosome (BAC) array-based methylated CpG island amplification on 126 tissue samples. The authors concluded that genome-wide alterations of DNA methylation may participate in hepatocarcinogenesis from the precancerous stage, and DNA methylation profiling may provide optimal indicators for carcinogenetic risk estimation and prognostication (Arai et al. 2009).

3.2 Integrative Systems Genomics to Identify Potential Driver Genes in HCC

As discussed above, array-based CGH studies have deeply improved the mapping of important genomic alterations which occur in HCC. However, amplified or deleted regions identified by this approaches eventually span on relatively large DNA sequences, from several megabases to entire arms of chromosomes. Therefore, even the smallest regions often include numerous genes, among those few of them may represent important candidate that play a leading role in hepatocarcinogenesis. In order to narrow down the list of candidate oncogenes and tumor suppressors in HCC, we applied an integrative systems genomics approach by evaluating the correlation between copy number alterations and gene expression levels across tumors with both data sets available (Woo et al. 2009). Fine-scale whole genome copy

number and gene expression profiles were generated from 15 clinically well-defined HCC samples using high-resolution CGH and pan-genomic expression microarrays. Using a regional pattern recognition approach the most probable copy number-dependent regions were identified. The systematic integration of genome-scale data of DNA copy numbers and gene expression profiles identified specific chromosomal regions with concordant gene expression which were thought to be largely due to DNA copy number alterations. In particular, 4 out of the 25 concordant segments (1q21, 1q42, 7q36, and 8q24) were highly predictive for patient survival. Interestingly, 30 out of the 50 genes located in this concordant regions resided in chromosomes 1q ($n = 8$) and 8q ($n = 22$), which were previously shown to have copy number gains at the early stage of HCC development. Deregulated genes in these regions may therefore have a high probability of functioning as potential driver genes. Potent cell growth inhibition induced by specific knock-down of these genes supported the potential driver role of these genes in HCC development and making them attractive therapeutic targets. In addition, systemic prediction of drug responses by connectivity map analysis identified rapamycin, metformin, and gefitinib as therapeutic molecules associated with the potential 50 driver gene signature. Accordingly, the combined treatment with these drugs was shown to potentiate the growth inhibition of cancer cells. In conclusion, this study illustrates the great potential of integrative system genomics consisting to screen for potential driver genes and to provide novel mechanistic and clinical insights into the pathobiology of hepatocellular carcinoma.

3.3 Toward a Personalized Medicine

The application of high-throughput technologies revolutionized the field of molecular oncology. These technologies have produced a huge amount of “omic” data, at RNA, DNA, and protein level, which may be integrated to build a knowledge base usable for a personalized medicine approach in HCC. By translating phenotypic characteristics of HCC into qualitative and quantitative measurements, gene expression profiling provided a unique opportunity to improve the stratification HCC patients at a molecular level. It is clear than in the near future these technologies will be progressively introduced into routine clinical management.

The success of the new analytical approaches, such as cross-species comparative functional and/or integrative systems genomics, suggests that more integration of independent data sets will enhance our ability to identify robust predictive markers. An important goal would be to classify HCC instances into subgroups based on the observed spectrum of genetic alterations and to determine treatment modalities that are most effective for each subgroup. Applying such innovative genome-based and bioinformatics approaches will certainly allow identifying new molecular targets that are uniquely expressed in cancer cells and use these targets to develop new prevention, detection, diagnosis, and treatment options for patients with HCC.

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Chapter 13

Molecular Signatures of Hepatocellular Carcinoma Metastasis

Anuradha Budhu and Xin Wei Wang

Abstract Metastasis is a significant contributor to morbidity and mortality among cancer patients. Such patients are often considered incurable with treatments offering either supportive care or aggressive management without curative intent. Over the last several decades, research in the metastasis field has expanded our knowledge of cancer progression mechanisms; however, the translation of this knowledge into effective anti-metastasis therapies has not been swift. In fact, to add to the complexity of metastasis, recent findings have challenged the classic notion of clonal evolution whereby liver metastases develop during late stages of carcinogenesis. In this chapter, we evaluate several metastasis models and where applicable, describe how high-throughput molecular profiling technology has shed light on and provided prognostic value for this multifaceted process, with emphasis on the liver. The resolution of metastasis will have a large impact on clinical advances, specifically in targeted anti-metastasis therapies to benefit patients.

Keywords Liver Cancer · Hepatocellular Carcinoma · Metastasis · Molecular signature · Microarray

1 Metastasis and the HCC Patient

Hepatocellular carcinoma (HCC) is a heterogeneous disease, a feature that affects patient outcomes, including primary cancer metastasis within the liver or to a distant site. Metastasis is a particularly challenging phenotype in cancer due to the extreme complexity of the process. Metastasis is largely responsible for mortality in epithelial malignancies including the liver, lung, colon, breast, prostate, stomach, and pancreas. Treatment regimens, such as conventional surgery, radiation therapy, and

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chemotherapy have not sufficiently alleviated metastasis and this phenotype often contributes to poor patient prognosis. Thus, approximately 90% of human cancer deaths are attributed to local and distant metastases (Hanahan and Weinberg 2000).

The accurate risk assessment for metastasis is relevant at the time of diagnosis and in the consideration of cancer patient treatment throughout their lifespan. This assessment is useful to evaluate patients for risk of developing metastasis, prognosis, and the potential benefit of systemic therapy. Ideally, patients at high risk for metastasis would be treated with metastasis-specific therapeutic agents targeting disease spread and growth at the distant site. In this chapter, the role of molecular profiling in aiding such patient classifications will be presented.

2 Metastasis Models

Over the last century, our understanding of the metastatic process has significantly expanded. A synopsis of several metastasis models, suggested by clinicians through their understanding of physiology and by researchers through experimentation, is described in this section (Fig. 13.1). Where applicable, the advances attained through microarray technology is noted and particular metastasis biomarkers and signatures for liver metastasis are described.

2.1 *Historical Metastasis Models*

Understanding the origins and mechanisms underlying metastasis is fundamental to the development of clinically useful therapies against this deadly phenotype. Such theories regarding the mechanisms of cancer spread have been studied for over a century. Stephen Paget's early observations in the late 1800s were the first significant insight into this complex process. Puzzled by the enigma of the tissue specificity observed in metastatic tumor cell colonization, Paget suggested that cancer spread was dependent on the cross-talk between cancer cells (seeds) and their specific metastatic organ site (soil) (Paget 1989). Metastasis would thus depend on the compatibility between the seed and the soil. More recently, this concept has been revisited and will be described in more detail later in this chapter. The seed and soil notion was eventually challenged by Ewing who proposed an anatomic mechanism for cancer spread (Ewing 1928). He proposed that tumor cell dissemination occurred through mechanical factors associated with the structure of the vascular system, whereby features of the circulation would trap tumor cells at certain sites. These concepts, which persisted for half a century, were followed by Bross' metastatic cascade theory in 1975, which demonstrated that the spread of metastasis was not random, but rather occurred in specified steps that required one or more disseminating sites (Bross et al. 1975).

In the twenty-first century, research has led to the concept of mutation-induced metastasis. Thus, cancer is thought to develop from normal human tissue via a series

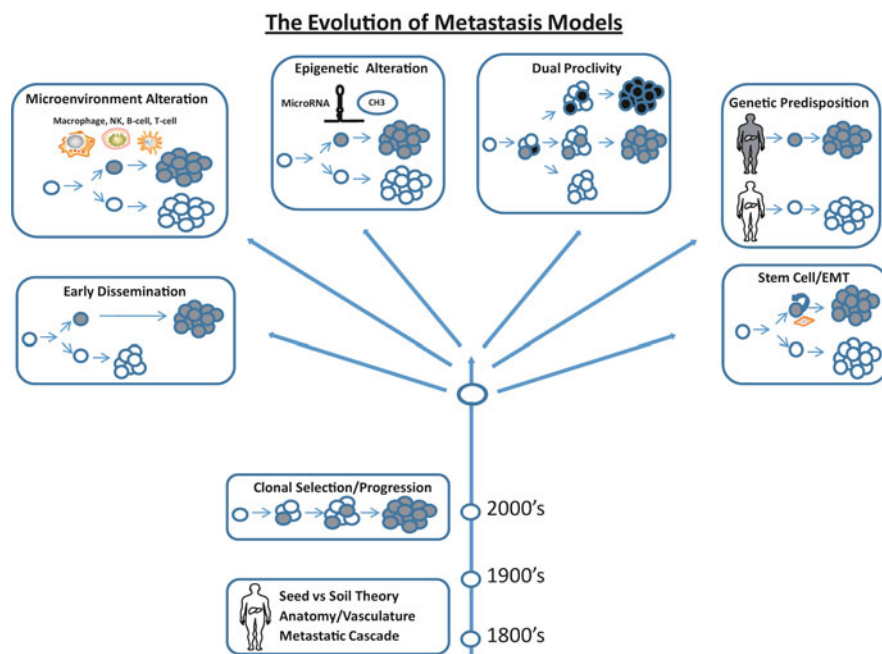


Fig. 13.1 *The evolution of metastasis models.* A schematic evolutionary tree is depicted of major models for metastasis. The models between the 1800s and 1900s described metastasis as a process dependent on the cancer cell (seed) as well as the environment for metastatic growth (soil). Further models described metastasis as an occurrence of the anatomy and as a stepwise rather than a random process. In the 2000s, the clonal selection/progression model asserted that metastasis occurred through the clonal growth of a tumor cell with specific mutation(s). In the last century, there has been a vast expansion of the models to describe metastasis: The early dissemination model states that metastasis cells arise and disseminate early in cancer. The microenvironment alteration model states that the tumor-surrounding environment, through the contribution of immune cells, affects the capacity to metastasize. The epigenetic alteration model states that factors such as microRNA activity or methylation events provide the stimulus for metastasis. The dual proclivity model states that the capacity to metastasize is an inherent quality of the primary tumor. The genetic predisposition model states that certain individuals are more prone to metastasis due to their genetic makeup such as the presence of single nucleotide variants (SNPs). The stem cell/epithelial–mesenchymal transition model states that certain populations of cells with progenitor features or alterations in EMT can lead to metastasis

of genetic and epigenetic events. In pioneering studies, Vogelstein and colleagues demonstrated that sequential mutations involving the loss of tumor suppressor genes and the gain of an oncogene are responsible for cancer development in the majority of colorectal adenocarcinoma patients (Vogelstein et al. 1988). These observations supported the model of carcinogenesis as the clonal expansion of mutant cells whose mutation allows them to acquire proliferative advantage and invasive properties (clonal selection/progression).

The metastatic phenotype has been thought to be a logical extension of this model, where at a late stage of disease, advanced clones acquire the capacity through mutations and selection, to systemically disseminate, invade, and grow at a distant site. Since the initial experiments by Vogelstein, the murine selection model has been used in conjunction with comparative genomics to identify the genetic mechanisms related to metastasis. Specifically, cancer cell lines implanted into host mice were allowed to grow and metastasize followed by isolation and expansion of metastatic cells *in vitro*. A repetition of this selection process resulted in cell lines with enhanced metastatic potential upon further implantation compared to the parental cell line. Clark et al. used this model to describe distinct gene expression patterns in high vs. low metastatic melanoma cell lines, including the metastasis-related enhancement of RhoC (Clark et al. 2000). These studies suggest that metastatic capacity is an intrinsic property of a subpopulation of malignant cells whose alteration(s) allows for selection, expansion, and spread.

Other studies have demonstrated that cancer cells isolated from metastases in a murine xenograft model not only have enhanced metastatic potential, but also retain specificity for the distant organ of metastasis. Massague and colleagues selected a subpopulation of MDA-MB-231 breast cancer cells with tropism for bone metastasis that had differential expression of a distinct set of genes with multiple functions (Kang et al. 2003). A unique gene expression pattern was also observed in an MDA-MB-231 subpopulation with tropism for lung metastasis (Minn et al. 2005). Interestingly, a subset of the differentially expressed genes from the *in vivo* selection for metastasis could also predict clinical lung metastasis based on gene expression profiles from primary cancers, suggesting that some of the genes involved in the selective and site-specific nature of metastasis may also be reflected in the genetics of primary cancer.

However, despite the accumulating evidence supporting this model, some inconsistencies remain. One is the existence of patients with metastatic lesions with unknown primary cancer (Kauffman et al. 2003; Steeg 2003; van de Wouw et al. 2002). The progression model suggests that a primary tumor should have sufficient primary tumor mass to achieve the necessary mutational events in the sequence leading to metastasis. Thus, the absence of a primary tumor in an individual with metastasis is a paradox. Second, it has been shown that highly metastatic variant clones identified in populations can revert to a low metastatic phenotype (Chambers et al. 1984; Harris et al. 1982). In the clonal progression hypothesis, somatic events would be stably inherited rather than rapidly lost. Although the current metastasis model suggests that multistage carcinogenesis is initiated by rare genetic alterations in a single cell, followed by clonal selection and population expansion (Kinzler and Vogelstein 1996), such stepwise and specific progression-related genetic changes have not been illustrated in HCC (Thorgeirsson and Grisham 2002). Thus, alternative models have been introduced to explain the metastatic process.

Since metastatic properties may not be selective for clonal proliferation, cells responsible for metastasis may be rare within the primary tumor. This viewpoint is supported by the clinical observation that metastatic lesions are rare events despite the continual dissemination of significant numbers of tumor cells into the circulation

(Komeda et al. 1995; Vona et al. 2004; Wong et al. 1997). To experimentally support this model, Fidler and Kripke developed subclones from melanoma cells in a murine model and first demonstrated metastatic heterogeneity, including the existence of a distinct subpopulation of highly metastatic cancer cells (Fidler and Kripke 1977).

Furthermore, recent experimental studies indicate that early-disseminated cancer cells may account for metachronous metastases (Husemann et al. 2008), suggesting that systemic dissemination may be an early event in cancer development. Using comparative genomic hybridization (CGH), multiple divergent clones were found to coexist within a primary tumor (Gorunova et al. 1998; Heim et al. 1997). Further genomic analyses showed that disseminated cancer cells often had fewer and different changes than in primary tumors, including those related to aneuploidy and passage through genomic “crisis” (DePinho 2000; Maser and DePinho 2002; Schmidt-Kittler et al. 2003). The rarity of the similarity between disseminated cells and the predominant clone of the fully established primary tumor might be due to a selection against the growth of variants capable of dissemination and metastasis at the primary site. These studies suggest that cells with metastatic capacity arise and disseminate early in the development of cancer.

2.2 Contemporary Metastasis Models

In more recent years, scientific advances have led to breakthroughs in technology, such as high-throughput molecular profiling and a multitude of new metastasis models. These models have examined the elements of the tumor, epigenetic mechanisms, the tumor microenvironment, genetic factors, and stem-cell activities and their contribution to metastasis. A discussion of these studies and the insight they have provided regarding the metastatic process is described in this section.

2.2.1 The Dual Proclivity Metastasis Model

A re-examination of the progression model has recently been made through microarray-based studies assessing metastatic signatures associated with primary liver tumors. These studies identified patterns of genes associated with liver metastasis and in certain instances, with outcome. A pair-wise comparison between primary and metastatic cancer tissue is possible with the availability of high throughput technology for gene expression profiling. Such experiments have challenged the clonal selection model of metastasis. Gene expression profiling analysis has shown that paired primary liver tumors and metastases are similar while a significant difference is observed when primary liver tumors with or without metastases are compared. Consistently, multiple reports have used gene expression profiles of primary tumor samples to predict metastasis and poor clinical outcome (Ramaswamy et al. 2003; van 't Veer et al. 2002; Ye et al. 2003). Direct comparisons of genetic profiles have been performed between primary tumors of the breast and liver and their matched metastases. When unsupervised clustering is performed, samples from the same patient almost always clustered together (Perou et al. 2000; Weigelt et al. 2003).

Moreover, identical expression patterns are observed between primary liver cancer and their extra-hepatic metastases (Roessler et al., unpublished data). The capacity of the molecular profile of the bulk tumor in predicting metastasis defies the theory that a rare variant within the tumor population is chiefly responsible for the spread of disease.

In our laboratory we have shown that osteopontin (OPN), a secreted phosphoprotein, is a significant factor in HCC metastasis. This protein is produced by macrophages and T cells and alters both pro-inflammatory and anti-inflammatory responses. The overexpression of OPN is correlated with metastatic potential of primary HCC and with invasiveness of liver-derived cell lines. Similar findings have been shown in metastatic tumor cell lines and breast cancer patients (Sharp et al. 1999; Singhal et al. 1997; Urquidi et al. 2002). More recently, we have identified a 5KD region of OPN, released through MMP9 activity, that is capable of invasive functions (Takafuji et al. 2007). Thus, factors inherent to the primary tumor mass are capable of metastatic properties.

2.2.2 The Epigenetic Metastasis Model

That a liver metastasis is more similar to its paired primary liver cancer compared to other metastases suggests that there may not be an integral set of changes that are selected for during the metastatic process. Rather, the genetics of the primary cancer may determine the capacity of the tumor to metastasize. Consistently, a recent study suggests that the genetic machinery that causes metastasis is hard-wired into the primary tumor since metastatic foci harbor few genetic alterations compared to their corresponding primary cancer (Jones et al. 2008). In addition, epigenetic mechanisms, such as methylation or small non-coding RNA activities may affect the ability of tumor cells to metastasize. Recent studies indicate that expression profiling with small non-coding RNA gene products (~22nt), known as microRNAs (miRNAs; miRs), is a superior method for cancer subtype classification and prognostication (Calin et al. 2004, 2005; Lu et al. 2005). miRNAs exist in many organisms and play key regulatory roles in mRNA translation and degradation by base pairing to partially complementary sites of the mRNA, predominantly in the 3' untranslated region (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). miRNAs are expressed as long-precursor RNAs that are processed by Drosha, a cellular nuclease, and subsequently transported to the cytoplasm by an Exportin-5-dependent mechanism (Gregory and Shiekhattar 2005; Yi et al. 2003). miRNAs are then cleaved by the DICER enzyme, resulting in ~17–24 nt mature miRNAs that associate with a RNA-induced silencing-like complex (RISC) (Hutvagner and Zamore 2002; Lee et al. 2002). The expression patterns, function, and regulation of miRNAs in normal and neoplastic human cells are largely unknown, but emerging data and their frequent location at fragile sites, common break-points or regions of amplification or loss of heterozygosity reveal that they may play significant roles in human carcinogenesis. The abnormal expression of several miRNAs have been observed in Burkitt's lymphomas, B cell chronic lymphocytic leukemia (CLL) and

in many solid cancer types, including breast, liver, lung, ovarian, cervical, colorectal, and prostate (Calin et al. 2002; Chanet et al. 2005; Cimmino et al. 2005; Iorio et al. 2005; Metzler et al. 2004; Michael et al. 2003; Sonoki et al. 2005; Takamizawa et al. 2004). Functional analysis has revealed the downregulation of PTEN by miR-21, the tumor suppressor function of the let-7 family and the oncogenic function of the miR17-92 cluster (Hayashita et al. 2005; Johnson et al. 2005; Meng et al. 2006). The biological and clinical relevance of miRNA expression patterns have been established in human B cell CLL and solid tumors, including breast cancers (Volinia et al. 2006). Each miRNA has a distinct capability to potentially regulate the expression of hundreds of coding genes and thereby modulate several cellular pathways including proliferation, apoptosis, and stress response (Ambros 2003). In addition, mature miRNAs are relatively stable. These phenomena make miRNAs superior molecular markers and targets for interrogation and as such, miRNA expression profiling can be utilized as a tool for cancer diagnosis (Yanaihara et al. 2006). In addition, the study of miRNAs is advantageous in improving our understanding of the mechanisms of cancer progression.

We investigated the miRNA expression profile of HCC specimens from radical resection. We identified 20 miRNAs that are associated with HCC venous metastasis. In contrast to HCC staging systems, this 20-miRNA-based signature was capable of predicting survival and recurrence of HCC patients with multinodular or solitary tumors, including those with early-stage disease. Moreover, this signature was an independent and significant predictor of patient prognosis when compared to other available clinical parameters. Our study suggests that these 20 miRNAs can assist in HCC prognosis and may have clinical utility for the advanced identification of HCC patients with a propensity toward metastasis. A recent study has shown that one of these 20 miRNAs, miR-122, functions as a tumor suppressor targeting ADAM17 (a disintegrin and metalloprotease 17) and thus affecting metastasis through suppression of angiogenesis (Tsai et al. 2009). Functional studies of these miRNAs may also help to elucidate the mechanism(s) leading to HCC metastasis.

Another possible avenue for metastasis capability suggests that all tumor cells have metastatic capacity, however, only a small fraction are capable of completing the process through the acquisition of positional or random epigenetic events. This model is supported by studies demonstrating that methylation inhibitors can modulate the activity of metastatic cell lines (Ishikawa et al. 1987; Kerbel et al. 1984; Trainer et al. 1988). Global methylation studies have recently been performed in HCC revealing distinct patterns of methylation potentially reflecting different stages of progression (Gao et al. 2008; Moribe et al. 2008). However, while global methylation may mimic epigenetic events, these agents can cause chromosomal aberrations and therefore metastatic capacity may be due to mutational rather than epigenetic effects.

2.2.3 The Microenvironment Metastasis Model

The intrinsic properties of the primary cancer may not confer the complete set of alterations for disseminated cells to complete the metastatic process. The liver is an

organ site housing multiple resident immune cells, such as stellate cells and kupffer cells, and the activities of these cell types within the microenvironment of the liver can have a large impact on metastasis (Fidler 2002; Liotta 1985). In fact, significant alterations of gene expression profiles in non-cancerous tissues from metastatic cancer patients have been observed (Budhu et al. 2006; Dave et al. 2004). Such alterations may be due to tumor infiltrating immune cells from the microenvironment, which may contribute to metastasis, either positively or negatively. This occurs due to tumor heterogeneity and the diversity of inflammatory cells that are located in the stroma or infiltrate the tumor. Examples of inflammatory cell infiltrates include tumor-associated macrophages, considered to be associated with angiogenesis and poor outcome and dendritic cell infiltration, typically associated with good outcome due to their induction of T-cell responses and presentation of tumor antigens (Pollard 2004; Talmadge et al. 2007). In addition, myeloid derived suppressor cells inhibit immune responses and facilitate tumor growth and metastasis, while T-cell infiltration is generally associated with a good immune response (Mantovani et al. 2008). Certain populations of T cells, however, such as the T-regulatory cells or those alternatively activated by Th2 cytokines are associated with metastasis and poor outcome (Vignali et al. 2008). Therefore, the accurate assessment of immune cell distribution, phenotypes, and the status of inflammation are critical factors for assessing metastasis proclivity and thus, inflammatory status may contribute to metastatic tendency. In addition, the alteration of the tumor microenvironment may reflect host genetics, imparting a high risk for metastasis as a result of inherited factors (Hunter 2006). It is possible that a few tumor-associated metastasis genes are necessary, but not sufficient to form a metastatic lesion unless they are supported by stroma-associated events, an underlying concept of Paget's metastasis model discussed earlier in this chapter.

To determine the role of the hepatic microenvironment in HCC metastasis, our laboratory compared the cDNA profiles of non-cancerous surrounding hepatic tissues from HCC patients with venous metastases which we termed as a metastasis-inclined microenvironment (MIM) sample to those without detectable metastases, which we termed as a metastasis-averse microenvironment (MAM) sample. We identified a unique change in the gene expression profiles associated with a metastatic phenotype, which was refined to 17 immune-related genes. This signature was inherently different from the HCC tumor signature found in our laboratory and was validated in an independent cohort. The non-tumor signature could successfully predict venous and extra-hepatic metastases by follow-up with >92% overall accuracy and was a superior and independent prognostic indicator when compared to other available clinical parameters for determining patient survival or recurrence. Dramatic changes in cytokine responses, favoring an anti-inflammatory microenvironmental condition, occur in MIM samples, where a predominant Th2-like cytokine profile, favoring a humoral response, was associated with MIM cases.

The microenvironment-based results indicated that the Th1 to Th2-like profile switch in livers bearing metastatic HCC were accompanied by an overexpression of leukocytes, including Kupffer cells. These findings are reminiscent of the

tumor-associated macrophage (TAM) phenotype whereby macrophages can potentially be “alternatively activated.” Consistently, we observed that an overexpression of colony stimulating factor 1 (CSF1), an activator and regulator of macrophages, accompanied the Th1 to Th2-like profile switch in livers bearing metastatic HCC and could induce this profile in peripheral blood mononuclear cells from healthy blood donors. Thus, CSF1 may play a pro-metastatic role in HCC. CSF1 may be one of the cytokines overexpressed in the liver milieu that is responsible for the cytokine shift that occurs in patients with HCC metastasis. An increase of CSF1 correlated with hepatic inflammation and necrosis (Itoh et al. 1999). Recently, this finding has been validated in an independent study showing that high peritumoral CSF1 is associated with HCC progression and poor outcome (Zhu et al. 2008b). Thus, the microenvironment plays a significant role in metastasis and CSF1 may be a promising target for adjuvant therapy.

2.2.4 The Genetic Predisposition Metastasis Model

Alterations of the liver microenvironment that promote metastasis may be due to the affects of tumor activity; however, genetic mechanisms may also be at play to drive metastasis susceptibility. A major source of genomic heterogeneity in human cancer patients is inherited polymorphism. Although polymorphic alterations have been associated with viral infection, particular variations have yet to be uncovered for HCC. The Hunter lab has shown using a highly malignant tumor transgenic mouse model that the genetic background upon which a tumor arises can significantly affect the ability of that tumor to metastasize to the lung. These studies led to the identification of a polymorphic metastasis efficiency gene, SIPA1, whose expression level was inversely proportional with metastasis (Park et al. 2005). In addition, these studies were translated to the human population, showing that SIPA1 polymorphisms were associated with poor outcome in a breast cancer cohort. Thus, metastasis signatures may not only be indicative of somatic mutations driving progression, but may also be a measure of inherited metastasis susceptibility segregating throughout the human population. Since constitutional polymorphisms are present in all tissues of an individual, the apparent metastasis risk could be interrogated not only from tumorous tissue.

As noted above, OPN was a lead tumor-associated gene in microarray-based and functional studies of HCC. Array CGH has been applied to measure and map copy number variations in OPN-positive and OPN-negative HCC cells (Sun et al. 2008). In this study, OPN-positive cancer cells showed more amplification of chromosomal regions on 4q and 13q covering several genes with 1.5-fold copy number increase. Array CGH has also been applied to cell lines derived from a metastatic HCC model in nude mice showing that chromosome 8p deletion (8p23) is associated with HCC metastasis (Tang et al. 2004). This region was also found in an array CGH study of paired metastatic lesions and primary HCC along with chromosome 9, 11, 13, 16, and 19 (Zhang et al. 2003). Whether polymorphic changes are associated with these genes and chromosomal regions in HCC remains to be determined. Although some

SNP array studies have identified players in HCC, specific alterations associated with metastasis have yet to be uncovered (Lee et al. 2009; Villanueva et al. 2008).

2.2.5 The Stem Cell/Epithelial–Mesenchymal Transition (EMT) Metastasis Model

Although detailed studies have not yet been conducted for liver cancer and its metastasis, another viewpoint comes from studies of stem cells and the critical role of the epithelial–mesenchymal transition (EMT) in metastatic progression. It has been shown that most carcinoma cells access and exploit components of the EMT program to acquire malignant cell traits (Scheel et al. 2007; Thiery 2002). It is possible that cancer cells may act autonomously by creating an EMT state or recruit stromal cells to induce the EMT program, although these two scenarios are difficult to distinguish. Accomplices in the scheme appears to be the embryonic proteins TWIST1 and TWIST2, which are transcriptionally active in tumors, suggesting a role in tumor development/progression. The TWIST proteins can inhibit senescence and apoptosis through downregulation of Rb and P53 while inducing EMT transitions and stem-like properties. Although array-based studies have not yet been conducted in HCC, a few analyses have shown that TWIST expression correlates with HCC metastasis and thus this mechanism needs to be further explored (Lee et al. 2006b; Zhu et al. 2008a). Other studies have shown that miRNAs, signal transducer and activators of transcription (STATs) as well as the HCV core protein can induce EMT (Battaglia et al. 2009; Gregory et al. 2008). In our laboratory, we have shown that cells positive for EpCAM and alphafetoprotein markers display stem-cell features and are associated with poor prognosis (Yamashita et al. 2007, 2008, 2009). Further studies showed that miRNA activity, particularly the miR-181 family, plays a critical role in EpCAM-positive hepatic cancer stem cells (Ji et al. 2009). In addition, hepatic progenitor-like cells associated with poor prognosis have also been identified in other laboratories (Lee et al. 2006a). Vander et al. recently explored the transcriptional profile of transporter genes in HCC and showed that the progenitor cell-related multidrug resistance-associated protein 1 (MRP1) may be associated with aggressive HCC (Vander et al. 2008). Such studies reveal the functional role of many factors in EMT-related and stem-cell-related processes, which share many common properties, and suggest that they may contribute to the metastasis (Reya et al. 2001). Further studies may shed light on the contribution of induction of EMT and stem-cell activities to HCC metastasis.

3 Metastasis and Clinical Treatment

Understanding the mechanisms underlying metastasis has important implications for basic science and clinical oncology. Several studies have established molecular expression profiles that are able to predict metastasis and outcome. These include metastasis profiles identified in our laboratory based on the expression of mRNA and miRNA patterns in primary hepatocellular carcinoma specimens and in their

surrounding non-cancerous tissues (Budhu et al. 2008; Van de Vijver et al. 2002). These molecular fingerprints may be useful for delineating mechanistic pathways associated with HCC metastasis and clinical prognosis including the identification and selection of specific patients who require systemic therapy. However, further validation of these biomarkers is necessary to establish their value when compared to existing clinical tools.

Of particular note, understanding the molecular events at various stages of metastasis may aid in identifying novel therapeutics for HCC. A patient who presents with metastasis is at a disease stage where intrahepatic or distant colonization has occurred and therefore the mechanisms associated with the metastatic site are of the most clinical significance to their treatment. The chief aim in therapeutic design should thus be geared toward the identification and development of agents with specificity for metastasis. Although many attempts have been made to develop HCC therapeutics, the agents utilized are constituents of processes shared between tumorigenesis and growth rather than a particular metastasis-specific event. These include inhibitors of matrix metalloproteinases, growth factors (Cetuximab), and tyrosine phosphorylation (erlotinib and lapatinib) for which HCC-related clinical trial results have not been encouraging (Philip et al. 2005; Thomas et al. 2007; Zhu et al. 2007).

In contrast, the use of an angiogenic inhibitor targeting VEGF (bevacizumab) has been more successful in clinical trials and is currently used as a first-line agent against metastatic colorectal cancer (Marshall 2005). Other VEGF-related agents include sunitinib and TSU-68 (oral anti-angiogenesis compounds) (Zhu 2008). Sorafenib, an oral multikinase inhibitor which blocks tumor cell proliferation/angiogenesis mainly by targeting Raf/MAPK-ERK kinase, has recently been described to improve survival in a randomized control trial in advanced HCC (Llovet et al. 2008). However, the survival benefit via Sorafenib therapy is fairly modest and thus other drugs, which provide longer prognosis are needed. Clinical trial results using individual or combined therapeutic agents suggests that synergistic effects may be achieved through the combination of drug regimens (Abou-Alfa and Venook 2008). Thus, drugs targeting more specific alterations associated with metastasis, such as angiogenesis are more likely to be beneficial in blocking this phenotype. However, further studies, addressing adjuvant therapy and validation efforts are needed.

Another important target for development of novel HCC therapeutics is the Wnt/ β -catenin signaling pathway. A large percentage of HCCs show activation of the Wnt/ β -catenin pathway, leading to nuclear translocation of β -catenin and upregulation of downstream transcription factors. Various strategies are being developed and tested in preclinical studies to target this signaling pathway, including blocking the interaction between β -catenin and TCF and monoclonal antibodies against Wnt-1 and Wnt-2. In our laboratory, we have found that an HCC subtype with stem-cell-like features and poor prognosis displays a molecular profile consistent with activation of the Wnt- β -catenin pathway (Yamashita et al. 2001). This suggests that certain patients with a stem-cell-like HCC subtype and activation of Wnt- β -catenin signaling may specifically benefit from Wnt- β -catenin inhibitor regimens.

This would allow for more effective patient stratification to provide the most relevant treatment regimens. Although Wnt-pathway antagonists have not been efficacious in HCC, the importance of this signaling pathway in aggressive HCC warrants future investigation and development of therapies geared toward modulating Wnt signaling.

A main feature of HCC is the sexual disparity of the disease, with a more common occurrence and aggressive nature in the male population when compared to females. In a miRNA-based expression profiling analysis conducted in our laboratory, we found that several tumor-related miRNAs were differentially expressed in men and women with HCC. In particular, tumors with reduced miR-26 had a distinct transcriptomic patterns, with activation of NF κ B and IL-6 signaling. Moreover, HCC patients with low miR-26 expression had a shorter overall survival and responded better to interferon therapy than patients with high expression of this miRNA. Thus, measurement of miRNA levels could be an important factor in stratifying patients for appropriate treatment regimens such as interferon, allowing for better efficacy of drugs already in the clinical arena. Whether the association of miR-26 expression and aggressive disease is related to features of metastasis remains to be determined.

Recurrence is another feature of HCC that contributes to patient mortality. Recurrence is thought to arise from two main factors: *de novo* tumor formation or metastasis. New tumors, independent of the primary resected mass, appear to be a consequence of impaired liver function, due to the presence of cirrhosis, arising from multiple factors including chronic viral hepatitis infection. This so called “field effect” is thought to create a suitable environment for carcinogenesis to occur and progress. Patients with this type of recurrence present more than 2 years post-surgery (late recurrence). Metastasis, on the other hand, is thought to contribute to the second type of recurrence, which presents at an earlier timepoint, within 2 years post-surgery (early recurrence). It is currently unclear how to distinguish patients who will develop recurrence and whether their recurrence will occur early or late. Although recent studies have explored the presence of outcome-related signatures in HCC, it is not yet clear whether both the microenvironment and the tumor contribute to HCC recurrence (Hoshida et al. 2008; Wang and Thorgeirsson 2009). The presence of microenvironment and tumor-based outcome signatures that are associated with early or late recurrence are currently being explored in our laboratory (Budhu et al., unpublished data). The biological mechanisms underlying early and late recurrence may be quite distinct and thus, the most effective manner of treating such patients may differ. In fact, early recurrence is associated with aggressive disease and prognosis tends to be quite poor. Therefore, the need for distinguishing patients who may suffer from early vs. late recurrence is urgent.

4 Summary

The current advances in technology, such as high-throughput molecular profiling, have expanded our ability to unravel the complex molecular mechanisms underlying

HCC and to identify potential clinically useful drug targets. A subsequent challenge is to identify the specific molecular events that distinguish a metastasis from its paired primary tumor. Due to the difficulty in obtaining metastatic tissue, current array-based studies comparing samples lack statistical power for complex analyses. Moreover, tumor/sample heterogeneity plays a significant confounding role in comparative tumor-metastasis studies. To more specifically ascertain metastasis changes, experimentation must be conducted in a paired/matched fashion. Despite these factors, profiling studies have shed tremendous light on the metastatic process and have opened new avenues for research and clinical applications. In particular, the events leading to metastasis have been ascribed to the alteration of rare metastasis promoting genes, changes in the nature of the microenvironment, genetic factors, epigenetic variations, and the effects of cells with progenitor features. Further analysis of this complex process will undoubtedly reveal the essential changes that allow for the survival, proliferation, and expansion of cells with metastatic capacity. The application of array technology to these studies has and will continue to provide important insight to unravel this aggressive phenotype. The resolution and specific targeting of metastasis pathways will revive the hope of significantly improving the survival status of patients afflicted with HCC.

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Chapter 14

Gene Mutations and Transcriptomic Profiles Associated to Specific Subtypes of Hepatocellular Tumors

Jessica Zucman-Rossi

Abstract Benign hepatocellular tumors mainly include focal nodular hyperplasia and hepatocellular adenomas whereas malignant hepatocellular tumors are essentially represented by hepatocellular carcinomas. Hepatocarcinogenesis is a complex multi-step process leading to the accumulation of a large number of genetic and epigenetic alterations both in benign and malignant hepatocellular tumors. These tumors are also characterized by a clinical broad diversity that includes a large spectrum of different risk factors, several pathological phenotypes, and clinical presentations. Recently, comprehensive genetic and transcriptomic profiling enabled the identification of new molecular classifications that are closely related to the clinical and phenotypic diversity of these tumors. Because almost all molecular classifications are closely related to mutations in oncogenes and tumor suppressor genes, these results revealed genetic events as major determinants of the benign and malignant tumor diversity. Finally, classifying tumors in molecular and clinical homogeneous subgroups is a promising tool to develop a personalized medicine in the future.

Keywords Hepatocellular carcinoma · Hepatocellular adenoma · Focal nodular hyperplasia · Oncogene · Tumor suppressor gene · Molecular classification · Transcriptomic profile · Beta-catenin · TP53 · GP130 · STAT3 · HNF1A · Micro-RNA

1 Introduction

1.1 Hepatocellular Carcinomas

A multi-step process of carcinogenesis is at the origin of hepatocellular carcinoma (HCC) development. Most of the HCC occurred during the evolution of a

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chronic hepatitis and/or the development of liver cirrhosis. Schematically, during cirrhogenesis, growing of macronodules, then, the development of a small-cell dysplasia frequently precedes the malignant transformation in HCC and dysplastic macronodules are well-characterized pre-neoplastic lesions (Edmondson and Peters 1983; Thorgeirsson and Grisham 2002). Altogether, HCC development resembles to other solid tumors because of its multi-step mechanism, but it is also a particular process due to the large number of different risk factors that are associated with HCC occurrence. They mainly include infection with the hepatitis B virus (HBV) or hepatitis C virus (HCV), heavy alcohol intake, obesity with metabolic syndrome, prolonged dietary exposure to AFB1 or vinyl chloride, and primary hemochromatosis. In the vast majority of the HCC cases, at least one of these risk factors can be identified; either alone or in combination (detailed in Donato et al. 2006) and the presence of each risk factor among patients varies according to the geographical origin of the patients (Seeff and Hoofnagle 2006). Although in a few cases, HCC can develop without chronic liver disease and these rare cases in which tumors develop in a healthy liver include cases of HCC that have resulted from the malignant transformation of a hepatocellular adenoma (Bralet et al. 2000; El-Serag 2004).

1.2 Benign Primary Liver Tumors

Focal nodular hyperplasia (FNH) is the most frequent benign hepatocellular tumors (Edmondson 1958). FNH usually developed in women between 20 and 50 years old (Nguyen et al. 1999). Relationship to oral contraceptive use is still under debate; however, some studies suggest that oral contraception may increase the size of the nodules (Heinemann et al. 1998; Mathieu et al. 1998; Scalori et al. 2002). It is proposed that FNH is a hyperplastic response of the hepatic parenchyma to a preexisting local arterial spider-like malformation, likely with a developmentally abnormal origin (Wanless et al. 1985). FNH is also related to well-known vascular diseases, such as the hereditary hemorrhagic telangiectasia (Rendu–Osler–Weber disease) or the congenital absence of the portal vein (Altavilla and Guariso 1999; Buscarini et al. 2004; De Gaetano et al. 2004). FNH usually occurs in normal liver and is multinodular, composed of normal hepatocytes arranged in 1–2 cell-thick plates. Increased arterial flow is thought to hyperperfuse the local parenchyma, leading to secondary hepatocellular hyperplasia. FNH is therefore considered the result of a hyperplastic response to increased blood flow (Fukukura et al. 1998; Gaiani et al. 1999; Wanless et al. 1985, 1989) and, accordingly, FNH usually does not bleed or undergo malignant transformation, justifying therapeutic abstention.

1.2.1 Hepatocellular Adenomas (HCA)

Hepatocellular adenomas (HCA) are rare benign tumors that usually develop in women who use oral contraceptives (Baum et al. 1973; Christopherson et al. 1977; Edmondson et al. 1976; Lansing et al. 1976; Rooks et al. 1979; Vana et al. 1977). HCA occurrence may also be related to androgenic-anabolic steroids use (Farrell

et al. 1975; Henderson et al. 1973; Johnson et al. 1972; Lesna et al. 1976; Sale and Lerner 1977), glycogenosis type I and III (Bianchi 1993; de Parscau et al. 1988; Labrune et al. 1997; Lee et al. 1994; Smit et al. 1990; Talente et al. 1994). HCA is a benign proliferation of hepatocytes with arterial vascularization in an otherwise normal liver. The tumor is also characterized by the lack of frequent mitosis, portal tract, and cholangiolar proliferation (Bioulac-Sage et al. 2007a, International Working Party 1995). A particular subtype of adenoma is characterized by the presence of inflammatory infiltrates and of telangiectasia (Bioulac-Sage et al. 2005). HCA nodules are generally solitary, but two or three nodules occasionally develop simultaneously. The development of more than ten HCA nodules is rare and has been specifically defined as adenomatosis by Flejou and collaborators in 1985. In this context, HCA development was described to be less significantly related to oral contraception and with women (Flejou et al. 1985). During its natural evolution, HCA may remain stable, increase in size, or regress (Buhler et al. 1982; Mariani et al. 1979; Steinbrecher et al. 1981; Svrcek et al. 2007). HCA occasionally bleeds and this risk increases with the nodule's size (Baum et al. 1973; Kent et al. 1978; Kerlin et al. 1983). Malignant transformation in hepatocellular carcinoma is considered to be extremely rare but has been consistently described (Foster and Berman 1994; Grigsby et al. 1987; Gyorffy et al. 1989; Korula et al. 1991; Tao 1991). This risk of malignant transformation seems to be more critical in HCA related to androgenic-anabolic steroid exposure or glycogenosis type I (Conti and Kemeny 1992; Franco et al. 2005; Henderson et al. 1973; Johnson et al. 1972).

Despite our growing comprehension of the different pathways altered in hepatocellular tumors, the molecular mechanisms that lead hepatocytes to undergo transformation and give rise to a hepatocellular carcinoma are still poorly understood. As in other solid tumors, a large number of genetic alterations accumulate during the carcinogenetic process and multiple genetic and epigenetic alterations are observed in HCCs. These changes are both quantitative (losses and gains of chromosome segments) and qualitative (point mutations) or epigenetic, and numerous cases of extinctions of gene expression secondary to the hypermethylation of their promoters have been reported (Laurent-Puig and Zucman-Rossi 2006). As proposed by Vogelstein in overall cancer, hepatocellular tumorigenesis is a DNA disease due to the accumulation of genetic and epigenetic alterations and more specifically of genes that control cell cycle and cell proliferation (Vogelstein and Kinzler 2004). Some of the observed genetic alterations are widely shared among the different tumor types, however, the association of the recurrent genetic alterations is highly specific of a tumor type. Therefore, the comprehensive knowledge of a broad field of genetic alterations in a tumor type and the study of the correlation between these alterations and the different clinical and histological parameters allow to refine the tumor classification and the understanding of the multi-step carcinogenesis process.

Recent comprehensive analysis of large number of genetic and epigenetic alterations together with transcriptome and systematic pathway analyses enabled to clarify the diversity of HCC and HCA, their molecular classification, and the identification of subgroups of tumors likely to be efficiently targeted by specific drugs.

In this chapter, we will summarize the most important molecular classifications that have been described and how these classifications could be useful in clinical practice (Lee et al. 2004, 2006; Villanueva et al. 2007).

2 Oncogene and Tumor Suppressor Gene Mutations Define Specific Subgroups Of Hepatocellular Tumors

2.1 TP53 is the Tumor Suppressor the Most Frequently Mutated in HCC

The mutational spectrum of *TP53* gene in HCC from Qidong and Mozambique where aflatoxin B1 (AFB1) exposure level is high, revealed G→T transversion at codon 249 in more than 50% of the tumors (Bressac et al. 1991; Hsu et al. 1991). This mutation at codon 249 of *TP53*, leading to the amino acid substitution R249S, is exceptionally found in HCC from geographical regions without AFB1 exposure. Usually, in a determined geographic area, the frequency of the R249S mutation paralleled the estimated level of AFB1 exposure, supporting the hypothesis that the carcinogen has a causative role in hepatocarcinogenesis. In western countries, where there is no exposure to AFB1, *TP53* mutations are found in approximately 20% of the HCC, without specific hotspot of mutations (Laurent-Puig et al. 2001). Finally, no *TP53* mutations were found in benign hepatocellular tumors (Bluteau et al. 2002a; Chen et al. 2002).

2.2 CTNNB1 Coding for β -Catenin is Frequently Mutated in HCC and HCA

Mutations activating β -catenin are found in 20–40% of hepatocellular carcinomas, showing that β -catenin is the most frequently activated oncogene in HCC by mutation (de La Coste et al. 1998; Laurent-Puig and Zucman-Rossi 2006; Miyoshi et al. 1998). The Wnt/ β -catenin pathway plays a key role in liver physiological phenomena, such as lineage specification, differentiation, stem cell renewal, epithelial–mesenchymal transition, zonation, proliferation, cell adhesion, and liver regeneration (Benhamouche et al. 2006; Cadoret et al. 2002; Micsenyi et al. 2004; Monga 2001, 2003; Nagafuchi and Takeichi 1989; Suksaweang et al. 2004; Tan et al. 2006). We showed that β -catenin mutations are associated with chromosome stability and this genetic alteration occurs more frequently in patients without HBV infection (Laurent-Puig et al. 2001; Legoix et al. 1999). In a recent study, Audard and collaborators found that β -catenin activated HCC exhibit specific features associating high differentiation with a homogeneous microtrabeculo-acinar pattern, low-grade cellular atypia, and cholestasis (Audard et al. 2007). In addition, β -catenin activated HCC are frequently developed in non-cirrhotic liver in absence of usual HCC risk factor (Audard et al. 2007; Cieply et al. 2009). Depending of the

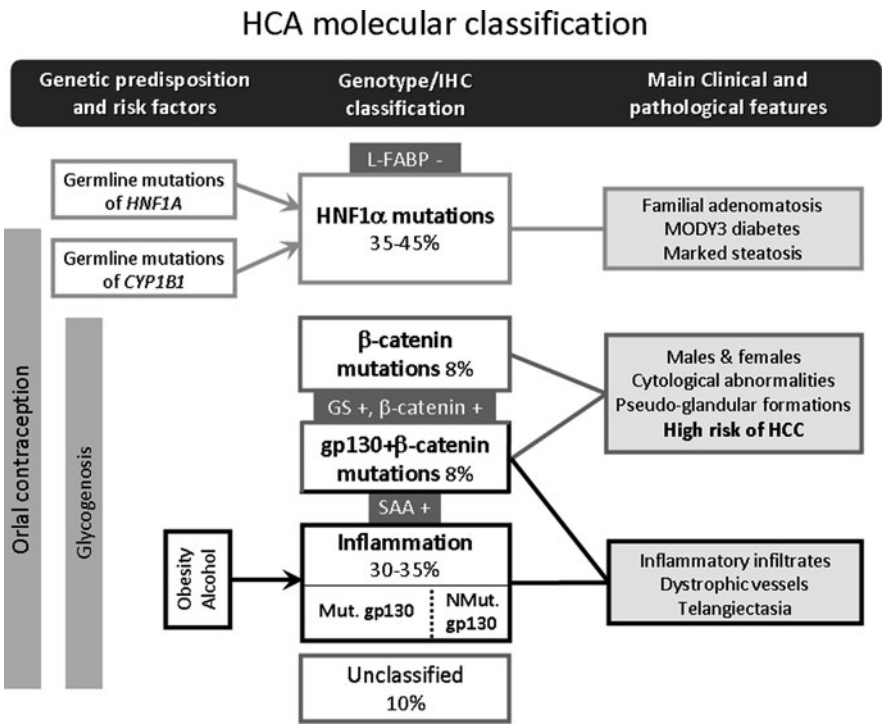


Fig. 14.1 Molecular classification of hepatocellular adenomas

series, β -catenin activating mutations were found to be associated with either good (Fujito et al. 2004; Hsu et al. 2000) or bad prognosis (Cieply et al. 2009) and its relation with prognosis remain debated.

In HCA, activating β -catenin mutation is found in approximately 15% of the cases (Fig. 14.1). β -catenin-mutated HCA are usually unique, they frequently exhibit cytological and architectural abnormalities such as pseudoglandular formations (Bioulac-Sage et al. 2007b; Zucman-Rossi et al. 2006). By immunohistochemistry, these HCA overexpressed glutamine synthetase, usually in a strong and diffuse pattern, associated with an aberrant cytoplasmic and nuclear expression of β -catenin (Bioulac-Sage et al. 2009, 2007b). β -catenin-mutated HCA are particularly frequent in specific etiologies (i.e., glycogenosis, male hormones administration); they are over-represented in male patients, in comparison with other HCA subtypes. Moreover, β -catenin-mutated HCA are associated with a major risk of HCC (Van der Borgh et al. 2007; Zucman-Rossi et al. 2006).

In FNH, we identified in all cases, a heterogeneous activation of the β -catenin pathway within the tumor, but without β -catenin or Axin1 mutation (Rebouissou et al. 2008). However, molecular mechanisms of this activation of β -catenin pathway remain questionable; increase in arterial blood flow could participate in

zonal/restricted β -catenin activation, contributing to tumor formation, as β -catenin may promote hepatocyte proliferation and regeneration.

2.3 Biallelic Mutations Inactivating HNF1 α Are Frequent in HCA

The *HNF1A* gene (also termed *TCF1*) encodes the hepatocyte nuclear factor 1 (HNF1) α , a transcription factor that is involved in hepatocyte differentiation. Biallelic inactivating mutations of this gene is found in approximately 35% of HCA (Bioulac-Sage et al. 2009; Bluteau et al. 2002b; Zucman-Rossi et al. 2006) (Fig. 14.1). In rare patients with a MODY3 (Maturity Onset Diabetes of the Young type 3), *HNF1A* germline mutations could participate in a genetic predisposition to develop adenomatosis by the inactivation of the second *HNF1A* allele leading to a complete HNF1 α inactivation in tumor cells (Bacq et al. 2003; Bluteau et al. 2002b; Reznik et al. 2004). Another gene could also participate to a genetic predisposition to the development of HNF1 α inactivated adenoma; *CYP1B1* germline heterozygous mutations has been found in 15% of the patients that developed sporadic or familial *HNF1A*-mutated HCA (Jeannot et al. 2007). *HNF1A*-mutated HCAs consist in a homogeneous group of tumors with marked and diffuse steatosis, no significant inflammation or cytological abnormalities. The down-regulation of LFABP, a gene positively regulated by HNF1 α and highly expressed in normal liver tissue, may contribute to the fatty phenotype through impaired fatty acid trafficking together with an activation of lipogenesis (Rebouissou et al. 2007).

2.4 gp130 Is Frequently Activated by Small in-Frame Deletions In Inflammatory HCA

Inflammatory HCA (IHCA) are characterized by an over-expression of proteins of the acute phase inflammatory response such as serum amyloid A protein (SAA) and C-reactive protein (CRP) at both the mRNA and protein levels (Bioulac-Sage et al. 2007b; Zucman-Rossi et al. 2006). We have recently identified in 60% of IHCA small in-frame deletions that target the binding site of gp130 for interleukin 6 (IL-6) (Rebouissou et al. 2009) (Fig. 14.1). Mutant gp130 constitutively activates the signal transduction and the activator of transcription 3 (STAT3) independently of the interleukin 6 signaling. In the non-mutated IHCA cases, the mechanism of STAT3 activation remains to be elucidated. Finally, both β -catenin mutations and in-frame deletions of gp130 may coexist in roughly 10% of IHCA. Activation of the two pathways, Wnt/ β -catenin and IL-6/STAT3, may cooperate for the malignant transformation of HCA since gp130 have been found in rare cases of HCC (<2%), but in all cases in association with a β -catenin activating mutation (Rebouissou et al. 2009). These tumors were particular; they were inflammatory HCC developed in normal liver.

2.5 Other Genetic Alterations Identified in Specific HCC Subgroups of Tumors

Exposure to different risk factors may be related to specific genetic alterations in HCC. For example, KRAS2 mutations are found in less than 2% of HCC, but it seems to be specifically associated to vinyl chloride exposure (Weihrauch et al. 2001). Axin 1 and TP53 mutations are more frequently identified in HCC related to HBV infection (Laurent-Puig et al. 2001). Deletion at chromosome 16q23 could be related to AFB1 exposure as homozygous deletions were observed in HCC tumors from exposed patients leading to the deletion of a chromosomal fragile site located in the WWOX tumor suppressor gene (Yakicier et al. 2001). Similarly, common region of deletion described on chromosome 4q differ in HCC related to HBV infection from those related to alcohol intake (Bluteau et al. 2002a). Recently, Schlaefer and collaborators identified numerous additional DNA copy number aberrations in HCC not only specifically associated with the different viral risk factors, but also with alcohol origin and cryptogenic cirrhosis (Schlaefer et al. 2008).

Altogether, including expression data, the main carcinogenetic pathways known to be deregulated in HCC are the inactivation of TP53 pathway observed in 10–50% of the cases (reviewed in Hussain et al. 2007); the Wnt/wingless pathway activation through β -catenin activated mutations and Axin1 inactivated mutations observed in 15–40%, and in 9% of the cases (de La Coste et al. 1998; Legoix et al. 1999; Miyoshi et al. 1998; Satoh et al. 2000; Zucman-Rossi et al. 2007) retinoblastoma pathway inactivation through RB1 and CDKN2A promoter methylation (Liew et al. 1999; Matsuda et al. 1999) and rare gene mutations (Lin et al. 1996); insulin growth factor pathway activation through IGF2 over-expression and rare IGF2R inactivating mutation (De Souza et al. 1995); PTEN inactivation related to tumor progression was also identified in HCC subsequently to frequent promoter methylation or rare gene mutation (Hu et al. 2003; Wang et al. 2007; Yao et al. 1999).

3 Comprehensive Molecular Profiling are Related to Genetic Alterations and Specific Tumor Subtypes

3.1 Genetic and Chromosomal Classification of Benign and Malignant Hepatocellular Tumors

Genetic alterations accumulated in HCC are numerous with over 20 different genes involved affecting at least four principal signaling pathways. First, to understand the underlying mechanisms between these various genetic alterations, we analyzed a series of 137 French HCC tumors related to various risk factors (Laurent-Puig et al. 2001). We found two main groups of HCC defined by the presence of chromosome instability or not: (1) Chromosome instability together with TP53 and AXIN1 mutations were closely related to HBV infection, (2) in contrast the second

hepatocarcinogenesis pathway defined by β -catenin mutation associated with chromosome 8p deletion in a context of chromosome stability is significantly associated with the absence of HBV infection (Laurent-Puig et al. 2001; Legoix et al. 1999). Furthermore, chromosome instability measured by the fractional allelic loss (FAL) was identified as an independent prognostic marker in resected HCC (Laurent-Puig et al. 2001) and in transplanted patients (Dvorchik et al. 2008; Schwartz et al. 2008).

In benign tumors, only a very few number of chromosome alterations have been identified. Almost all benign hepatocellular tumors are chromosome stable. The most recurrent chromosome deletion is located at the long arm of chromosome 12 and it is closely associated to the biallelic inactivation of HNF1 α (Bluteau et al. 2002a). In FNH, only rare and non-recurrent chromosome alterations have been described (Bioulac-Sage et al. 2005).

3.2 Transcriptomic Classification of Benign and Malignant Hepatocellular Tumors

In a more recent study, we performed a genome-wide transcriptomic analysis of 60 HCC tumors together with an exhaustive characterization of structural genetic alterations and clinical parameters (Boyault et al. 2007). In this study, unsupervised transcriptomic analysis identified six robust subgroups of HCC (termed G1–G6) associated with clinical and genetic characteristics. The main classification divider was the chromosome stability status. Tumors from group G1 to G3 were chromosome unstable whereas tumors from G4 to G6 were chromosome stable. Indeed, tumors presenting chromosome unstable phenotype demonstrated a transcriptomic profile strikingly different from chromosome stable ones (Fig. 14.2). Once again chromosome instability appears as the main driver of tumor classification. In addition, genetic alterations and pathways analyses allowed for a refined transcriptomic classification: G1 tumors were related to a low copy number of HBV and over-expression of genes expressed in fetal liver and controlled by parental imprinting; G2 included HCC infected with a high copy number of HBV-, *PIK3CA*-, and *TP53*-mutated cases; G3 tumors were *TP53* mutated without HBV infection, a frequent P16 methylation and showed over-expression of genes controlling cell-cycle; G4 was a heterogeneous subgroup of tumors including *TCF1*-mutated adenomas and carcinomas; G5 and G6 were strongly related to β -catenin mutations leading to Wnt pathway activation; G6 tumors presented satellite nodules, higher activation of the Wnt pathway, and a E-cadherin under-expression. This six-group classification has clinical application regarding the development of targeted therapies for HCC because specific pathway activations, particularly AKT and Wnt pathways, are closely associated to subgroups G1–G2 and G5–G6, respectively. Therefore, we identified and validated a robust 16-gene signature to classify HCC tumors into the six-group transcriptomic classification. This signature should be very useful to determine alterations of specific pathways and to predict putative response to targeted drugs (Boyault et al. 2007).

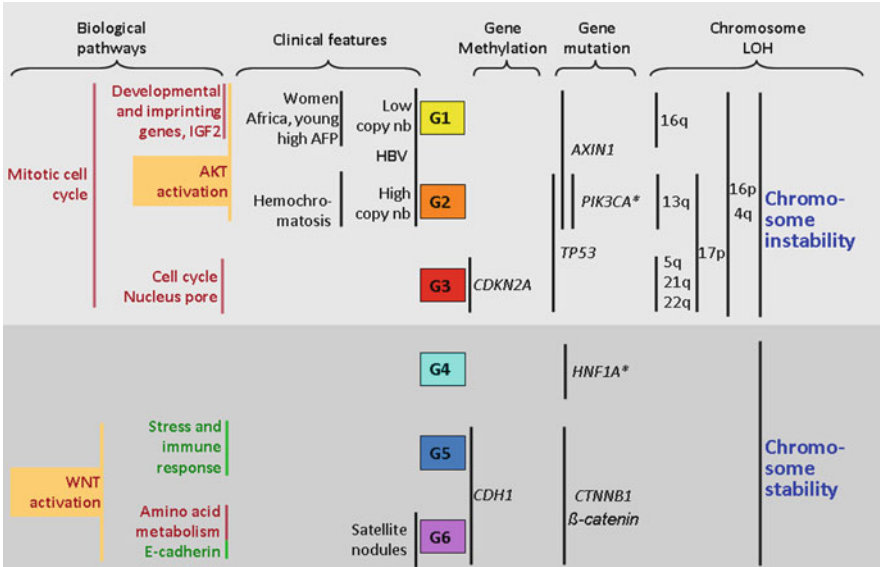


Fig. 14.2 Significant associations with the transcriptomic classification adapted from Boyault et al. (2007). The six robust subgroups found in 120 HCC (termed G1–G6) are shown with their significant relationships with clinical, genetic, and oncogenic pathway features

Concerning HCA, tumor transcriptomic profiles are closely associated with the tumor suppressor gene and oncogene mutations. HNF1 α inactivated adenomas showed a specific profile characterized by a repression of gluconeogenesis coordinated with an activation of glycolysis, citrate shuttle, and fatty acid synthesis predicting elevated rates of lipogenesis (Rebouissou et al. 2007). Moreover, the strong down-regulation of L-FABP suggests that impaired fatty acid trafficking may also contribute to the fatty phenotype. In inflammatory adenoma, demonstrating a gp130 activating mutation or not, we identified a specific activation of the JAK/STAT cascade associated with an over-expression of the type and two interferon pathways (Rebouissou et al. 2009). Finally, in β -catenin activated adenomas, we identified an over-expression of genes known to be targeted by β -catenin in hepatocytes such as genes involved in activation of the glutamine metabolism as previously described in mice models and HCC human tumors (Boyault et al. 2007; Cadoret et al. 2002; Cavard et al. 2006).

In focal nodular hyperplasia, we identified an over-expression genes encoding proteins of the extracellular matrix with an activation of the TGF β pathway localized around the central fibrous scar a typical feature of these lesions (Rebouissou et al. 2008). We also observed a zonated activation of the perivascular pattern and a down-regulation of the genes normally expressed in the periportal area. Finally, we showed a abnormally zonated β -catenin activation in typical FNH that may result in the slight polyclonal over-proliferation of hepatocytes at the origins of the lesions (Rebouissou et al. 2008).

3.3 Micro-RNA Profiling in Hepatocellular Tumors

Micro-RNAs (miRNAs) are small non-coding RNAs that regulate gene expression. Many studies show that they are implicated in essential physiological functions and particularly in tumors (Cullen 2004). Specific alterations of miRNA expression have been identified directly involved in carcinogenesis. Indeed, miRNAs could act as oncogenes or tumor suppressors. In addition, some miRNAs deregulations seem to be associated to specific tumors subtypes, suggesting that they could be used as tumor biomarker. Recently, we performed microRNA (miRNA) profiling in two series of fully annotated liver tumors to uncover associations between oncogene/tumors suppressors' mutations, clinical, and pathological features (Ladeiro et al. 2008). Expression levels of 250 miRNAs in 46 benign and malignant hepatocellular tumors were compared to four normal liver samples

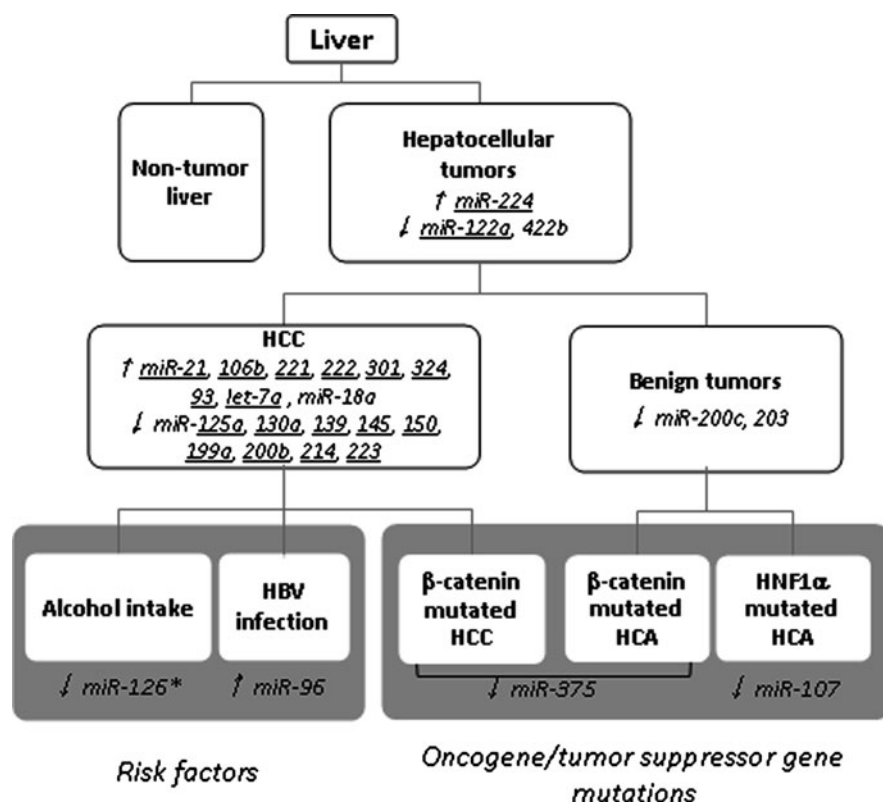


Fig. 14.3 miRNA recurrently altered in HCC and benign liver tumors (Connolly et al. 2008; Gramantieri et al. 2007; Jiang et al. 2008; Kutay et al. 2006; Ladeiro et al. 2008; Meng et al. 2007; Murakami et al. 2006; Varnholt et al. 2008; Wang et al. 2008). Major deregulated miRNAs validated in at least two different published studies are indicated

using quantitative RT-PCR. miRNAs associated to genetic and clinical characteristics were validated in a second series of 43 liver tumor and 16 non-tumor samples. miRNAs profiling unsupervised analysis classified samples in unique clusters characterized by histological features (tumor/non-tumor; benign/malignant tumors, inflammatory adenoma, and focal nodular hyperplasia), clinical characteristics (HBV infection and alcohol consumption), and oncogene/tumor suppressor gene mutations (β -catenin and HNF1 α). Our study identified and validated miR-224 over-expression in all tumors, miR-200c, miR-200, miR-21, miR-224, miR-10b, and miR-222 specific deregulation in benign or malignant tumors 5 (Fig. 14.3). Moreover miR-96 was overexpressed in HBV tumors, miR-126* downregulated in alcohol related HCC. Down-regulations of miR-107 and miR-375 were specifically associated with HNF1 α and β -catenin gene mutations, respectively. miR-375 expression was highly correlated to that of β -catenin targeted genes as miR-107 expression was correlated to that of HNF1 α in a siRNA cell line model. Thus, strongly suggesting that β -catenin and HNF1 α could regulate miR-375 and miR-107 expression levels, respectively. All together, hepatocellular tumors may have distinct miRNAs expression fingerprint according to malignancy, risk factors, and oncogene/tumor suppressor gene alterations. Dissecting these relationships provides new hypothesis to understand the functional impact of miRNAs deregulation in liver tumorigenesis and their promising use as diagnostic markers (Ladeiro et al. 2008).

4 Conclusion

Benign and malignant hepatocellular tumors demonstrate a broad diversity at the genetic and epigenetic levels leading to robust classification closely related to clinical features and carcinogenesis pathways. Molecular classifications are closely related together. In each of them, tumor suppressor and oncogene mutations are frequently major drivers of these classifications. Finally, classifying tumors in homogeneous subgroups using gene signature is a promising tool to construct rational protocols with targeted therapies and to refine prognosis.

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Part VII

Cancer Stem Cells

Chapter 15

Cancer Stem Cells and Liver Cancer

Jens U. Marquardt and Snorri S. Thorgeirsson

Abstract The cancer stem-cell hypothesis proposes that tumors contain a distinct subpopulation of cells which are exclusively responsible for the initiation and maintenance of the cancer. These cells are referred to as either tumor-initiating cells or cancer stem cells (CSC). CSC were first identified in hematologic malignancies, but there is increasing evidence that variety of solid tumors (e.g., in breast, brain, pancreas, colon, and liver) are hierarchically organized and sustained by a distinct subpopulation of CSC. The frequency of CSC in solid tumors is variable indicating both biological variation and technical issues. The existence of CSC also poses a significant clinical challenge since CSC have been shown to be more resistant to chemotherapy and radiotherapy. Consequently, identification and prospective characterization of the CSC in liver cancer promises to have an enormous impact on the development of both, novel diagnostic and therapeutic approaches. This chapter will include a background on the general concept of CSC; a presentation of different isolation methods for CSC; a discussion on the putative origin of the CSC; and a critical discussion of selected recent publications dealing with the existence, identification, and elimination of human liver CSC.

Keywords Liver cancer · HCC · Liver stem cells · Cancer stem cells · Tumor-initiating cells

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1 Cancer Stem Cells (CSC)

The adult stem cells are defined by at least three different properties: self-renewal, differentiation into various cellular lineages, and nearly unlimited capacity to proliferate. Since cancer share number of these stem-cell properties the parallels between cancer and stem cells have often be noted. In fact, the first known description of the connection between cancer and stem cells was made already in 1855, when Virchow formulated the embryonal rests theory (Virchow 1863). Subsequently, several other investigators have alluded to the similarities between cancer and stem cells. However, it is only in the last decade that significant interest in CSC emerged (Al-Hajj 2007; Huang et al. 2007; Jordan et al. 2006; Li et al. 2007b).

It has long been recognized that the capacity of cancer cells to form colonies is restricted to a small subpopulation of cells within each tumor (Bergsagel and Valerieo 1968; Heppner 1984; Weisenthal and Lippman 1985). In addition, in vivo autologous and xenogeneic transplantation assays demonstrated that only certain subsets of cancer cells are able to propagate tumors (Hewitt 1958; Southam and Brunschwig 1961). These results suggest that only a minor subpopulation of cells (<1% of the total tumor) within each tumor possesses the ability to initiate and propagate tumors (Hamburger and Salmon 1977; Reya et al. 2001). These findings led to two general theories of carcinogenesis, i.e., the stochastic and the hierarchical models (Bonnet and Dick 1997; Campbell and Polyak 2007; Nowell 1976; Reya et al. 2001) (Fig. 15.1). In the stochastic model, cancer is composed of a relatively homogenous population of cells, and only few cells that sustain stochastic events (mutations) are able to proliferate extensively, and form new tumors. The hierarchical model, postulates that a subpopulation of stem-cell like cells in cancer (i.e., cancer stem cells) generates a hierarchical organization, with the CSC occupying the apex of the hierarchy (Fig. 15.1). The CSC share important properties such as self-renewal (both symmetric and asymmetric divisions) and differentiation (even if it is abnormal) with the normal tissue stem cells. However, only a cell with stem-cell like properties possesses the capacity to initiate and maintain new tumors.

Recently, several groups reported on the existence of self-renewing cells (referred to as “tumor-initiating” cells or CSC) in various solid organ cancers (e.g., breast, glioblastoma multiforme, prostate, thyroid, and liver). In addition to the capacity for self-renewal, these cells could generate all the different subtypes of cancer cells within a given tumor (Al-Hajj et al. 2003; Sell 2006). We will refer to these cells as cancer stem cells (CSC). It is important to emphasize, that the name “cancer stem cells” in this context is purely based on the functional properties of the cells and does not make any assumptions regarding their origin.

One of the fundamental problems that have emerged from the investigations on the properties of CSC is the problem of heterogeneity (Visvader and Lindeman 2008). This is reflected in discordant results (including both antigenic and functional properties) obtained by several investigators characterizing CSC from the same cancer (O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Wright et al. 2008). The inevitable conclusion to be drawn from these results is that the putative cancer stem cells constitute a rare subpopulation of cells within the cancer and display

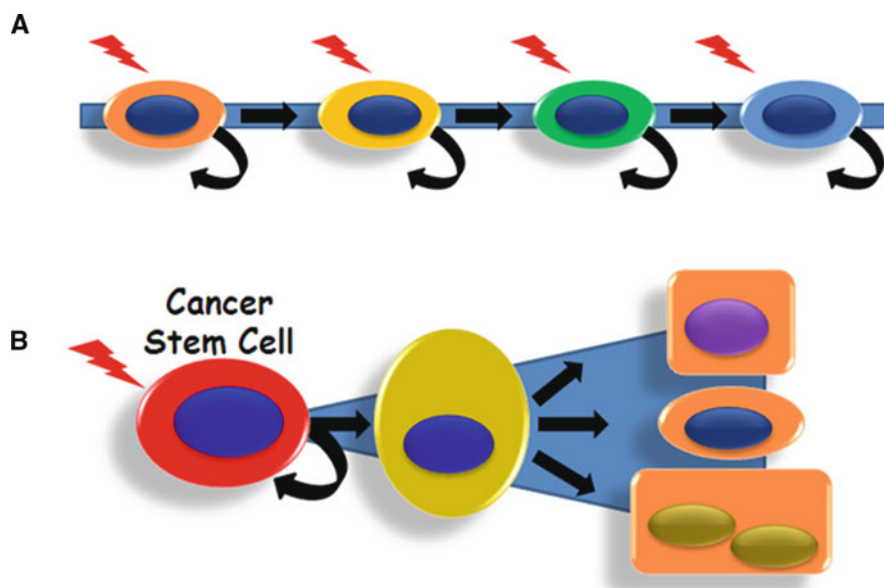


Fig. 15.1 *Two general concepts of Cancer Origin.* The stochastic model (a) postulates that any given cells could initiate tumor growth and proliferate extensively upon accumulation of stochastic events and that tumor cells per se are heterogeneous. In the hierarchal cancer model(b), cancer initiation is exclusively driven by propagating CSC. The CSC are responsible for the tumor heterogeneity that arises through their descending progenies

considerable heterogeneity among the same cancer types and thereby dictate, at least to significant extend the commonly observed tumor heterogeneity. However, as attractive as the concept of CSC is for accounting for the heterogeneity in tumor biology, it is important to revise this issue; at least in some tumors (Quintana et al. 2008).

Nevertheless, there are several reasons why definition and characterization of the putative cancer stem cells and their heterogeneity would be of utmost importance not only for the tumor biology, but more importantly from the therapeutic point of view. Since current therapeutic modalities mainly targeting highly dividing cells and are, therefore, unlikely to target the CSC, which are thought to be quiescent (Dean et al. 2005; Ganguly and Puri 2007; Zajicek 1986). In order to effectively target the CSC it is essential to understand the heterogeneity among cancer stem cells.

There are different hypotheses as to the cellular origin of cancer stem cells (Jordan et al. 2006; Reya et al. 2001). One hypothesis postulates that they arise from normal stem cells that undergo a transformation event(s). Another hypothesis, posits that either stem cells or their progenitors sustain a transformation event(s) during tissue regeneration or healing, in particular after chronic inflammatory stimuli. A third hypothesis suggests that CSC are not derived from normal stem cells but from any cell, including somatic cell, that undergoes transformation, degeneration,

and acquisition of stem-cell like properties. Lastly, a forth hypothesis is a combination of the previous three. Each one of the first three hypotheses can explain the origins of cancer stem cells. The relative contribution of each postulated cellular origin will most likely depend on number of factors including the type of cancer, tissue microenvironment, and the contributing mutagen(s).

There are several different circumstances in which cancer stem cells' heterogeneity could impact cancer biology. Cancer stem cells are thought to be the primary source of all cells in a malignant tumor, however, depending on the circumstances (e.g., type of mutagen(s), specific disease, etc.) different populations of stem cells or progenitors will undergo transformation, resulting in different cancer stem cells. Knowing which kind of stem cells underwent a transformation event under specific circumstances might provide us with better tools for targeting the perpetrator "cell," and possibly, better means for early detection. The stem-cell biology is the biology of hierarchical systems, and studying cancer stem cells' heterogeneity might provide insight into this system. It is plausible that "local tumor initiating cells," "distant tumor initiating cells," "chemotherapy resistant cells," and "radiotherapy resistant cells" are all part of the cancer stem cells subpopulation (Fig. 15.2). However, each

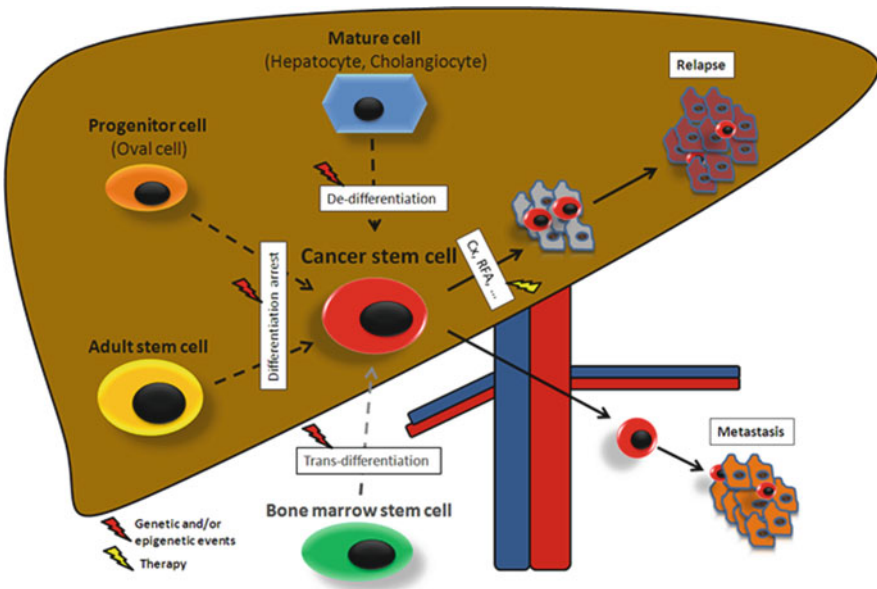


Fig. 15.2 *Biology of liver CSC.* The cellular origin of liver CSC is still unknown. Generally, at least four different scenarios are possible: Stem cell/progenitor cells of the specific tissue could be transformed while remaining in an undifferentiated state and originate tumor growth. Mature cells (i.e., hepatocytes and/or cholangiocytes) could accumulate genetic or epigenetic events and gain stem-like properties. Less likely, also bone marrow-derived stem cells could undergo trans-differentiation upon transformation. Besides initiation of the tumors, CSC are suspected to be the source of metastatic spread and recurrent disease after therapy

cancer stem cell that possesses one or a combination of these traits may reside at a different position on the hierarchical tree and establish its own niche environment. This in return might be reflected in the innate heterogeneity. Lastly, studying cancer stem cells heterogeneity might help us understand, which subpopulation of cells within each cancer is responsible for the three fundamental tumor traits, i.e., local recurrence, distant recurrence (metastasis), and therapy resistance that constitute the major challenges in clinical oncology.

2 Definition and Isolation of Putative Cancer Stem Cells

In order to understand the different methods used to isolate CSC it is important to summarize and define the characteristics of the putative CSC (Clarke et al. 2006b). Again emphasizing that cancer stem cells are defined by the functional characteristics of the cells. From the current perspective, cancer stem cells are defined by at least three distinct properties: (1) *self-renewing capacity* (the ability to undergo asymmetric division and thereby replenish itself “infinitely.” Self-renewal can be addressed by single-cell re-plating assays and by serial transplantation experiments); (2) *differentiation capacity* (the ability of the CSC to recapitulate all cell types of a tumor. The heterogeneity of the original tumor should be reflected in the tumors resulted from the transplantation of the isolated cancer stem cells); (3) *tumor-initiating capacity* (the ability of the isolated CSC to propagate tumors when transplanted into the proper environment. The tumor-initiating capacity is generally tested in xenotransplantation assays) (Fig. 15.3).

Based on these definitions, two general approaches for the isolation of CSC have been established. The first, and most commonly used, is the antigenic labeling approach. This approach relies on the prospective isolation of CSC based on cell surface markers. These markers are mainly adapted from isolation methods of hematopoietic stem cells. The other approach is the functional approach. This approach relies on surrogate characteristics that the CSC share with normal stem cells such as anchorage independent growth, chemo-resistance, self-renewal, asymmetric division and “stemness” gene signatures. In general, a cancer stem cell is not defined by the expression of a single marker rather a combination of different functional and antigenic approaches should be applied to identify and isolate these cells.

2.1 Antigenic Markers

A number of different markers have experimentally been shown to be useful for the isolation of a cancer stem-cell enriched cell fraction. Most of the markers relied on the detection of surface proteins from hematopoietic stem cell and/or on markers specifically expressed on the normal adult stem cells of the tissue in which the cancer occurred. Some of the most commonly used markers for the isolation of human CSC in solid tumors are summarized in Table 15.1. From this table, it becomes obvious that some of these markers are used to isolate CSC from different organs. It is

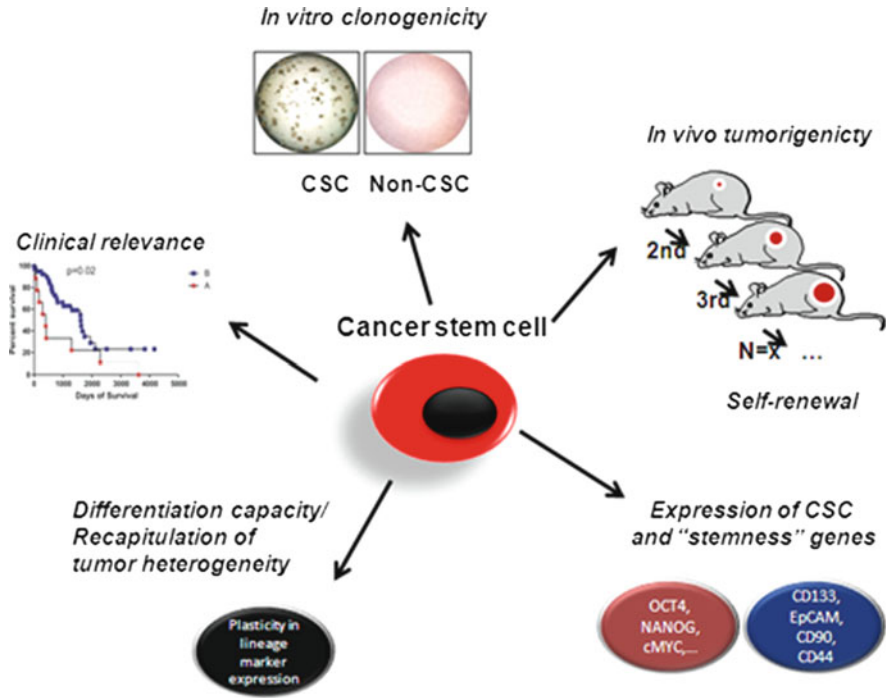


Fig. 15.3 *Properties of CSC.* Per definition, a CSC is judged by its ability to (1) initiate tumors, (2) self-renew, and (3) form differentiated progenies. To prospectively identify cells with enriched CSC characteristics, isolated cells should be experimentally reflected in different surrogate methods: high clonogenicity in vitro, tumorigenicity and self-renewal in vivo, and enrichment of stemness genes on the molecular level should be tested. Finally, they should recapitulate the tumor heterogeneity and proof relevance for the outcome of the cancer patients

yet unclear if this phenomenon relates to a functional role of these markers for CSC or is just a coincidence.

2.2 Functional Approaches

The use of functional isolation techniques is a helpful tool for stem cell studies, especially when there is a lack of other specific markers. Since most of the putative CSC, lack marker specificity, functional methods might be more unbiased approaches, since they only rely on the functional properties of the cells. However, it is unclear what cells exactly are extracted and how heterogeneous and reproducible these methods are.

2.3 Side Population Approach (Hoechst-33342-Dye Staining)

Drug resistance is a common characteristic of cancer cells and stem cells, and is thought to depend on the increased expression of the ABC membrane transporters.

Table 15.1 Antigenic markers of CSC

Marker	Characteristics	Organ	Reference
CD133 (PROM1)	Pentaspans transmembrane glycoprotein. Specific function unclear. Originally identified on hematopoietic and neuronal stem cells	Prostate, Breast, Colon, Liver, Brain, Pancreas	Wright et al. (2008), Collins et al. (2005), O'Brien et al. (2007), Ricci-Vitiani et al. (2007), Singh et al. (2004), Bao et al. (2006), Hermann et al. (2007), and Ma et al. (2007)
CD44 (PGP1)	Surface glycoprotein with multiple isoforms. Multifunctional roles in adhesion, migration and homing	Breast, Prostate, Head and Neck, Pancreas, Colon	Al-Hajj et al. (2003), Li et al. (2007a), Patrawala et al. (2006), and Prince et al. (2007)
CD24 (HSA)	Glycosylated cell surface adhesion molecule. Detected on pancreas CSC. Low levels in mammary CSC	Breast, Pancreas	Al-Hajj et al. (2003) and Li et al. (2007a)
CD90 (THY-1)	Membrane glycoprotein. Potential role in stem-cell differentiation and adhesion of thymocytes in the thymus	Liver	Yang et al. (2008a, b)
EpCAM (ESA, TACSTD1)	Onco-fetal adhesion molecule. Unclear function. Potentially involved in activation of WNT-signaling pathway	Pankreas, Colon, Liver	Li et al. (2007a) and Yamashita et al. (2009)

A high expression supports the energy-dependent substrate exportation against steep concentration gradients across membranes. In 1996, Goodell et al. used the capacity of cells with a high expression of ABC membrane transporters to efflux agents in order to isolate hematopoietic stem cells (Goodell et al. 1996). Using HOECHST 33342 vital dye staining and subsequently flow cytometry analysis, a distinct population of cells actively effluxing the HOECHST dye via the ABCG2 membrane transporter were discovered to be highly enriched for hematopoietic stem cells. This population is known as the side population (SP) (Fig. 15.4a).

2.4 ALDEFLUOR-Approach (ALDH-Activity)

ALDEFLUOR is another non-immunological way for the isolation of stem/progenitor cells, based on their ALDH expression. ALDH is a ubiquitously expressed family of enzymes that has a role in the conversion of retinol to retinoic acid. It is, therefore, important for the proliferation, differentiation, and survival of cells. ALDH was first recognized to be highly expressed in hematopoietic stem and progenitors. Subsequently, high expression was also shown in different stem

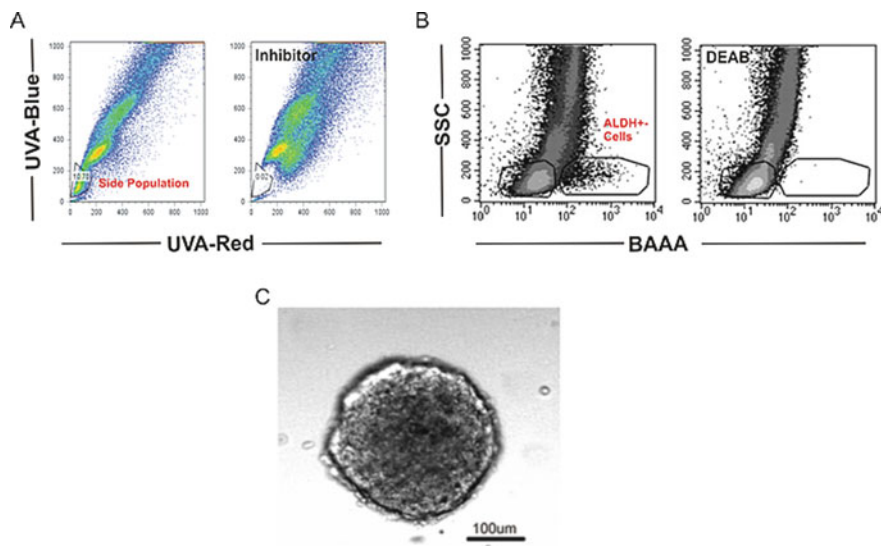


Fig. 15.4 *Functional isolation of CSC.* Different ways for the functional isolation of CSC have been established. Commonly used methods are the side-population approach (a), the ALDEFLUOR approach (b), and different sphere-formation assays (c)

and progenitor cells of other lineages. In the ALDEFLUOR assay ALDH converts the ALDH substrate, BAAA (BODIPY[®]-aminoacetaldehyde), into the fluorescent product BAA (BODIPY[®]-aminoacetate). Cells that highly express ALDH become brightly fluorescent (ALDH^{hi}) and can be identified and isolated using by standard flow cytometry (Fig. 15.4b) (Ma et al. 2008).

Both, the SP and the ALDEFLUOR approach has been successfully used for the isolation of CSC from different tissues including the liver (Ma et al. 2008; Chiba et al. 2006).

2.5 Sphere Formation

Sphere formation is an increasingly used method for the enrichment of stem cells that relies on the stem-cell property of anchorage independent growth. It has been widely used for the isolation of neuronal stem cells. Most of the assays use serum-free culture conditions and are performed under clonal conditions (Fujii et al. 2009). Alternatively, a matrix like Matrigel can be used for the investigation of 3D growths. Current results for the use of sphere formation in CSC are unclear and need further evaluation (Fig. 15.4c).

All of these methods have shortcomings. It became evident, that, as attractive as the isolation of CSC by a single marker might be, it is not clear what these markers really “mark” (Shmelkov et al. 2008). Antibody and/or dye dependent

toxicity clearly biases investigations when used for a negative selection (Kiechle and Zhang 2002; Taussig et al. 2008); and cross-reactivity negatively influences markers used for positive selection (Potgens et al. 2002). Additionally, stochastic activation of several markers by, e.g., experimental conditions and/or epigenetic events impacts the validity of these approaches (Baba et al. 2009; Tabu et al. 2008; Bidlingmaier et al. 2008; Griguer et al. 2008). On the other hand, the isolation based on functional characteristics like sphere formation does also not provide definite information which cells are really propagated and how heterogeneous they are. Furthermore, it might not reflect *in vivo* characteristics of the cells. This might cause over-interpretation of the obtained results (Visvader and Lindeman, 2008). In addition, *in vivo* tumor models also pose problems. Besides the well-known xenotransplantation bias, not only related to the animal models and application methods (e.g., with/without Matrigel, hormone-pellets, etc.), but also the transplantation sites have huge impact on the outcome of the transplantation (Kelly et al. 2007). This may significantly compromise experimental reproducibility. There are also fundamental question that relates to the use of tumor-derived cell lines. Cell lines that originated from a single clone and maintained under culture conditions for decades might be inappropriate for the isolation of CSC and this may explain contradictions between different observations (Visvader and Lindeman, 2008). Nevertheless, these concerns should not undermine the usefulness of the different methods, but rather underlines the complexity of the topic and the necessity for the development of better and more stringent ways to prospectively isolate CSC. Additionally, it emphasizes the need for critical evaluations and interpretation of the biological relevance of data obtained.

3 Cancer Stem Cells and Hepatocellular Carcinoma (HCC)

The liver is a versatile organ composed of many different cell types that interact to maintain the homeostasis of the tissue. Three different primary liver cells are potential targets for cancer initiation, i.e., hepatocytes, cholangiocytes, and adult stem/progenitor cells. It is also possible that a bone marrow-derived stem cell might be a target. Experimental evidence for the likelihood of each of these cells might be the source of potential CSC in hepatocarcinogenesis has been discussed (Sell 2002, 2003, 2004).

Mature hepatocytes have a long life span and extensive proliferative capacity, and could, therefore, be a target of transformation events (Roskams 2006). Overturf et al. demonstrated in fumarylacetoacetate hydrolase (FAH) deficient mice that a limited number of hepatocytes could repopulate host livers over several transplantation cycles, and undergoing at least 69 doublings, without any functional deficits. The results of these elegant serial transplantation experiments clearly demonstrate the enormous proliferation capacity of mature hepatocytes (Overturf et al. 1997). Characteristics, such as longevity, extensive capacity to proliferate, and self-renewal (clonogenic) are fundamental stem-cell properties. Hence, mature hepatocytes, with their intrinsic stem-cell like traits, could, via dedifferentiation, be a source of cancer stem cell in HCC.

The current notion of stem cells as the origin of hepatocellular carcinoma is not a new idea. Experimental induction of liver stem/progenitor cells in rodents has been extensively studied in liver injury models, as well as in liver carcinogenesis. Examples of how cancer arises in these models provide a crucial evidence for the existence of HCC cancer stem cells, and may be the very first modern evidence for the solid tumor cancer stem-cell hypothesis. These early studies demonstrated the effects of various experimental protocols in rodents on the kinetics of adult liver stem-cell (often referred to as oval cells) activation and proliferation. Oval cells are putative liver stem/progenitor cells, first described by Opie in 1944, and later by Farber who coined the name Oval Cell (Farber 1956; Opie 44 A.D.). Oval cells are thought to originate from the terminal branches of the biliary epithelium, so called Canals of Herring, and, at a minimum, give rise to both cholangiocytes and hepatocytes (Evarts et al. 1987; Popper and Schaffner 1961; Ruben and Balls 1964). In case of the liver it is very well-known that precancerous conditions for the development of HCC are chronic inflammation such as Hepatitis-B and C, alcoholic hepatitis, and steatohepatitis. In most of these liver diseases the chronic liver damage is accompanied by progenitor cell activation (Roskams and Desmet 1998; Hsia et al. 1992, 1994). Therefore, liver stem/progenitor cells could be another source of CSC in HCC.

Albeit controversial, there is some evidence that bone marrow-derived cells could take part in liver regeneration through differentiating into oval cells and/or hepatocytes (Fiegel et al. 2003; Fukuda et al. 2004; Minguet et al. 2003). The most striking evidence for this observation was again performed on FAH deficient animals (Lagasse et al. 2000). Injecting adult bone marrow cells into deficient recipients completely restored the damaged liver tissue and function. However, recent studies demonstrated that the functioning liver cells in these models emerge from fusion between the bone marrow cells and the host hepatocytes (Willenbring et al. 2004). Taken together, there is yet no convincing evidence available to support this notion (Thorgeirsson and Grisham, 2006).

It is at least conceivable that all of these cell types might be a target of a transforming event(s), resulting in HCC harboring heterogeneous cancer stem cells, and explaining the diversity of this disease. If the progenitors and/or stem cells are the prime targets of the transformation event(s), then the biological characteristics of the transformed cells will likely be directly related to the state of differentiation at which these cells were transformed. Transformation at a less differentiated point might result in a more heterogeneous phenotypes and aggressive behavior of the CSC.

Several studies have recently tried to address the crucial question of the cellular origin of cancer stem cells in the liver. Chemical and oncogene induced carcinogenesis of oval cells cultures resulted in the formation of variety of liver tumors including HCC upon transplantation into recipient animals (Coleman et al. 1993; Dumble et al. 2002; Garfield et al. 1988; Tsao and Grisham 1987). Several rodent models further provided in vivo evidence for a progenitor cell origin in rodent HCC (Alison et al. 1996). These studies clearly show that HCC can originate from adult liver stem/progenitor cells. Evidence for a stem/progenitor cell origin also exists in

human HCC. As already mentioned, progenitor cell proliferation can be observed in preneoplastic conditions of the liver such as chronic inflammation. This activation is also called “ductular” reaction and is the equivalent to the oval cell activation in rodents. Elegant histological investigations could demonstrate that the amount of ductular reaction correlates with the severity of the underlying liver disease, e.g., chronic inflammation (Libbrecht et al. 2000a, 2000b; Lowes et al. 1999). After progression to HCC a substantial number of the tumors phenotypically show characteristics of progenitor cells. Close to 50% of the HCC had a range of tumor cells that co-express biliary and hepatocytic markers such as CK7, CK19, OV6, AFP, and albumin indicating progenitor cell characteristics of these tumors (Roskams 2006; Durnez et al. 2006). These tumors also displayed a more aggressive phenotype and a worse prognosis (Wu et al. 1999). Other studies suggest that disruption of TGF- β signaling pathway in adult liver tissue stem cells is a key event leading to the transformation of pluripotent liver stem cells into cancer stem cells (Tang et al. 2008). The most convincing evidence for a stem/progenitor origin in human liver cancer originates from a study of Lee et al. investigating the transcriptional characteristics of human HCC. The investigators integrated gene expression data from rat fetal hepatoblasts and adult hepatocytes with gene expression data from human HCC. The authors were able to demonstrate that HCC patients who shared a gene expression pattern with fetal hepatoblasts (fetal liver progenitor cells) had a poor prognosis. The gene signature of this subtype of patients was clearly different from other types of HCC, and included markers of hepatic oval cells, confirming earlier studies and strongly suggesting that this subtype of HCC arose from hepatic progenitor cells. However, more studies are needed that directly address the question of the CSC origin in human liver cancer (Lee et al. 2006).

Recent investigations have focused on characterization and isolation of potential cancer stem cells in human HCC, regardless of their cellular origin. The first isolation of a potential CSC population from human liver cancer was reported by a Japanese group in 2006 (Haraguchi et al. 2006). Using the functional SP approach to isolate cells from various human cancer cell lines derived from the gastrointestinal system (including the liver), a subset of cells comprising 1–2% of the total population was identified. These SP populations differentially expressed markers of differentiation and chemo-resistance like ABC transporters and CAECAM6. Further investigations demonstrated both the potential of these cells to generate both SP and non-SP cells and in vitro resistance to chemotherapeutic agents. Chiba et al. later confirmed the existence of stem-like cells in the SP population isolated from liver cancer cell lines and showed that these cells possess higher proliferative potential and anti-apoptotic properties in vitro compared with those of non-SP cells. The SP cells also expressed markers of stemness, and xenograft transplantation experiments in immunodeficient animals revealed that 1000 SP cells were sufficient to form tumors in the animals, while injection of 1 million non-SP cells consistently failed to generate tumors (Table 15.2). The SP cells further demonstrated self-renewal capacity in serial transplantation experiments (Chiba et al. 2006). Subsequent work from the same group determined a critical role of BMI, a key molecule for stem-cell self-renewal, in the maintenance of cancer stem cells in liver cancer cell lines (Chiba

Table 15.2 Isolation methods of liver CSC

Marker/Method	Frequency	Tumor-initiating cell number (minimum)	Reference
SP	0–28%	1000	Chiba et al. (2008), Haraguchi et al. (2006), Shi et al. (2008)
CD133	0–65%	1000	Ma et al. (2007)
CD133/ALDH1	0–56%	500	Ma et al. (2008)
CD90	0–2.5%	500	Yang et al. (2008a, b)
EpCAM	0–99%	200	Yamashita et al. (2009)

et al. 2008). Also, SP cells isolated from liver cancer cell lines are associated with the metastatic potentials and therapeutic-resistance of HCCs (Shi et al. 2008; Haraguchi et al. 2006).

Several studies have reported the isolation of liver CSC based on the antigenic properties of the cells. Numbers of reports described isolation of potential liver CSC based on the expression of prominin1 (CD133) (Ma et al. 2007; Rountree et al. 2008). CD133(+) cells possessed greater colony-forming efficiency and higher tumorigenicity in vitro and in vivo, although tumor growth was also present in the CD133– population, but to a far less extend. Again, the isolated CD133+ cells harbored other stem-cell characteristics including the expression of “stemness” genes involved in WNT/b-catenin, NOTCH and Hedgehog signaling pathways, the capacity to both self-renew, and differentiate into non-hepatocyte lineages (angiomyogenic cells). Combining of CD133 with the functional ADLH approach revealed that the majority of ALDH(+) were also CD133(+), but not vice versa (Ma et al. 2008). The combination of these two approaches significantly enhanced the stem-cell characteristics of the isolated cells and could potentially facilitate the isolation of a more “homogeneous” CSC fraction. Similar results were obtained for CD133+ cells in murine studies, using models of endogeneous HCC development. These studies confirmed the potential of this marker for the isolation of liver CSC, even without the frequently criticized “xenotransplantation bias” (Rountree et al. 2008).

The mesenchymal stem-cell marker CD90 (Thy-1) has also been used to study and characterize CSC in HCC, and only the CD90+ cells from HCC cell lines were tumorigenic (Yang et al. 2008a, 2008b). The importance of CD90 expression was also evaluated in fresh tumor specimens and blood samples from HCC patients. All the tumor specimens and most of the blood samples contained CD45–/CD90+ cell populations, which could generate tumor nodules in immunodeficient mice, while the markers were not expressed in normal and cirrhotic liver. Additionally, isolated cells positive for CD90 and CD44 demonstrated a more aggressive phenotype than the CD90+ and CD44– counterpart. These cells also formed metastasis to the lung after transplantation into immunodeficient mice and propagated tumors in serial transplantation experiments. CD44 blockade by anti-human antibodies diminished the formation of local and metastatic tumor nodules and induced apoptosis in the CD90+ cells. Additionally, to the finding of CD90 as a potential marker for CSC

in liver, the results of the study suggest that a combination of several markers for isolation of CSC in liver is promising. This approach is already commonly used for the isolation of CSC in other malignancies, as described in the earlier paragraphs.

Another putative marker recently established for the isolation of liver cancer stem cells is EpCAM (Epithelial Cell Adhesion Molecule), also known as ESA and TACSTD1. EpCAM is also used in studies on cancer stem cells from variety of tumors (Al-Hajj et al. 2003; Ricci-Vitiani et al. 2007; Li et al. 2007a; Munz et al. 2009). In the liver, Yamashita et al. recently demonstrated that EpCAM is potentially helpful in the classification of HCC patients (Yamashita et al. 2008, 2009). Subsequent studies validated the differential expression of AFP and EpCAM in tumor specimens from two of their identified HCC subtypes. FACS isolation of EpCAM positive cells from two HCC cell lines displayed CSC like characteristics including self-renewal potential. EpCAM positive cells were highly tumorigenic in vivo and formed substantially more spheres in anchorage independent growth experiments, compared to their EpCAM⁻ counterpart. The high tumorigenic potential in vivo was then confirmed on freshly isolated HCC specimens. Moreover, activation of Wnt/b-catenin signaling enriched the EpCAM⁺ cell population, while

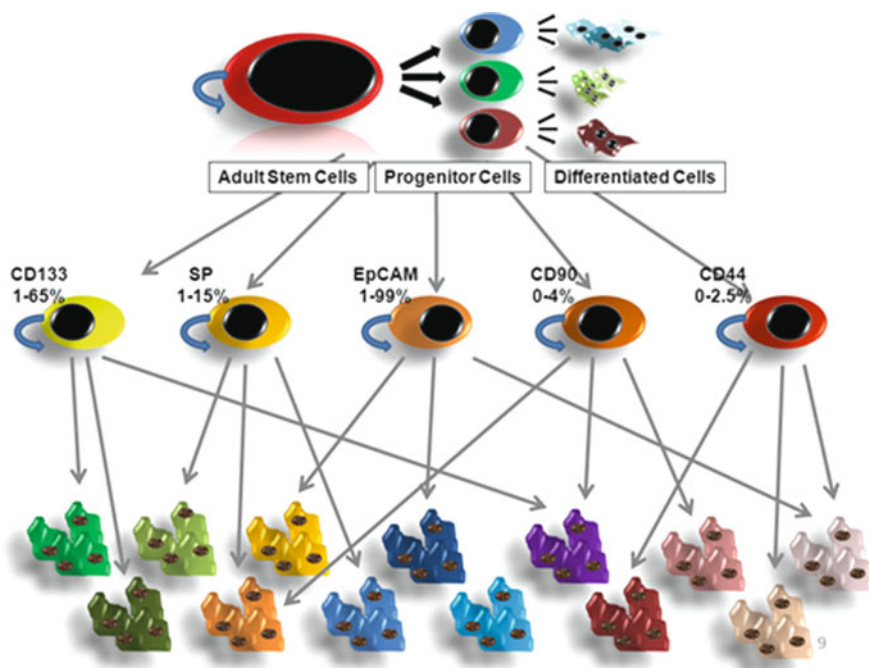


Fig. 15.5 *Heterogeneity of liver CSC.* Different functional and antigenic assays were used to isolate cells with enriched CSC properties (*center row*). These putative CSC appeared to be as heterogeneous as the tumor itself (*lower row*). In a hierarchal cancer concept, heterogeneity among the differentiated tumor cells is driven exclusively by the CSC. Therefore, liver CSC are still undefined and their cellular origin remains unknown (*upper row*)

the blockage of EpCAM attenuated the activities of these cells. These results demonstrated a potential role for the EpCAM in the isolation of liver cancer stem cells.

It is worth emphasizing that the CD90 and EpCAM studies investigating potential CSC in liver cancer did not only rely on cell lines, but also investigated the relevance of their CSC markers in fresh human HCC specimens. Indeed these were the first studies to prove the significance of the isolated putative cancer stem cells in a clinical setting of human HCC patients.

To summarize, all these studies demonstrated cancer stem-cell characteristics in the isolated cells including self-renewal, multipotency, and extensive proliferation capacity, regardless isolation procedures (Fig. 15.3). Consistent with the CSC hypothesis and in agreement with observations from other types of tumor, the liver CSC comprise only a minor fraction of the total cell population of the tumor. Unfortunately, there was little or no overlap between the gene expression pattern and antigenic characteristics of the cells isolated in the different studies. Therefore, the current “state of the art” strongly suggests that the heterogeneity of human liver cancer disease may also be reflected in the liver cancer stem cells (Fig. 15.5).

4 Cancer Stem Cells and Metastasis/Circulating CSC

The ability of a tumor to form local and distant metastasis is major cause of cancer-related deaths. The generation of metastasis is tightly connected to the ability of a cell to migrate and invade through the extracellular matrix into the blood vessels, escape from immunosurveillance, and extravasate at the target tissue (Nguyen et al. 2009). Although the metastatic mechanisms are not fully understood the epithelial–mesenchymal transition appears to play a pivotal role in this process (Polyak and Weinberg 2009). The CSC are posited to be the culprit for these events (Fig. 15.2) (Jordan et al. 2006). Therefore, several studies tried to identify circulating and metastatic CSC and study their significance in different tumors. Some of them were able to reveal the metastatic potential of CSC, their contribution to neovascularization, or mechanisms leading to immune-escape (Jaiswal et al. 2009; Lu et al. 2009; Ahn et al. 2009; Hermann et al. 2007). Others demonstrated that CSC and/or their gene signatures are highly correlated with the prognosis of patients (Aktas et al. 2009; Liu et al. 2007). Although the contribution of liver CSC to the formation of hepatic metastasis is not well-defined, early recurrence occurs frequently even after curative resection or transplantation and is *inter alia* caused by metastatic tumor cells (Hoshida et al. 2008; Llovet et al. 2005; Zimmerman et al. 2008). Experimental evidence that these metastatic cells are indeed CSC comes from the study by Yang et al. (2008b). The authors were able to identify CD90+ and CD45– cells in most of their investigated HCC patients, but not in cirrhotic patients or disease free control patients. These cells, after subsequent isolation, were highly tumorigenic in xenotransplantation assays and could be serially propagated. Prospective studies, directly investigating the role of CSC for the course of HCC patients are needed to answer this question.

5 Clinical Implications of CSC for the Liver Cancer

There exists, as discussed above, strong evidence for CSC in human liver cancer. The fundamental clinical implication of this observation is that the CSC have by definition to be the key targets for effective therapy of liver cancer. This notion is further supported by the outcome of current cancer therapies (i.e., chemotherapy, irradiation, and immunotherapy). All of these therapies primarily target rapidly dividing generally well-differentiated tumor cells. Although the majority of these tumor cells are killed after these traditional therapies, relapse is a frequent event (Llovet et al. 2003). These observations indicate that a highly resistant, potentially quiescent population of cells is left behind that, once activated, propagate the relapse of the tumor. These cells are most likely to be the cancer stem cells.

The high efficiency and approval of Sorafenib, a multi-tyrosine-kinase inhibitor targeting RAF/VEGF/PDGFR, for the therapy of advanced HCCs was a strong evidence for the effect of a target therapy for multi-resistant cancer entities like HCC (Llovet et al. 2008; Clarke et al. 2006a). Future therapies should, therefore, explore susceptibility of the CSC to existing therapies in combination with disruption of key pathways responsible for the stem-cell traits, such as self-renewal, pluripotency radio-chemo-resistance, and angiogenesis of the CSC (Clarke et al. 2006b). Also, there is experimental evidence that the disruption of the tumor niche is an effective approach for the elimination of CSC in some tumors (Gilbertson and Rich 2007; Meads et al. 2008; Gokmen-Polar and Miller 2008). Another attractive approach to deplete the liver CSC population might be a forced differentiation. For brain tumors and leukemia, the use of BMP and retinoic acids already obtained promising results (Endo et al. 2008; Nishanian et al. 2004; Zhao et al. 2008). Indeed, these results were recently confirmed by a study that used high-throughput screening to identify specific therapies for breast CSC. Highest efficiency was achieved by an agent that led to forced differentiation of the tumor cells (Gupta et al. 2009). Also, experimental evidences indicate that forced HNF4 α expression induces differentiation, decreases “stemness” gene expression and the percentage of CD133⁺ and CD90⁺ in hepatoma cells. Moreover, it abolished the tumorigenicity and metastatic potential of these cells (Yin et al. 2008). However, to accurately predict the effect of these strategies and avoid inadequate differentiation in non-target cells, the cancer stem cell in liver and its differentiation pathways needs to be further defined (Mishra et al. 2009).

Direct targeting of specific surface markers of liver CSC like CD133, CD90, EpCAM, and CD44 might be a powerful approach for the development of new and specific therapy strategy for HCC. For example, RNAi targeting EpCAM significantly decreased the CSC fraction, as well as their tumorigenicity and invasive capacity (Yamashita et al. 2009). Since EpCAM expression is a direct WNT/ β -catenin target gene, this might have further implication for a novel target therapy. In addition, there is experimental evidence that targeting pluripotency genes like OCT4 and NANOG could be potentially useful for the specific eradication of CSC (Glinsky 2008; Hu et al. 2008; You et al. 2009). However, all of these molecules are shared with the normal stem-cell population and, therefore, need to be critically evaluated in future investigations. The combination of traditional used anticancer

therapies with some of these new specific target strategies may be the most promising approach. Recent studies further indicate the relevance of microRNAs for the specific eradication of CSC (Dirks 2009; Shimonon et al. 2009). So far, only one study focused on the role of microRNAs in liver CSC, indicating a potential regulatory role of micro-RNA181 for EpCAM+ liver CSC (Ji et al. 2009). Promising results from investigations on these emerging molecules can be expected from future studies.

Another important issue is the relevance of CSC as potential markers for diagnosis and/or prognosis in liver cancer. It is already known that histopathological evaluation of CSC markers in HCC patients failed to accurately predict prognosis (Salnikov et al. 2009), again highlighting the clear prognostic power of the progenitor cell signature in liver cancer patients (Lee et al. 2006).

Taken together, the field of cancer stem cells in liver cancer is a rapidly evolving field that has the potential of significantly impacting the future management of this deadly disease. We have highlighted some of the milestones in this field, as well as potential caveats that investigators might face when tackling the many unresolved questions that have to be addressed in future investigations on liver CSC. Additionally, we have emphasized the urgent need for new integrative strategies and improved in vitro and in vivo models to better define the CSC in the liver and to improve our limited understanding of the CSC biology.

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Chapter 16

Heterogeneity of Liver Cancer Stem Cells

Taro Yamashita, Masao Honda, and Shuichi Kaneko

Abstract Hepatocellular carcinoma (HCC) is an aggressive disease with a dismal outcome. Although considered to be monoclonal in origin, HCC has heterogeneous pathologies and genetic/genomic profiles, suggesting that HCC may initiate in different cell lineages. Recent advances in cancer and stem-cell biology have revealed similarities between organogenesis and tumorigenesis, including hierarchical organization dictated by a subset of cells with stem-like features termed cancer stem cells (CSCs). Several hepatic stem/progenitor markers have been shown to be useful for the isolation of putative CSCs from HCC, although the expression patterns and phenotypic diversity of CSCs purified by these markers are still elusive. Here, we summarize the current knowledge of liver CSCs and discuss their heterogeneity and commonality.

Keywords Cancer · Stem Cell · Liver Development · Hepatocellular Carcinoma · Epithelial-Mesenchymal Transition

1 Introduction

Embryogenesis and tumorigenesis share similar features including autonomous cell proliferation, motility, homing, dynamic morphologic changes, cellular heterogeneity, and interactions with the microenvironment. Indeed, carcinogenesis could be described as deregulated malignant organogenesis mediated by abnormally proliferating and/or metastatic cancer cells and activated stromal cells that trigger angiogenesis, fibrosis, and inflammation at the site. Cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and generation of heterogeneous cell populations. These observations have resulted in the hypothesis that cancers are transformed stem cells with arrest of maturation (Potter 1978; Sell 1993). Although the origin of cancer is still a controversial issue, the

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cancer stem-cell (CSC) concept, i.e., that a subset of cells bearing stem-cell like features are indispensable for tumor development and perpetuation, has recently been revived and supported by accumulating evidence (Clarke et al. 2006). Because both embryogenesis and tumorigenesis are a continuous process of self-renewal, asymmetric division, and differentiation, various molecules are thought to be concomitantly and gradually regulated, which results in the generation of heterogeneous populations expressing various stem/maturation markers. In this chapter, we would like to discuss the heterogeneity and phenotypic diversity of liver CSCs as well as hepatic stem/progenitor cells.

2 Liver Development and Stem-Cell Marker Expression

2.1 Early Stages of Embryogenesis

Embryogenesis is characterized by the ordered emergence of an organism made up of a multitude of stem and differentiated cells, and various signaling pathways play crucial roles in organogenesis where dynamic cell proliferation and motility arises (Slack 2008). The first differentiation event during mammalian development is the formation of the inner cell mass at the blastula stage (Fig. 16.1). Embryonic stem cells (ES cells) are located and can be successfully isolated from the inner cell mass of the blastula (Murry and Keller 2008). Before the blastula stage, early embryonic cells appear to have no capability for self-renewal; therefore, the most primitive markers of fetal tissue stem cells should be expressed first in this inner

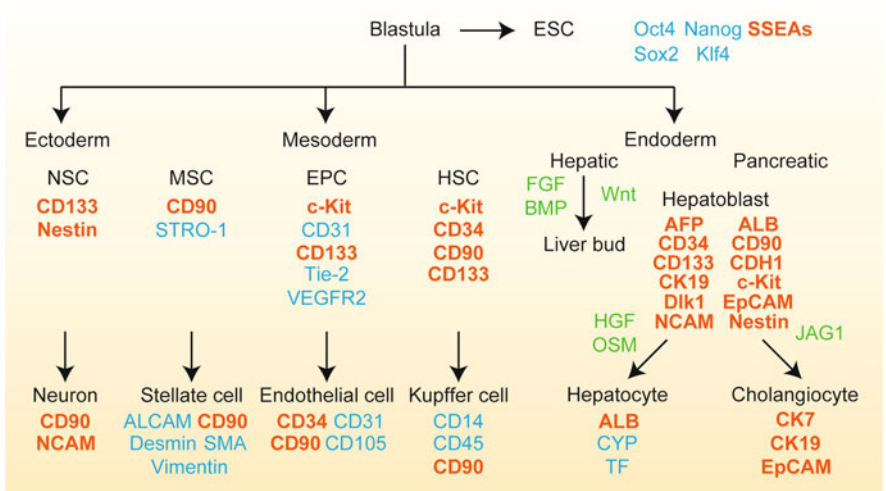


Fig. 16.1 Liver development, stem/progenitor cell markers, and activated signaling. Stem/progenitor cell markers expressed in various cells constituting the liver (neurons, stellate cells, endothelial cells, Kupffer cells, hepatocytes, and cholangiocytes) are shown. Reported hepatic stem/progenitor cell markers are indicated as bold. ESC: embryonic stem cell; NSC: neural stem cell; MSC: mesenchymal stem cell; EPC: endothelial progenitor cell; HSC: hematopoietic stem cell

cell mass at the blastula stage (Slack 2008). Several markers are reported to be expressed at this stage by ES cells, including OCT4, Klf4, Sox2, SSEAs, and Nanog (Graf and Stadtfeld 2008). The importance of these genetic regulators was dramatically demonstrated by their induction of pluripotency in fibroblasts (Takahashi et al. 2007a, 2007b).

2.2 Hepatic Specification

The formation of tissue-specific stem cells is believed to occur at the stage when the three germ layers (endoderm, mesoderm, and ectoderm) are developed from the blastula (Fig. 16.1). The primitive endodermal cells are thought to generate both hepatic and pancreatic stem cells (Murry and Keller 2008). Liver specification signaling is activated at the ventral endoderm (hepatic endoderm) by paracrine secretion of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from the cardiac mesoderm and septum transversum, respectively (Calmont et al. 2006; Rossi et al. 2001; Zaret and Grompe 2008). Recent findings suggest that Wnt/ β -catenin signaling also induces hepatic specification (Ober et al. 2006). Activation of these signaling pathways results in the formation of the liver bud from the hepatic endoderm. The liver bud is considered to be the earliest developmental stage of liver organogenesis, and albumin and alpha-fetoprotein (AFP) are known to be expressed at this stage (Dabeva and Shafritz 2003). Once the hepatic endoderm is specified and the liver bud begins to grow, the cells are called hepatoblasts and have the ability to differentiate into hepatic and biliary lineages. Thus, hepatoblasts are at least bipotent progenitors developed from hepatic endoderm (Fausto 2004).

2.3 Hepatocytic Differentiation

Several cytokines/growth factors are known to be involved in the differentiation of hepatoblasts into hepatocytes (Kinoshita and Miyajima 2002). Oncostatin M (OSM), an interleukin 6-related cytokine produced by CD45+ hematopoietic cells, enhances glucocorticoid mediated hepatocytic differentiation through the activation of the signal transducer and activator of transcription 3 (STAT3) pathway (Kamiya et al. 1999). Hepatocyte growth factor (HGF) is also known to be activated during the process of liver regeneration; and treatment with HGF induces hepatocytic differentiation in hepatoblasts, although the detailed mechanism is still unclear (Kinoshita and Miyajima 2002).

Hepatocytes adjacent to the periportal area are more likely to be responsible for the regeneration and spread of the liver into the pericentral area, and hepatocytes adjacent to the centrilobular region are considered to have a more mature hepatocyte-like phenotype that includes production of serum proteins, e.g., albumin (ALB), alpha-1 antitrypsin, and transferrin (TF), and activation of enzymes involved in xenobiotic metabolism, e.g., cytochrome P450 (CYP) (Fig. 16.1). Thus, markers associated with hepatic liver function, such as production of serum proteins and metabolism of various substrates, are generally used to evaluate liver maturation.

2.4 Hepatocytes as Stem Cells

Once fully developed, hepatocytes in the adult liver have a life expectancy of over a year; and most of hepatocytes remain quiescent and stay in the G0 phase. However, when parenchymal cells are lost, hepatocytes exit the G0 phase and start to proliferate. Hepatocytes are known to have the ability to proliferate almost indefinitely in rodents (Oertel and Shafritz 2008; Overturf et al. 1997). Furthermore, hepatocytes have the potential to differentiate into biliary lineages under special conditions with activation of hepatocyte growth factor/epidermal growth factor (HGF/EGF) signaling (Limaye et al. 2008; Michalopoulos et al. 2005). Thus, in light of the definition of stem cells, hepatocytes have similar features in terms of the potential for self-renewal, differentiation, and unlimited cell proliferation. However, transplanted hepatocytes cannot repopulate the liver without injury and do not behave as stem cells do under normal conditions (Oertel and Shafritz 2008).

2.5 Biliary Differentiation

Cholangiocytes are bile duct epithelial cells developed from hepatoblasts, and defects in bile duct formation result in the impairment of bile flow, or cholestasis. Previous studies demonstrated that mutations in the Jagged1 (JAG1) gene cause Alagille syndrome, which is characterized by cholestasis and jaundice due to intra-hepatic bile duct abnormalities (Li et al. 1997; Oda et al. 1997). Consistently, several studies have demonstrated that the JAG1–Notch signaling pathway plays a crucial role in the differentiation of hepatoblasts into cholangiocytes (Lozier et al. 2008; Tanimizu and Miyajima 2004). Once developed, cholangiocytes are considered to be mitotically dormant and express specific cytokeratins such as CK7 and CK19. However, in the course of chronic liver diseases, cholangiocytes as well as hepatic progenitor cells may start to proliferate in response to stimuli to form ductular reactions in the periportal area (Roskams et al. 2004b), although phenotypes as well as markers expressed on cells of the various cholangiocyte lineages are largely unknown.

3 Heterogeneity of Stem-Cell Marker Expression in Hepatic Progenitor Cells

3.1 Putative Hepatic Stem/Progenitor Cell Markers

Hepatoblasts are considered to have the features of stem cells with respect to self-renewal and asymmetric division, and can repopulate normal and injured liver. Similar small primitive epithelial cells are known to emerge in the periportal area of the injured adult liver when hepatocyte replication is blocked, and these cells are called oval cells (in rodents) or hepatic progenitor cells (Roskams et al. 2003).

Hepatoblasts and hepatic progenitor cells express the biliary markers cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EpCAM) as well as the hepatocyte markers albumin and AFP (Oertel and Shafritz 2008; Schmelzer et al. 2006, 2007; Sell 2003). In addition, numerous studies have demonstrated that hepatic progenitor cells express a variety of markers putatively detected in various ectodermal or mesodermal lineages, including nestin [neural/mesenchymal] (Koenig et al. 2006; Niki et al. 1999; Roskams et al. 2004a), NCAM [neural/mesenchymal] (Roskams et al. 2004a; Schmelzer et al. 2006), CD34, and c-Kit [hematopoietic] (Crosby et al. 2001), CD133 [neural/hematopoietic/mesenchymal] (Kordes et al. 2007; Suzuki et al. 2008), CD90 (Thy-1) [hematopoietic/mesenchymal] (Masson et al. 2006; Weiss et al. 2008), E-cadherin [epithelial] (Nitou et al. 2002), and Dlk1 [epithelial/hematopoietic] (Jensen et al. 2004; Khurana and Mukhopadhyay 2008; Oertel et al. 2008; Tanimizu et al. 2003) (Fig. 16.1). Indeed, these markers are expressed in neural, hematopoietic, mesenchymal, and epithelial stem/progenitor cells that can give rise to neurons, stellate cells, Kupffer cells, endothelial cells, hepatocytes, and cholangiocytes (Dudas et al. 2009; Escribano et al. 1998; Gangenahalli et al. 2006; Gilyarov 2008; Wauthier et al. 2008). Thus far, it is unclear how these markers are expressed in hepatic stem/progenitor cells at a particular developmental stage or whether the expression status of these markers is associated with their functional phenotypes. It is also unclear which would be the most primitive marker detected in hepatic stem cells that can generate relatively differentiated hepatic progenitor cells.

3.2 Heterogeneity of Hepatic Progenitor Cells

Hepatic stem/progenitor cells are considered a heterogeneous population (Jelnes et al. 2007) that is potentially organized in a hierarchical manner with various degrees of differentiation that may be related to their expression of stem-cell markers. Furthermore, the expression status of the stem/maturation markers would be altered to form a gradient, which potentially makes any isolated cell population arbitrary and heterogeneous. Therefore, identification of hepatic progenitor cells using a robust marker is crucial to understanding the heterogeneity of liver lineages during the development/regeneration processes. However, there is a great deal of controversy about the status of marker expression and the cellular phenotypes of stem/progenitor cells.

For example, AFP is one of the earliest markers detected in the liver bud, suggesting that AFP is detected in the most primitive hepatic stem cells. However, recent publications indicate that AFP might not be expressed in EpCAM-positive putative hepatic stem cells from adult and fetal liver that can give rise to AFP-positive hepatoblasts (Schmelzer et al. 2006, 2007). CD90 is a marker detected in both hepatic and hematopoietic stem/progenitor cells, and a recent report suggested that CD90+ cells from adult human livers showed the immunophenotype of CD34+ c-Kit+ CK14+ CK19+ HepPar1+ hepatic progenitors with the capability to engraft in the liver of immunodeficient mice (Weiss et al. 2008). However, another recent study investigating the characteristics of oval cells using EpCAM and CD90

reported that CD90+ cells are more likely to have the features of hepatic stellate cells or activated myofibroblasts in a 2-acetylaminofluorene/partial hepatectomy rat model (Yovchev et al. 2008). Another group also raised a question about the capability of CD90-positive embryonic day (ED) 14 fetal liver cells to repopulate the liver (Oertel et al. 2007). Intriguingly, a recent paper identified a human fetal liver cell population of CD90+ EpCAM+ cells that can give rise to both mesenchymal and hepatic lineages (human fetal liver multipotent progenitor cells (hFLMPCs)) with the immunophenotype of CD34+, CD90+, c-kit+, EpCAM+, c-met+, SSEA-4+, CK18+, CK19+, albumin–, AFP–, CD44h+, and vimentin+ (Dan et al. 2006). Thus, even with the robust stem-cell markers that are widely used for the isolation of hepatic progenitor cells, there are many controversial issues including the differentiation status and the repopulation capability of hepatic progenitors expressing a certain stem-cell marker.

3.3 Factors Affecting the Heterogeneity of Putative Hepatic Stem/Progenitor Cells

Several factors may contribute to the controversy over the phenotypes of the hepatic stem/progenitor cells described above. First, the expression status of stem-cell markers is thought to be gradually altered in hepatic progenitor cells of various developmental stages in the fetal liver. Given this situation, purification of stem-cell marker-positive cells in the fetal liver could be arbitrary and may produce mixtures of hepatic stem/progenitor cells if different thresholds are used. Therefore, the cellular phenotypes of isolated cells could be stem- or progenitor-like depending on the abundance of each cell type, which may be related to the developmental stage of the fetal liver used for isolation.

Second, hematopoiesis takes place in the fetal liver, and various stem/progenitor cells including hematopoietic and mesenchymal cells have emerged and could be co-isolated to various degrees according to the developmental stage of the fetal liver. Indeed, all markers currently used for the isolation of hepatic stem/progenitor cells are not specific to a certain lineage (e.g., c-Kit and CD34 serve as hematopoietic markers; CD90, as a hematopoietic and mesenchymal marker; nestin, as a neural marker; AFP, as a hepatocytic marker; and EpCAM, as a biliary marker) (Fig. 16.1). Therefore, purified putative hepatic stem/progenitor cells may include cells of various lineages, making analysis of the phenotypes of the isolated cells difficult and controversial.

Third, hepatic stem/progenitor cells as well as hematopoietic/mesenchymal stem cells may exhibit cellular plasticity. If hepatic/mesenchymal/hematopoietic stem cells can trans-differentiate into one another, a hepatic stem-cell population isolated using a certain marker could easily be a mixture of hepatic, mesenchymal, and hematopoietic lineages depending on their culture conditions. Consistently, recent papers have suggested the phenotypic reversion of fetal human liver epithelial cells and hematopoietic cells into mesenchymal and epithelial lineages, respectively (Inada et al. 2008; Khurana and Mukhopadhyay 2008).

Fourth, although stem cells' self-renewal and differentiation are tightly regulated by the microenvironment (called the stem-cell niche), the phenotypes as well as potential markers of niche cells (presumed to be located in the "canals of Hering," bile canaliculi lined partially by hepatocytes and partly by cholangiocytes) are still under debate (Kuwahara et al. 2008). The phenotypes of isolated hepatic stem/progenitor cells may be different if these cells are isolated from different niches.

Taken together, the expression patterns of stem-cell markers currently used for the isolation of hepatic stem/progenitor cells are very heterogeneous and may be distinct and time-dependent at the various developmental stages of the liver lineages. Cellular plasticity of isolated stem/progenitor cell populations as well as co-purification of mesenchymal/hematopoietic stem cells may further complicate the results of liver transplantation experiments even if rigorously purified cells are used. Accurate phenotyping of fetal liver cells using robust markers and reliable isolation/culture systems may provide more detailed information about the process of human liver lineages differentiation.

4 Liver Cancer as a Disease of Deregulated Stem Cells

4.1 The CSC Concept

Although considered monoclonal in origin, tumor cells are heterogeneous in terms of morphology, clinical behavior, and molecular profiles (Fialkow 1976; Vogelstein and Kinzler 2004). This heterogeneity has been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of multiple genetic changes (Hanahan and Weinberg 2000). However, recent data have suggested that heterogeneity may also be due to derivation of the tumor cells from stem/progenitor cells residing in the organ (Jordan et al. 2006). The concept of cancer as an abnormal stem-cell disease was proposed many years ago on the basis of the similar capabilities of cancer cells and normal stem cells to self-renew, produce heterogeneous progeny, and divide in an unlimited fashion (Wicha et al. 2006).

The CSC concept, that a subset of cells bearing stem-cell like features are indispensable for tumor development, has recently been revived by the advancement of stem-cell biology (Clarke et al. 2006). Accumulating evidence suggests the involvement of CSCs in the perpetuation of various cancers including leukemia, breast cancer, brain cancer, and colon cancer (Al-Hajj et al. 2003; Bonnet and Dick 1997; Lessard and Sauvageau 2003; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004). Experimentally, putative CSCs have been purified using cell-surface markers specific for normal stem cells, and stem-cell like features have been confirmed by *in vitro* clonogenicity and *in vivo* tumorigenicity assays. CSCs are considered more metastatic and resistant to drugs and radiation than non-CSCs within the tumor, and these findings warrant the development of treatment strategies that can specifically eradicate CSCs (Dean et al. 2005; Rich, 2007).

4.2 Hepatocellular Carcinoma as a Disease of Stem Cells

Recent results of cancer genome sequencing revealed that about ten or more genes are mutated and may be responsible for the development of colorectal cancers (Sjoblom et al. 2006; Wood et al. 2007), which could hardly happen in differentiated colonic epithelial cells because the majority of these cells are exfoliated in a short period of time (Boman and Huang 2008). Thus, it is now widely believed that gastrointestinal cancer is a disease of stem cells with aberrant genetic/epigenetic changes (Takaishi et al. 2008; Zou 2008). However, the cellular origin of HCC is still a controversial issue because both hepatocytes and stem/progenitor cells may reside in the injured liver for a long period to acquire genetic/epigenetic changes.

In rodents, accumulating evidence suggests that HCC may originate from oval cells as well as hepatocytes. For example, HCC developed in the “Solt-Farber” model is thought to originate from oval cells, whereas HCC arising from diethylnitrosamine (DEN) treatment appears to originate from hepatocytes (Sell 2002). Transgenic models that used the albumin promoter to express c-Myc, E2F1, TGF- α , or c-Myc/TGF- α resulted in the formation of HCC in mice, suggesting a role for these genes in the development of HCC that mainly originates from hepatocytes (Calvisi and Thorgeirsson 2005). On the other hand, when fetal hepatoblasts were isolated from TP53 $^{-/-}$ mice by cell sorting using E-cadherin antibodies and transformed by c-Myc, Akt, or Ras, they developed HCCs with a mixture of HCC and CC cellular types (Zender et al. 2006). Furthermore, single hepatoblasts transformed by β -catenin or Bmi1 also developed HCC with mixed cellular types in immunodeficient mice (Chiba et al. 2007).

Altogether, the above observations strongly suggest that HCC may originate from stem/progenitor cells as well as hepatocytes. HCC developed from progenitor cells appears to be a mixed population of HCC and CC with various degrees of expression of hepatic and biliary lineage markers, suggesting that these HCCs continue to maintain the ability to differentiate into both hepatic and biliary lineages.

4.3 Putative Liver Cancer Stem-Cell Markers

The generally acknowledged definition of a CSC is as a cell within a tumor that possesses the capability to self-renew and to give rise to the heterogeneous lineages of cancer cells that comprise tumors in immunodeficient mice (Clarke et al. 2006). Thus far, the expression of six markers, i.e., side population (SP), ATP-binding cassette protein (ABC) G2, CD133, CD90, OV6, and EpCAM, has been experimentally proven in the population of CSCs in human HCCs; and these markers have been used for the isolation of putative liver CSCs.

4.3.1 SP Fraction

The ability to effectively carry out efflux of dyes was first demonstrated in bone marrow cells, and these cells were termed SP cells because they were found to the side

of the peak containing the bulk of dye-positive cells in fluorescent activated cell sorting (FACS) analysis plots (Goodell et al. 1996). These bone marrow cells are highly enriched for long-term repopulating hematopoietic stem cells, and since then, SP cells have been identified in a variety of normal and tumor tissues (Challen and Little 2006). The ability to regulate the efflux of Hoechst dyes appears to be conferred in part through the expression of ATP-binding cassette protein (ABC) transporters because treatment with the ABC transporter inhibitor verapamil reduces the number of cells in the SP fraction (Wu and Alman 2008). The degree of efflux activity appears to correlate inversely with the maturation state, and the cells exhibiting the highest efflux activity appear to be the most primitive. Chiba et al. investigated the existence of the SP fraction in four HCC cell lines using Hoechst 33342 dye and identified the SP fraction in HuH7 and PLC/PRL5 cells (Chiba et al. 2006). They demonstrated that AFP+ CK19+ cells are enriched in the SP fraction and form tumors more efficiently compared with non-SP cells in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. SP cells isolated from HCC cell lines may be related to the metastatic and chemoresistant capability of these tumors (Shi et al. 2008), and may have activation of anti-apoptotic signaling through Bcl-2 and Bax regulation (Fan et al. 2007).

4.3.2 ABCG2

Resistance to chemotherapeutic agents is one of the hallmarks of CSCs (Dean et al. 2005), and ABC transporters are believed to play a central role in the efflux of chemical reagents. Especially, the ABC transporter ABCG2 is believed to be essential to pump out Hoechst dyes and maintain the SP fraction (Zhou et al. 2001). Zen et al. (2007) investigated the expression of ABCG2 in two human HCC cell lines and showed a hierarchy of cancer cells with respect to ABCG2 expression. Expression of AFP and CK19 is mainly detected in ABCG2+ HCC cells, and ABCG2+ cancer cells are detected in clinical HCC specimens. ABCG2 expression may be related to doxorubicin resistance, and Akt signaling may affect chemoresistance through alteration of the subcellular localization of ABCG2 (Hu et al. 2008).

4.3.3 CD133 (Prominin 1)

CD133 was the first identified member of the prominin family of pentaspan membrane proteins recognized by an AC133 monoclonal antibody and was originally classified as a marker of primitive hematopoietic stem cells (Yin et al. 1997). In addition, CD133 is known to be expressed in neural, hepatic, colonic, and endothelial stem/progenitor cells (Mizrak et al. 2008). Discrepancies exist concerning the gene expression status (AC133 mRNA) and the protein levels recognized by AC133 antibody, possibly because of the glycosylation status of CD133 and the existence of a splice variant (termed as AC133-2).

Expanding evidence highlights the utility of CD133 as a marker for CSCs in various human tumors including brain, colon, pancreas, and prostate cancers (Visvader and Lindeman 2008). Ma et al. (2007) investigated the processes of liver regeneration using a partial hepatectomy model to identify the emergence of CD133+ cells

in the resected liver. They further discovered that CD133+ cells act as CSCs that are chemoresistant in HCC (Ma et al. 2007, 2008b). Similar findings were reported by several groups (Suetsugu et al. 2006; Yin et al. 2007), and a recent paper further suggested that an aldehyde dehydrogenase (ALDH) + cell population among the CD133+ cells may be more tumorigenic CSCs (Ma et al. 2008a).

4.3.4 CD90 (Thy-1)

CD90 is a glycosylphosphatidylinositol (GPI)-anchored protein that is particularly abundant on the surface of thymocytes and T cells; but CD90 is also expressed in various cell types including fibroblasts, endothelial cells, neurons, and hematopoietic cells (Rege and Hagood 2006). Yang et al. (2008b, 2008c) recently investigated the expression of CD90 and CD44 in CD45-depleted primary HCC cells and peripheral blood mononuclear cells and identified that CD45– CD90+ tumor cells were more tumorigenic if they expressed CD44, Oct4, Bmi1, Albumin, AFP, Wnt3a, stat3, and HIF-1 α . They further demonstrated that CD90+ CD44+ cells were more aggressive than CD90+ CD44– cells, and CD44 blockage prevented tumor formation by the CD90+ cells. On the basis of these data, the authors suggested that the presence of CD45– CD90+ cells in a population could be used as a marker for human liver cancer and as a target for the diagnosis and therapy of HCC.

4.3.5 OV6

Anti-OV6 is one of the monoclonal antibodies previously developed against cells isolated from carcinogen-treated rat liver (Dunsford and Sell 1989) and is known to react with oval cells and normal bile duct epithelial cells (Van Den Heuvel et al. 2001). The antigen recognized by anti-OV6 monoclonal antibodies in human liver has not yet been determined (Strain et al. 2003). Yang et al. (2008a) recently isolated OV6+ cells from human HCC to demonstrate that OV6+ cells have CSC-like features such as high tumorigenic capability and chemoresistance (Yang et al. 2008a). The authors further showed that OV6+ cells were characterized by the activation of Wnt/ β -catenin signaling and inactivation of this signaling pathway resulted in a decrease in the OV6+ cell population. These results suggest that Wnt/ β -catenin signaling may be a good target for eradication of OV6+ liver CSCs.

4.3.6 EpCAM (Epithelial Cell Adhesion/Activating Molecule, CD326)

EpCAM is one of the first tumor-associated antigens identified (Herlyn et al. 1979) and has numerous synonyms including 17-1A, HEA125, MK-1, GA733-2, EGP-2, EGP34, KSA, TROP-1, ESA, and KS1/4. EpCAM is expressed in a large variety of human adenocarcinomas and squamous cell carcinomas (Went et al. 2006), but the function as well as the regulatory mechanisms of EpCAM expression remained largely unknown to date (Balzar et al. 1999). We recently showed that the expression of the EpCAM gene (TACSTD1) is activated by Wnt/ β -catenin signaling in a cis-regulatory mechanism (Yamashita et al. 2007). Furthermore, a very recent paper

suggested that the EpCAM intracellular domain is cleaved at the cell membrane and associates with β -catenin and Lef-1 in the nucleus to activate Wnt/ β -catenin signaling (Maetzel et al. 2009). These data suggest that EpCAM is not just a cell-surface molecule, but a signal transducer regulated by Wnt/ β -catenin signaling in a positive-feedback manner.

EpCAM is used for the isolation of CSCs from various tumors including colonic and pancreatic cancers (Visvader and Lindeman 2008). We recently used EpCAM and AFP to identify the novel prognostic HCC subtypes related to a certain developmental stage of human liver lineages (Yamashita et al. 2008). Furthermore, we isolated EpCAM+ HCC cells from primary HCC samples and cell lines to show that EpCAM+ cells have the features of CSCs (Yamashita et al. 2009). Activation of Wnt/ β -catenin signaling enriched the population of EpCAM+ CSCs, and blockage of EpCAM expression resulted in the inhibition of tumor formation by EpCAM+ cells in NOD/SCID mice. Thus, EpCAM seems a potentially useful marker and a good target for isolation and elimination of liver CSCs.

4.4 Heterogeneity of Liver Cancer Stem Cells

Although the markers listed above have been shown to be useful for the isolation of putative CSCs, it is unclear how these markers are expressed in primary HCC tissues or in HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all or are restricted to a certain subtype of HCCs. Furthermore, primary HCC tissues are composed of mixtures of mesenchymal/endothelial/inflammatory cells as well as tumor epithelial cells, and isolation of CSCs using such markers may result in the isolation of mixtures of tumor epithelial and stromal cells, a problem similar to that observed in the isolation of normal hepatic stem/progenitor cells (Fig. 16.2). Because recent findings have suggested the significance of stromal cells in tumorigenesis and metastasis of cancer (Dome et al. 2009; Karnoub et al. 2007; Mishra et al. 2008), it is possible that co-isolation of stromal cells may result in an enhanced tumorigenicity in immunodeficient mice that may not be related to the stem-like traits of tumor epithelial cells.

We recently investigated the expression of EpCAM (epithelial), CD133 (epithelial/hematopoietic/neural/endothelial), and CD90 (hematopoietic/mesenchymal/endothelial) in six HCC cell lines (Yamashita et al. 2009). Interestingly, AFP+ HCC cell lines (Hep3B, HuH1, and HuH7) have a subpopulation of EpCAM+ CD133+ cells but no CD90+ cells. In contrast, AFP- HCC cell lines (SK-Hep-1, HLE, and HLF) have a subpopulation of CD90+ cells but no EpCAM+ or CD133+ cells. Thus, AFP+ HCC cells may be more likely to have a subpopulation of epithelial CSC-markers+ cells (epithelial CSCs), whereas AFP- HCC cells may have a subpopulation of mesenchymal-markers+ cells (mesenchymal CSCs) (Fig. 16.2). These data suggest that CSC markers may not be equally expressed in all HCCs. Instead, the expression patterns of CSC markers in liver CSCs may be different in each HCC subtype, possibly due to the heterogeneity of activated signaling

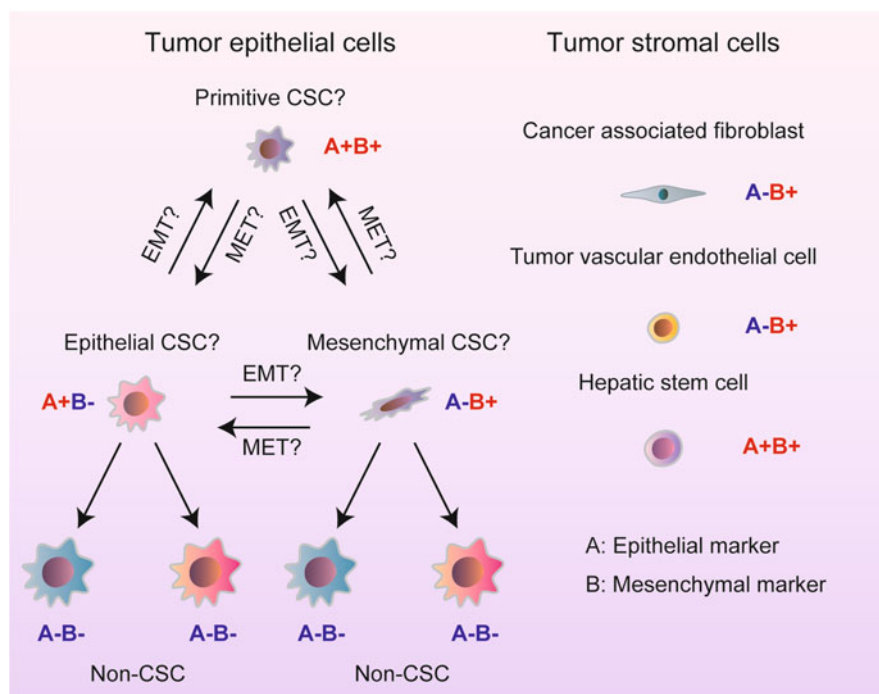


Fig. 16.2 A hypothetical model of CSC heterogeneity and hierarchy. Both tumor epithelial cells and stromal cells may express the same markers currently used for the isolation of CSCs. Although CSCs are considered to be metastatic (mesenchymal CSCs?), tumorigenic (epithelial CSCs?), and drug/radiation-resistant (primitive CSCs?), these phenotypes may be distinct in each CSC subtype having distinct stem-cell markers that may be associated with EMT/MET signaling

pathways and stem-cell marker expression in normal hepatic stem/progenitor cells where these tumor-initiating cells may originate. Although primitive hepatic stem cells expressing both epithelial and mesenchymal markers (e.g., CD90+ EpCAM+) do exist in the fetal liver (Dan et al. 2006), it is unclear whether HCC subtypes containing primitive CSCs are present in primary HCC as well as in HCC cell lines that express epithelial and mesenchymal markers. Investigation and characterization of HCCs containing such primitive CSCs will be of interest in the future.

The epithelial–mesenchymal transition (EMT) and the reverse process, termed mesenchymal–epithelial transition (MET), are known to play a crucial role in embryonic development (Hugo et al. 2007). Accumulating evidence indicates that EMT also confers some important malignant traits of cancer, especially metastasis (Turley et al. 2008). CSCs are considered to be more metastatic than non-CSCs, and recently the concepts of CSC and EMT have emerged in the field of breast cancer research (Mani et al. 2008; Morel et al. 2008). In breast cancer, a population expressing low levels of CD24 (an epithelial marker) and high levels of CD44 (a

mesenchymal marker) is known to be representative of breast CSCs (Al-Hajj et al. 2003). Interestingly, activation of the EMT program by the induction of Snail or Twist genes or addition of recombinant TGF- β resulted in the enrichment of a CD24-low CD44-high population that had a high capability to form spheroids in vitro and subcutaneous tumors in vivo (Mani et al. 2008). Induction of oncogenic Ras also induced EMT and enriched the CSC population in breast cancer cells (Morel et al. 2008). In the liver, TGF- β signaling appears to induce the differentiation of hepatic stem/progenitor cells and suppress the development of HCC (Mishra et al. 2009), suggesting that it may not work in the same manner observed in breast cancers. Regardless, the association between the liver CSC phenotypes and the induction of EMT/MET programs is completely unclear and should be pursued in future studies (Fig. 16.2).

5 Conclusions

There is accumulating evidence that liver CSCs play a key role in the development and perpetuation of HCC, and the relevance of targeting CSCs has also become clear. Yet, experimental models for the treatment of HCC are still in the preliminary stages. Identification of useful CSC markers and exploration of their roles in maintaining stem-like traits are critical steps toward the clinical application of the CSC hypothesis for the improved diagnosis and the treatment of HCC.

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Chapter 17

Cancer Stem Cells in Liver Carcinoma

Tania Roskams

Abstract According to the cancer stem-cell concept, hepatocellular carcinoma (HCC) consists of a hierarchy of cell populations, of which the very small cancer stem-cell population is the one that has the growth and metastatic potential of the tumor. The other neoplastic cells are offspring of the cancer stem cells and each can be differentiated a little differently, according to the local microenvironment in each part of the tumor, hence explaining the enormous phenotypic heterogeneity of a neoplasm. Current therapeutic strategies mostly target rapidly growing differentiated tumor cells. However, the results are often unsatisfactory because of the chemoresistance of HCC. New therapies targeting cancer stem cells should therefore be developed. A prerequisite is a good understanding of the mechanisms of activation and differentiation of normal stem/progenitor cells in normal and diseased liver. Hepatocytes and cholangiocytes not only have stem-cell features, but also have progenitor cells, located in the smallest branches of the biliary tree. These cells are especially activated in the cirrhotic stage of chronic liver diseases, the stage in which HCC develops. HCC with progenitor cell features, possibly reflecting a progenitor cell origin, have a very bad prognosis and therefore should be recognized and treated accordingly.

Keywords Liver stem cells · liver progenitor cells · hepatocellular carcinoma · cholangiocarcinoma · chemoresistance · cancer stem cells · side population

1 Introduction

In the nineteenth century, pathologists like Virchow observed that some tumors exhibit features of a whole range of different organs (teratocarcinoma) and hypothesized that these tumors originated from embryonal rests (Sell 2004). Today's stem

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cells are the modern equivalent of embryonal rests: tumors can originate from malignant transformation of a stem cell on its way to a differentiated mature cell type in a given organ (maturation arrest). In continuously renewing systems like the epidermis, the gut, and the haematopoietic tissue, it is widely accepted that cancer arises from stem cells, since stem cells are the only cells which have a life span long enough to acquire the requisite number of genetic changes for neoplastic development. The liver however is a silent organ in which several cell types not only have longevity and proliferative potential: hepatocytes, cholangiocytes, but also have bipotential progenitor cells residing in the most terminal branches of the biliary tree, the ductules, and/or canals of Hering (Roskams et al. 2004). This implies that in the liver several cell types can be carcinogen targets (Fig. 17.1).

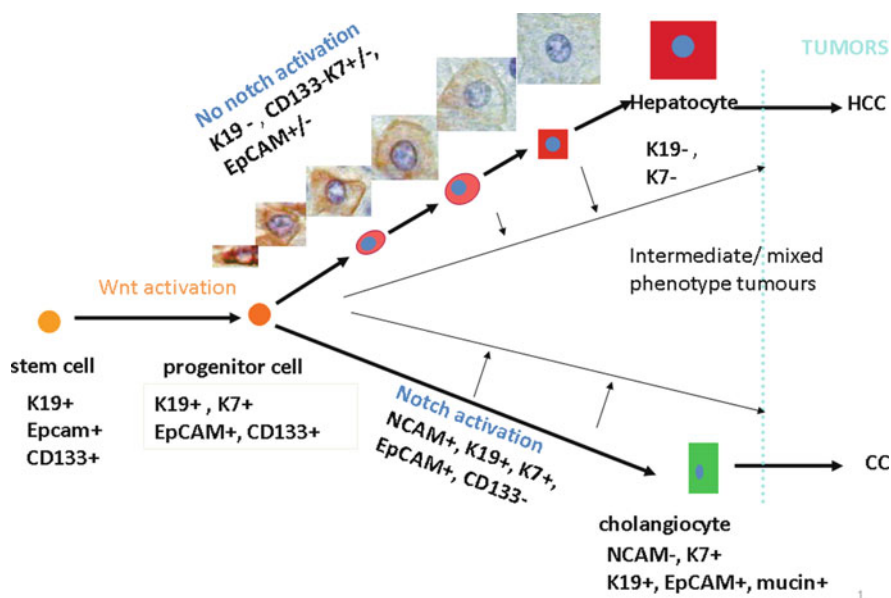


Fig. 17.1 Liver stem/progenitor cells can differentiate into hepatocytes and cholangiocytes, both during embryogenesis and regeneration after injury. After injury the differentiation of stem/progenitor cells depends on the site of injury: after hepatocytes loss, progenitor cells differentiate into hepatocytes, after bile duct loss, they form new cholangiocytes. On the way to the differentiated cell type, intermediate cell types are formed. Each of these intermediate cell types can become a cancer stem cell according to the maturation arrest theory. This explains the huge diversity of phenotypes within a tumor. A whole range of phenotypes with traits of progenitor cells, hepatocytes, and cholangiocytes can be formed. Also, mature hepatocyte or cholangiocytes can act as cancer stem cells, since these cells also have stem cell properties. They then form mature hepatocellular carcinomas or cholangiocellular carcinomas

A re-emphasized concept is that of the cancer stem cell, which is the cell renewal source of a neoplasm and the seed for metastasis (Hamburger and Salmon 1977), expressing similar toxic drug exporting protein pumps as the non-neoplastic stem cell. According to this concept, a tumor consists of a hierarchy of cell populations,

of which the very small cancer stem-cell population is the one that has the growth and metastatic potential of the tumor. The other neoplastic cells are offspring of the cancer stem cells and each can differentiate a little differently, according to the local microenvironment in each part of the tumor, hence explaining the enormous heterogeneity of the phenotype of a neoplasm in different areas (Fig. 17.2). Targeting the liver cancer stem cells sounds like the ideal treatment for liver cancer.

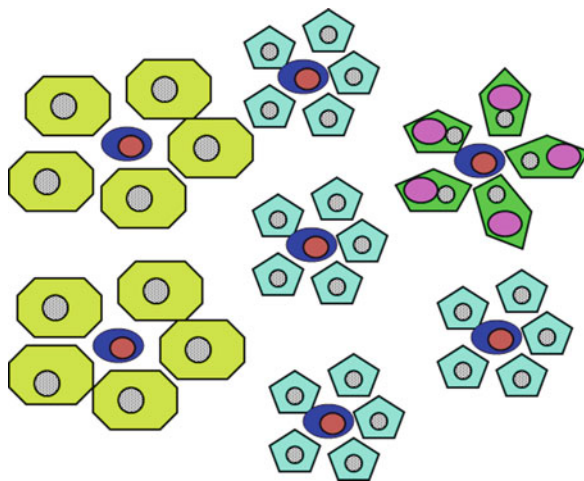


Fig. 17.2 Schematic representation of a clonal expansion of progenitor cells (small blue oval cells) and their offspring. In the center of the tumor, where there is more ischemia, the cancer stem cells (in this instance a progenitor cell) can, e.g., differentiate into cholangiocytes, because these are more resistant to chemotherapy. At the border of the tumor, where there is contact with surrounding non-neoplastic hepatocytes, the microenvironment can make the cancer stem cells differentiate into hepatocytes (yellow cells). Even mucus producing cells can be formed (cells with pink “mucus” droplet). Such heterogeneous tumors are also what a pathologist sees in clinical practice

2 The Cancer Stem-Cell Concept

The classical model for cancer proposes that genetic alterations transform mature, differentiated cells into malignant tumor cells. Conversely, according to the concept of cancer stem cells, a tumor consists of a hierarchy of cell populations, of which the very small cancer stem-cell population is the one that has the growth and metastatic potential of the tumor. The other neoplastic cells are offspring of the cancer stem cells (Hamburger and Salmon 1977). The cancer stem-cell concept can help to explain two of the most challenging aspects in cancer therapy: remission and relapse. Common clinical experience shows that radiation and/or chemotherapy often induces regression of a tumor to a minimum where it no longer can be detected, yet, unfortunately, the tumor returns. The concept of cancer stem cells has aroused the hope that specifically targeting these cells will allow more effective cancer treatment and complete tumor regression.

Research focused on the cancer stem-cell hypothesis started more than 10 years ago with an intriguing discovery by Bonnet and Dick (1997). They demonstrated that human acute myeloid leukemia (AML) arises from the malignant transformation of primitive hematopoietic cells, rather than from committed and thus differentiated progenitor cells. Importantly, these tumor-initiating “bad stem cells” possessed the capacity to differentiate and proliferate as expected for normal hematopoietic stem cells. Moreover, leukemic cancer stem cells expressed cell-surface markers typically found on normal hematopoietic stem cells. To date, a number of cancer stem-cell specific markers have been reported for a variety of human cancers including leukemias and colon, brain, pancreas, and breast cancers.

What are the common properties of cancer stem cells and normal stem cells and – even more important – are there differences, which would allow to specifically target cancer stem cells? In fact, normal and cancer stem cells seem to have a lot in common (Table 17.1). Evidence that hematopoietic stem cells reconstitute and sustain adult tissues had been already provided by pioneering work in the 1950s and 1960s when McCulloch and Till (1960) demonstrated the survival of irradiated recipient mice after transplantation of marrow cells from non-irradiated donor mice. Today we know that injection of a single hematopoietic stem cell might be sufficient for reconstituting hematopoiesis (Cao et al. 2004). In analogy, only rare human cancer stem cells have been shown to propagate and sustain leukemia in immune-compromised mice (Bonnet and Dick 1997), and injection of a single human melanoma stem cell was sufficient for tumor formation in murine recipients (Quintana et al. 2008). One additional common denominator for normal and cancer stem cells is that although both have the capacity of unlimited self-renewal, a small population of “dormant stem cells” is very rarely dividing throughout our lifetime (Hope et al. 2004; Wilson et al. 2008). A possible consequence of dormant cancer stem cells is therefore that these cells are possibly more resistant to toxic insults and thus might successfully survive treatment with chemotherapeutic drugs. Recent insight into the regulation of normal as well as cancer stem cells points to the stem-cell niche, a specific microenvironment that protects these cells and imposes

Table 17.1 Properties shared by *normal* and *cancer* stem cells

1. <i>Capacity for self-renewal</i>
2. <i>Ability of multilineage differentiation</i> Heterogeneous offspring
3. <i>Active telomerase expression</i> Longevity
4. <i>Activation of anti-apoptotic pathways</i> Longevity
5. <i>Increased membrane transporter activity</i> Drug- and toxin resistance
6. <i>Ability to migrate and metastasize</i> Anchorage independence: Anoikis

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regulatory cues on their behavior. For instance, endothelial cells closely interact with self-renewing brain tumor stem cells and also secrete factors that maintain these cancer cells in a stem cell-like state (Calabrese et al. 2007). Importantly, depletion of blood vessels from orthotopic brain tumor xenografts ablated self-renewing cells from these tumors and arrested tumor growth. These findings suggest that vascular niches might be important targets for therapeutic anti-cancer strategies. Last but not least, normal stem cells are present in many adult tissues, and thus do represent a potential life-long reservoir for malignant transformation (reviewed in (Ailles and Weissman 2007)).

However, although the concept of cancer stem cells is intriguing and a large number of experimental studies support the cancer stem-cell hypothesis, there are still open questions and plenty of room for caution. One debate concerns the origin of cancer stem cells. Does the cancer stem cell derive initially from a normal stem cell or from a dedifferentiated cell during tumor progression? Their functional similarities with normal stem cells and the observation that they often share specific surface markers at least argue for mutated stem cells as their origin. Furthermore, in contrast to data obtained with human leukemic stem cells, nearly all leukemic mouse cells were proficient in initiating leukemia after transplantation into congenic mice. This raises the important question if cancer stem cells really exist or if they are only the result of trans-species transplantation (Kelly et al. 2007). One argument for the existence of cancer stem cells is the very recent success in specifically targeting them. Yilmaz and coworkers demonstrated that normal hematopoietic stem cells can be distinguished from leukemia-initiating cells by their dependence on the expression of the tumor suppressor PTEN. In these experiments, conditional deletion of PTEN in mice led, on the one hand, to myeloproliferative disease within days and, on the other hand, initially promoted proliferation of normal hematopoietic stem cells (Yilmaz et al. 2006). However, this finally led to a depletion of normal hematopoietic stem cells due to a compensatory mechanism. Hence, hematopoietic stem cells need PTEN for normal function. Mechanistically, these effects were mainly mediated by mTOR because Rapamycin, an inhibitor of mTOR, did not only deplete leukemia-initiating cells, but also restored the function of normal hematopoietic stem cells. Unambiguously demonstrating the role of cancer stem cells for maintaining different cancers and identifying mechanistic differences between normal and cancer stem cells will be an important and challenging task and might eventually improve the efficiency of cancer therapy.

3 Hepatocytes Have “Stem Cell” Properties

In normal circumstances, the liver hardly proliferates. Hepatocytes in normal adult liver have a life span of over a year. After partial hepatectomy however proliferation of the main epithelial compartments (hepatocytes and cholangiocytes), followed by proliferation of the mesenchymal cells (hepatic stellate cells and endothelial cells), quickly restores the liver. In rodents, the liver can restore its original volume after two-thirds hepatectomy in approximately 10 days (Michalopoulos and DeFrances

1997; Fausto 2004). Serial transplantation experiments have shown that hepatocytes have a near infinite capacity to proliferate (Overturf et al. 1999). Since at least 69 doublings can occur, the clonogenic potential of hepatocytes, one of the crucial properties of a stem cell, is confirmed. In the diethylnitrosamine (DEN) rat model, a direct lineage relationship between mature hepatocytes and hepatocellular carcinoma has been shown (Bralet et al. 2002).

4 Hepatic Progenitor Cells are Activated in the Majority of Chronic Liver Diseases and form a Target Cell Population for Carcinogenesis

When the mature epithelial cell compartments of the liver, hepatocytes, and/or cholangiocytes are damaged or inhibited in their replication, a reserve cell compartment is activated (Roskams et al. 2003). This compartment, in human called the progenitor cell compartment and in rodents the oval cell compartment, resides in the smallest and most peripheral branches of the biliary tree, the ductules, and canals of Hering (Roskams et al. 2004). These progenitor cells can differentiate into hepatocytes and cholangiocytes via intermediate cell types (Fig. 17.1). Wilson and Leduc were the first to describe this activation of a “reserve cell compartment” in mouse after severe dietary injury (Wilson and Leduc 1958). Subsequently, several models of so-called oval cell reaction in rodents have been described. Mostly, these models employed potential carcinogenic agents to inhibit the proliferation of mature hepatocytes after a regenerative stimulus. For example, acetaminofluorene intoxication of rats, after partial hepatectomy or after administration of a necrogenic dose of carbon tetrachloride (Alison et al. 1996) or ethionine intoxication in mice. Also, models of fatty liver disease like ObOb-mice or PARP1(-/-) mice are characterized by inhibition of the replication of mature hepatocytes, caused by oxidative stress and show a striking oval cell response (Roskams et al. 2003; Yang et al. 2004).

In parallel to what we know from these rodent models, also in human liver diseases there is inhibition of replication of mature hepatocytes. Recently it has been shown that hepatocytes are senescent due to telomere shortening, in the cirrhotic stage of a wide variety of chronic human liver diseases. Intriguingly, mesenchymal cells like endothelial cells and hepatic stellate cells do not show this replicative senescence (Wiemann et al. 2002; Marshall et al. 2005). Probably this hepatocyte replicative senescence is in part the result of ongoing proliferation during 20–30 years of chronic liver disease. Chronic inflammation, presence of growth factors, DNA-damaging agents like reactive oxygen species and nitrogen species also play a role. So, similar to rodent models, replicative senescence of hepatocytes triggers progenitor cell activation also in man.

The activation of oval cells or in human liver called “ductular reaction,” comprises expansion of a transit amplifying cell compartment of small biliary cells which can differentiate into at least biliary epithelial cells and hepatocytes. The progenitor cells are labeled by biliary type cytokeratins (CK) CK7 and 19, oval cell markers OV6 and OV1, neuroendocrine markers chromogranin-A, neural cell

adhesion molecule and parathyroid hormone-related peptide, and connexin 43. A subpopulation of ductular/progenitor cells express markers of haematopoietic cells (CD34, C-kit, flt-3, CD133), which raised the question whether progenitor cells would be directly derived from bone marrow stem cells. Most studies however are concordant with a progenitor cell niche in the ductules/canals of Hering, at the interface between the parenchyma and the portal tract mesenchyme. This is also the location where during embryonic development, bipotential hepatoblasts form the primitive ductal plate, which has the same phenotype as progenitor cells in adult life: ductal plate cells express biliary markers and (immature) hepatocytic markers like alpha-fetoprotein and in addition haematopoietic markers like CD34.

The majority of chronic human liver diseases are characterized by progenitor cell activation or ductular reaction, which is the human equivalent of rodent oval cell activation. Reactive ductules form strands of small cholangiocytes with an oval nucleus and a small rim of cytoplasm (Roskams and Desmet 1998). The degree of progenitor cell activation increases with the severity of the disease (Lowe et al. 1999). In chronic hepatitis, progenitor cell activation correlates with the degree of inflammation (Libbrecht et al. 2000). In a variety of chronic liver diseases like chronic hepatitis C, haemochromatosis, and (non)alcoholic steatohepatitis, the degree of progenitor cell activation also correlates with the degree of fibrosis, the stage of the disease (Lowe et al. 1999; Roskams et al. 2003). In moderate and severe degrees of inflammation, intermediate hepatocytes occur, having a phenotype intermediate between progenitor cells/ductular cells and mature hepatocytes. The number of these intermediate hepatocytes gradually increases with higher degrees of inflammation, and also with higher degrees of necrosis in necrotising hepatitis and with more advanced stages of (non)alcoholic steatohepatitis (Lowe et al. 1999; Libbrecht et al. 2000; Roskams et al. 2003; Katoonizadeh et al. 2006). This highly suggests a higher degree of differentiation of progenitor cells into hepatocytes when there is more hepatocyte damage. In cirrhotic livers, especially in (non)alcoholic steatohepatitis, whole cirrhotic nodules can be composed of intermediate hepatocytes, which ultrastructurally look strikingly “normal,” without Mallory body formation and without fatty change, suggesting that these intermediate hepatocyte-nodules originate from progenitor cells (Roskams et al. 2003). In parallel, Falkowski et al. (2003) showed in a 3D reconstruction study that sequestered hepatocyte “buds” in cirrhosis are always in continuity with reactive ductules, highly suggesting a progenitor cell origin. As already stated above, cirrhotic stages of a wide variety of chronic liver diseases are characterized by hepatocyte senescence, being a classical trigger for progenitor cell activation in rodents. In an environment with reactive oxygen species, growth factors, chronic inflammation, the progenitor cell compartment is a target cell population for carcinogenesis.

There have been a number of papers illustrating the role of bone marrow stem cells in producing hepatocytes, both in animal models and in human. Their precise role is not clear, since in several models, it has been shown that cell fusion of bone marrow stem cells with damaged hepatocytes took place (Thorgeirsson and Grisham 2006). A role for bone marrow stem cells in cancer formation has not yet been shown, so a detailed discussion on these cells is beyond the scope of this review.

How do liver stem cells drive cancer initiation? By using microdissection and analysis of the molecular pathways of activated progenitor cells, we could show that Wnt signaling plays an pivotal role in expansion of non-neoplastic human progenitor cells, while notch activation is important for biliary differentiation and notch inhibition is necessary to have hepatocyte differentiation (Spee et al. 2010) (Fig. 17.1). An excessive and persistent self-renewal signal, involving Wnt/beta-catenin and BMI-1 is also one of the key events in early carcinogenesis (Wicha et al. 2006; Taniguchi and Chiba 2008; Yang et al. 2008a; Armengol et al. 2009).

5 Liver Cancer with Progenitor Cell Phenotypical Features: Maturation Arrest or Dedifferentiation?

In adult life, the two major primary liver cancers are hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CC). In addition mixed forms of HCC and CC are described. When more detailed immunohistochemical phenotype is performed, a whole range of phenotypical traits of hepatocytes, cholangiocytes, and progenitor cells can be seen in tumors, being consistent with a progenitor cell origin (Figs. 17.1 and 17.2).

Several studies have shown that liver tumors are monoclonal, i.e., derived from a single cell. The question is of course which cell: hepatocytes, cholangiocytes, progenitor cells, or all three, since all these different cell types have the required longevity and long-term repopulating potential.

Animal models show that hepatocytes are implicated in some models of HCC, while other models, using direct injury to the essentially unipotent cholangiocytes induce CC. Progenitor cells (oval cells in rodents) are activated in many animal models, just like in many instances of human liver damage, irrespective of etiology, making such cells very likely carcinogen targets. If progenitor cells do give rise to cancer during their maturation/differentiation process (maturation arrest theory), one would expect a range of neoplastic phenotypes, recapitulating stages in normal development, a prediction supported experimentally (Hixson et al. 2000), as well as in human liver tumors (see below). Direct evidence for the involvement of oval cells (progenitor cells) in the histogenesis of HCC was obtained by Dumble et al. (2002) who isolated oval cells from p53 null mice. When these cells were transplanted into athymic nude mice they produced HCCs.

In **human**, chronic viral hepatitis B and C, alcoholic and non-alcoholic steatohepatitis, metabolic diseases, and mutagens like aflatoxins (toxic metabolites of the food mould *Aspergillus* sp.) are the most important risk factors for the development of HCC. Chronic inflammatory biliary diseases like primary sclerosing cholangitis, hepatolithiasis (gall stones) and liver fluke infection by *Opisthorchis viverrini* and *Clonorchis sinensis* are known risk factors for the development of cholangiocarcinomas. This underscores that oxidative stress and chronic inflammation form common carcinogenic risk factors in all primary liver cancers.

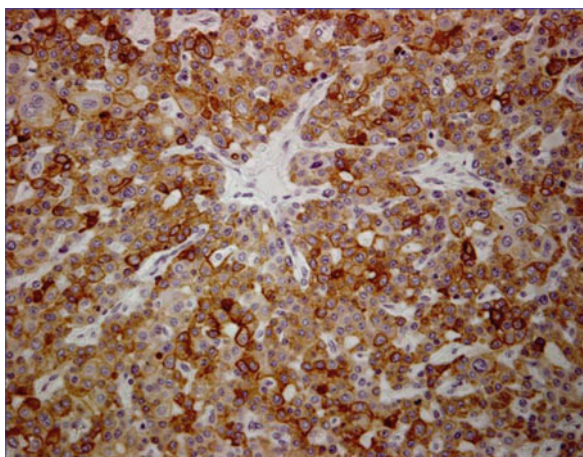
As in rodents, HCC and CC evolve from focal precursor lesions that reflect the stages of multistep carcinogenesis (Libbrecht et al. 2005).

Since progenitor cells are activated in most chronic liver diseases which are known risk factors for the development of hepatocellular carcinoma as well as cholangiocellular carcinoma, progenitor cells are potential target cells for carcinogenesis.

Most tumors show still phenotypical features of their cell of origin and the histopathological classification of tumors is largely based on this. Several studies using detailed immunophenotyping of HCCs, showed that a substantial number (ranging from to 28–50%) of human HCCs express markers of progenitor/biliary cells like cytokeratin 7, 19, OV6 (Van Eyken et al. 1988; Hsia et al. 1992; Wu et al. 1999; Yoon et al. 1999; Uenishi et al. 2003).

Morphologically, these tumors consist of cells with a very immature phenotype and a range of cells with intermediate phenotypes in between progenitor cells and hepatocytes. Especially cytokeratin 19 expression in HCC (Fig. 17.3) has been associated with a worse prognosis and faster and more recurrence after surgical treatment. Wu et al. (1999) observed a significantly shorter survival in patients with HCCs expressing AE1-AE3 and CK19 without any treatment. Uenishi et al. (2003) recently reported that HCCs expressing CK19 and CK7 have a lower tumor free survival rate after curative resection and demonstrated that CK19 expression was an independent predictor of postoperative recurrence (Uenishi et al. 2003). A recent study by Ding et al. (2004) correlated overexpression of CK19 with HCC metastasis. In our own consecutive series of 109 HCCs, CK19-positive tumors (in more than 5% of tumor cells) had a higher rate of tumor recurrence after liver transplantation, as compared to CK-negative HCCs (Durnez et al Histopathology 2006).

Fig. 17.3 K19-positive HCC, consisting of small oval cells in continuity with a range of intermediate cells with larger cytoplasm and a submembranous staining pattern of keratin, such as is seen in non-neoplastic intermediate hepatocytes



CK19 expression was significantly associated with elevated serum alpha-fetoprotein (> 400 ng/ml), expression of alpha-fetoprotein by the tumor, the presence in serum of anti-hepatitis B core, a lower degree of fibrosis in the non-tumoral parenchyma, and less nuclear beta-catenin expression. The association with

alpha-fetoprotein, being also a marker of progenitor cells, is compatible with a progenitor cell origin of these tumors.

Aishima et al (2007) studied 35 small HCC (<3 cm) with biliary differentiation based on morphology, K19 immunoeexpression, and mucin secretion and compared them with 61 ordinary HCC. They found that extrahepatic recurrence was more common in K19(+) HCC, and that K19(+)/mucin(+) HCC had worse survival. More recently, Zhuang et al. (2008) recognized two pathologic types of HCC with lymph node metastasis based on their K19-immunophenotype. They studied 172 HCC with or without positive lymph nodes (LN) and showed that K19 immunoreactivity was an independent prognostic factor for development of LN metastasis. Moreover, in the subgroup of LN(+) HCC, patients with K19 expressing HCC had shorter overall survival.

Of course, the presence of progenitor cell features in a tumor can be explained in two ways: either the cell of origin is a progenitor cell (maturation arrest theory) or alternatively, tumors dedifferentiate and acquire progenitor cell features during carcinogenesis (dedifferentiation theory). When progenitor cells are the cell of origin of a subtype of primary liver tumors, one would expect that the earliest premalignant precursor lesions also would consist of progenitor cells and their progeny. This is indeed the case: 55% of small cell dysplastic foci (smaller than 1 mm), the earliest premalignant lesions known to date in humans, consist of progenitor cells and intermediate hepatocytes. This is a very strong argument in favor of the progenitor cell origin of at least part of the HCCs. Large cells “dysplastic” foci, on the other hand, consist of mature senescent hepatocytes being a result of continuous proliferation in chronic liver diseases and not the true precursor lesions of HCC.

Also in hepatoblastomas, the most common liver tumor in childhood, cells resembling progenitor cells have been noted (Ruck et al. 1996; Xiao et al. 2003; Fiegel et al. 2004). This tumor is widely believed to be of stem cell origin, since it can be composed of both epithelial and mesenchymal tissue components. A recent study identified two major subclasses of hepatoblastomas, resembling early and late phases of prenatal liver development and presenting distinctive chromosomal alterations. It has been shown that the molecular signature of Wnt/beta-catenin signaling in hepatoblastoma is mainly imposed by liver context, but differs according to developmental stage. Finally, the differentiation stage of tumor cells strongly influences their invasive and metastatic properties, therefore affecting clinical behavior. Tumors with worse clinical behavior also express K19(Armengol et al. 2009).

The prognostic value of K19 expression in HCC was further supported by microarray-based gene expression profiling. Study of the global gene expression pattern in 91 HCCs from China and Belgium revealed two distinctive subclasses (A and B) that were highly associated with patient survival (Lee et al. 2004). A subsequent integrative functional genomics study compared the gene expression data of an independent set of 61 HCCs with rat fetal hepatoblasts and adult hepatocytes, and with mouse HCCs from different experimental models. Individuals with HCC who shared a gene expression pattern with fetal hepatoblasts (hepatoblast signature) belonged to subclass A with poor prognosis and the profile included markers of hepatic progenitor cells (K19, K7, vimentin), suggesting that HCC of this subtype may

arise from progenitor cells (Lee et al. 2006). Yamashita et al. (2008) used cDNA microarrays to study 238 HCC and identified a subset of EpCAM-positive HCC with worse prognosis, which displayed a unique molecular signature with features of HPC including the presence of K19 expression.

Aberrant expression of p63, a marker for basal/stem cells in several organs including prostate and skin, and of ckit, a stem cell marker, has been described in CCs, also suggesting a progenitor cell origin of part of the CCs (Nomoto et al. 2006).

6 Potential Liver Cancer Stem Cell Markers

Several liver stem cell markers have been proposed. Given the similarities between normal stem cells and cancer stem cells, it is reasonable to assume that the phenotype of liver cancer stem cells resembles that of normal stem/progenitor cells.

As described above, **K19** is a very early hepatoblast marker and marker of adult normal liver progenitor cells and is associated with worse prognosis of HCC. OV6, a rodent progenitor cell marker, recognizes K19 and K14 in rat and as such is the rodent equivalent of K19. Yang et al. (2008a) have demonstrated that OV6+ HCC cells possess greater tumorigenic ability and chemoresistance to standard chemotherapy when compared to OV6– cells. The Wnt/beta-catenin pathway plays a key role in the expansion of progenitor cells in human HCC, similar to its role in expansion of non-neoplastic progenitor cells in acute and chronic liver diseases (Spee et al. 2010).

Ma et al (2007) discovered that **prominin-1**, the murine homologue of human **CD133** was significantly upregulated in liver regeneration. In subsequent functional studies on sorted HCC cells, CD133+ cells displayed cancer stem cell properties. CD133+ cells had greater tumorigenicity in immunodeficient mice, higher colony-forming capacity, and proliferation ability in vitro and could be induced into non-hepatocyte-like lineages, showing multipotency. The expression of “stemness” genes was higher in CD133+ cells than in CD133– cells and further studies showed CD133+ cells are resistance to conventional chemotherapy (Ma et al. 2008).

CD90 has been suggested as a putative marker for liver cancer stem cells. This marker is expressed on hepatic progenitor cells during development (Dan et al. 2006) and CD90 expression correlated with tumorigenic potential in a panel of liver cell lines (Yang et al. 2008b). Further characterization with cell sorting for CD45 resulted in a population of CD90+/CD45– cells of which the majority also expressed **CD44** (Yang et al. 2008c). CD44 is a cell-surface receptor for hyaluronic acid and its blockade with neutralizing antibodies induced apoptosis of CD90+ cells and prevented tumor formation in mice (Yang et al. 2008c).

EpCAM has been suggested as cancer stem cell marker since EpCAM+ HCC cells were found to be more tumorigenic than EpCAM– cells (Yamashita et al. 2009). Interestingly, EpCAM is a direct transcriptional target in the Wnt/beta-catenin pathway and hence could serve as a biomarker for the activation of this pathway (Yamashita et al. 2007).

Normal human progenitor cells have certain subtypes of active transmembrane adenosine triphosphate-binding cassette (**ABC**) **transporters**, such as MDR1, ABCG2, and ABCC2 (Roskams et al. 2001; Ros et al. 2003; ; Vander Borgh et al. 2006), rendering them resistant to oxidative stress, toxic substances, including chemotherapy. These transporters also efflux the DNA-binding dye Hoechst 33342 and hence “side sort” in fluorescence activated cell sorting. It was shown that **side population** cells from HCC cell lines harbor cancer stem-cell like properties: they were highly proliferative and chemoresistant in vitro (Chiba et al. 2006) non-side population cells did not. The increased tumorigenic potential of side population cells was shown in vivo in SCID mice where 10^3 side populations’ cells consistently led to tumor formation whereas 10^6 non-side population did not. In addition, it was found that side population cells isolated from HCC cell lines may be related to the metastatic potential of HCC (Shi et al. 2008). MRP1 expression correlated with a more aggressive tumor phenotype and with K19 expression (Vander Borgh et al. 2008). High expression of ABC transporters render the cells resistant to chemotherapy, including cisplatin and doxorubicin. Inhibition of MDR1 (Wakamatsu et al. 2007), ABCG2 (Hu et al. 2008), and ABCC2 (Folmer et al. 2007) by inhibitors or by the antisense approach, can reverse this chemoresistance.

7 Conclusions

A better understanding of signaling pathways in HCC pathogenesis has led to targeted therapies against HCC, using drugs like sorafenib, erlotinib, and bevacizumab (Siegel 2008). These therapies target the major cell populations of rapidly growing differentiated tumor cells (Siegel 2008). However, increasing evidence for the existence of cancer stem cells suggests the possibility of targeting the undifferentiated cancer stem cells, which only constitute a small proportion of the tumor. The identification of liver cancer stem cell markers and their related pathways is one of the most important goals of liver cancer research. Understanding the mechanisms of activation and differentiation of non-neoplastic stem/progenitor cells and especially the differences with their malignant counterparts should therefore also be a top priority. New therapies should ideally target cancer stem cells and NOT normal stem/progenitor cells, since these are very important in regeneration and repair mechanisms.

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Part VIII
Liver Cancer Genetics in the Clinic

Chapter 18

Molecular Signaling in Hepatocellular Carcinoma

Hong Yang Wang and Jin Ding

Abstract Hepatocellular carcinoma (HCC) is usually observed in patient with chronic liver inflammation, which is characterized by persistent liver injury and chronic hepatocellular proliferation. Accumulating studies have clarified that development of HCC are closely associated with the dysregulation of several signaling pathways including MAPKs, Wnt/ β -catenin and IKK/NF- κ B, etc. Aberrant activations of these signaling cascades usually lead to the over-expression of tumor-promoter genes and down-regulation of tumor-suppressor genes. Disruption of the expression of these genes promotes cell-cycle progression, apoptosis evasion, dedifferentiation, etc. and thus contributes to HCC initiation, promotion and progression. Therefore, targeting the key molecules in the oncogenic signaling pathway might be a promising strategy for HCC therapy.

Keywords Hepatocellular carcinoma · Signaling pathway · Cytokine · Receptor · Kinase

1 Overview

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with a particularly high prevalence in Asia and Africa due to the chronic hepatitis virus infection. In most cases, HCC emerges on a background of persistent liver injury and hepatocellular proliferation, which is a characteristic of chronic and nonresolving inflammation. There is accumulating evidence showing that the viral proteins of hepatitis B and C can directly elicit oncogenic effects or contribute to enhanced risk of hepatocellular transformation in cooperation with the hyperproliferative

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response induced by chronic inflammation. Therefore, a proinflammatory and proliferative tissue microenvironment is a common feature of the preneoplastic liver regardless of the etiology. Increasing evidence has revealed that aberrant activation of several signaling cascades closely correlate with HCC occurrence, such as growth factors/MAPKs (mitogen-activated protein kinases), Wnt/ β -catenin, phosphoinositol 3-kinase (PI3-K)/Akt, (TGF- β transforming growth factor β)/STAT, IKK (I κ B kinase)/NF- κ B (nuclear factor- κ B), steroid hormones/nuclear receptors, and apoptotic signaling (Fig. 18.1). Two main outcomes resulted from these aberrant activations include: (1) the overexpression of protooncogene (e.g. c-myc, β -catenin), growth factor (e.g. IGF-II, TGF- α), or telomerase enzyme inducing cellular immortalization, and (2) the inactivation of tumor-suppressor genes (e.g. p53, PTEN, and Rb). Signaling pathway disruption in HCC can cause disturbances of cell-cycle regulation, evasion of apoptosis, and aberrant angiogenesis. Moreover, development-associated signaling cascade including hedgehog pathway (Hh), Notch pathway, and HGF/MET pathway have been implicated in human HCC, suggesting that some HCCs may arise from liver stem cells.

2 P53 and Rb-Associated Signaling Pathways

p53 is one of the most critical tumor-suppressor molecules, which functions at multiple levels in HCC development. Mutation of p53 has been identified in 30–60% of HCC cases. MDM2 (murine double minute 2) plays a critical role in modifying p53 level and activity. Elevated MDM2 accounts for the immortalization of cells by promoting ubiquitination and proteolysis of p53, which can be attenuated by the interaction of P14^{ARF} and MDM2. However, P14^{ARF} is frequently deregulated by the hypermethylation and mutations in HCC. Numerous evidence linking p53 with human HCC arises from the finding that HBx and HCV core protein are able to interact with p53 leading to the inactivation of p53 activity, which includes attenuating DNA repair, deregulation of cell-cycle check point controls and p53-mediated apoptosis.

The reduced expression of p21^{WAF1/CIP1}, a downstream target of p53, is frequently observed in HCC cases. p21 functions like a tumor suppressor, which binds to and inhibits cyclin-dependent kinases (CDK2, CDK4), leading to G₁ cell-cycle arrest. Disruption of p53–p21 cell-cycle pathway has been widely accepted as a key factor associated with the progression and poor prognosis of HCC.

It is reported that even brief reactivation of endogenous p53 in p53-deficient hepatocarcinoma can result in complete tumor regressions (Xue et al. 2007). The major response is not apoptosis, let alone necrosis, but the induction of a cellular senescence program, which is linked with cell differentiation as well as the upregulation of inflammatory cytokines. These will trigger cell-cycle arrest and innate immune activation targeting the tumor cells, thereby contributing to tumor clearance.

The retinoblastoma protein (Rb) checkpoint is another primary pathway in hepatocarcinogenesis, which attenuates cell proliferation, telomere shortening, and oncogene activation in response to DNA damage. Dephosphorylated Rb recruits

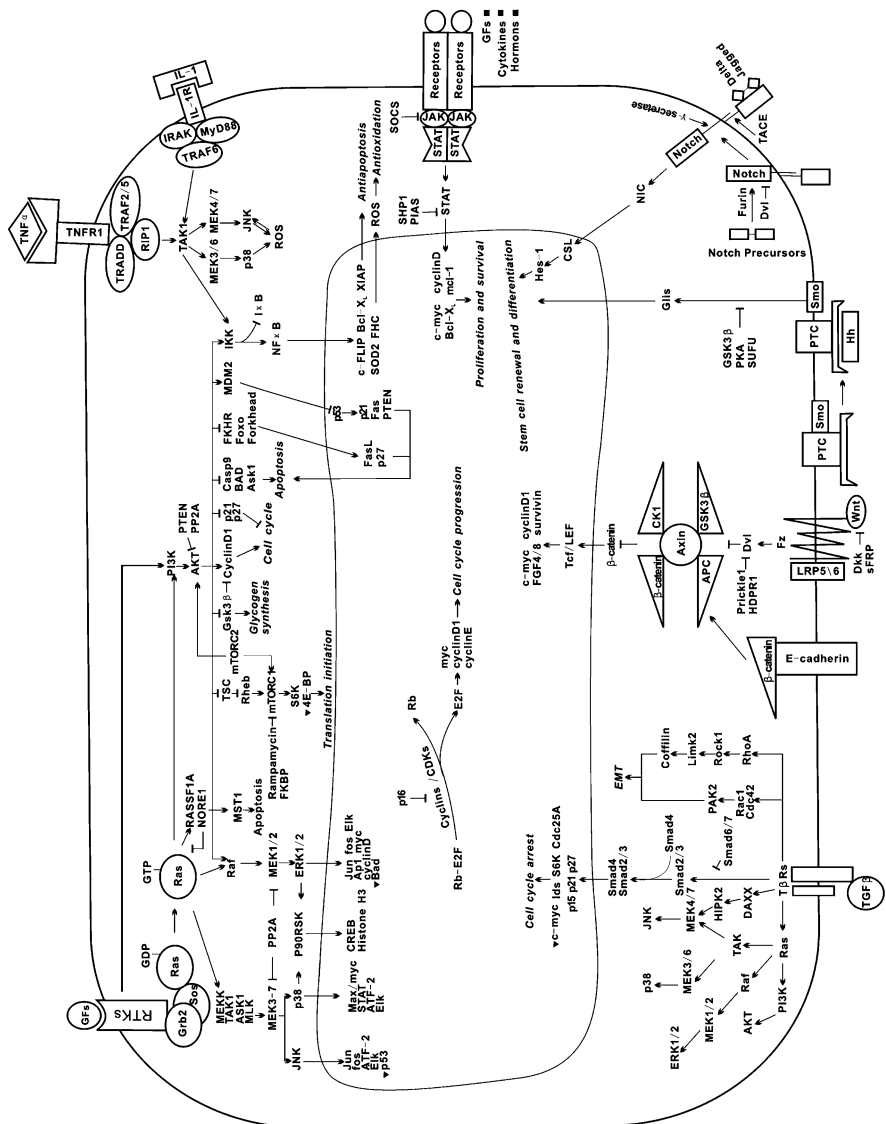


Fig. 18.1 Signaling network of hepatocellular carcinoma

the transcription factor E2F, and subsequently prevents the expression of E2F downstream genes, which mediate cell-cycle transition. Therefore, when Rb is phosphorylated and inactivated by complexes of CDKs and cyclins, repression of cell-cycle progression would be relieved and the cells tend to overgrow.

Rb pathway is disrupted in more than 80% of human HCC, while inactivation of Rb occurs in about 28% of the cases. Simultaneously, other members in Rb network also aberrantly express in HCC. For example, cyclin D1/CDK4 is overexpressed in around 58% of HCC. The vast majority of human HCC also overexpress gankyrin, which inhibits Rb-checkpoint. Furthermore, P16, P21, and P27, which inhibit CDK activity, are dysregulated thus contributing to carcinogenesis in nearly 90% of HCC cases (Aravalli et al. 2008). Therefore, disruption of Rb network is common in HCC development.

3 MAPKs Signaling Pathway

Ras/Raf/MEK/MAPK pathway is one of the most critical signaling cascades in HCC development. A number of growth factors including EGF, IGF, VEGF, PDGF, FGFs, or HGF, are able to activate the autophosphorylation of the tyrosine residues in receptor tyrosine kinases (RTKs) and recruit Ras to the activated receptor. Activation of Ras leads to a set of phosphorylation events, starting with the activation of Raf, a serine/threonine kinase also named as MAPKKK. Phosphorylated Raf, in turn, activates MEK (MAPKK), which then phosphorylates and activates its downstream kinase MAPKs, including extracellular signal-regulated protein kinases (ERKs), JUN NH2-terminal kinase (JNK), and p38 (Georg 2007).

Activated ERK by MEK1/2 shuttles to the nucleus, where it triggers the activation of transcription factor ELK1, AP-1, and TCF, which account for HCC progression by modulating the expression of ETS, c-Jun, c-Fos, c-Myc, cyclin D, etc. Moreover, ERK activation links MAPK pathway with survival response by mediating the phosphorylation of histone H3, the proapoptotic protein Bad as well as the transcription factor CREB through ribosomal S6 protein kinase-2 phosphorylation. Unrestrained ERK activation is usually observed in HCC patients with poorer prognosis.

Depending on its context, JNK signaling exhibits two different functions. On one hand, activated JNK facilitates cell-cycle progression by activating transcription factor c-JUN and synergize with the ERK signaling. On the other hand, JNK may promote apoptosis via two distinct mechanisms. First, phosphorylation and transactivation of c-Jun/ATF-2 heterodimers by JNK can activate transcription factor AP-1, which up-regulates the expression of the proapoptotic genes CD95L and TNF α . Second, JNK mediates the phosphorylation of BCL-2 and BCL-X_L, which attenuates their anti-apoptotic ability. Moreover, the evidence that JNK pathway determines the metastatic capability of MHCC97H cells suggests its potential role in HCC metastasis (Liu et al. 2008).

P38 mainly participates in the inflammatory responses and cell proliferation. P38 pathway can down-regulate cyclin D1 expression, thus arresting cell cycle at the

G1–S checkpoint. P38 can also contribute to the G₂/M arrest, probably by regulating the transactivation of Gadd45a, which is a downstream target gene of p53. Activated c-Jun is also reported to prevent apoptosis by antagonizing p53 activity, contributing to the early stage of HCC.

Mutations in Ras or MAPK upstream signaling deregulation contributes to the HCC development. Overexpression of Ras proteins and RTKs (such as EGFR, IGFR-I) has been usually found in human HCC and cirrhotic livers, which is consistent with the fact that antisense treatment against H-Ras can significantly inhibit hepatocarcinogenesis. Furthermore, overexpression of TGF α , BTC, HB-EGF, IGF-II, and AR has been demonstrated in over 60% of HCC cases. In addition, decreased level of the physiological inhibitors of Ras/Raf/MEK/MAPK pathway has been frequently observed in human HCC.

4 Wnt/ β -Catenin Signaling Pathway

Activation of Wnt/ β -catenin in hepatocyte is usually observed in the development of HCC. In normal cells, β -catenin is targeted by ubiquitin-proteasome-mediated proteolysis via phosphorylation at serine and threonine residues by casein kinase I α (CKI α) and its downstream protein, glycogen synthase kinase 3 β (GSK3 β). This process requires β -catenin to form a complex with axin and adenomatous polyposis coli (APC), which allows the subsequent degradation. When Wnt proteins bind to cell-surface frizzled receptors (Fz), Dishevelled (Dvl) is activated to antagonize the axin-GSK3 β -APC complex formation, thereby stabilizing cytoplasmic β -catenin. Some of the accumulated β -catenin is able to enter nucleus and interact with lymphoid enhancing factor/T-cell factor (LEF-1/TCF-4) family transcription factors and regulates the expression of a set of oncogenes, including c-Myc, cyclin D, and survivin.

Accumulating evidence implies that Wnt/ β -catenin pathway plays a critical role in HCC development. Mutations of β -catenin occur in up to 30% of human HCC, preventing β -catenin from being phosphorylated, therefore escaping from degradation. HCV-associated HCCs tend to have higher frequency of β -catenin mutations than HBV-associated ones. In HBV-positive HCC, HBx protein decrease E-cadherin expression via hypermethylation of its promoter, leading to releasing of β -catenin from cell membrane and a steady pool of β -catenin in the cytoplasm. Besides, dysregulation of other upstream mediators of Wnt/ β -catenin pathway has been observed in HCC as well. APC, a tumor-suppressor protein, is required for the nuclear export and degradation of β -catenin. Over 67% of mice with liver-targeted disruption of APC develop HCC accompanied with β -catenin signaling activation. Negative mediators such as Wnt antagonist, sFRP1, and Dvl inhibitors, HDPR1 and Prickle-1, are frequently reduced in HCC, whereas positive regulators such as Wnt ligands, Fz-7, Dvl-1, Dvl-3, and PIN1 are often increased. All these changes above will lead to the accumulation of β -catenin in the nucleus, where it regulates the transcription of various target genes. Among those, survivin accelerates cell proliferation through promoting cell-cycle progression by releasing p21^{WAF1/CIP1} from CDK4. Another

target gene of β -catenin is cyclin D1, which interacts with CDK4/6 to phosphorylate Rb, thereby facilitating cell-cycle transition from G1 to S phase. In liver cancer cells, c-Myc is closely associated with the expression of the hepatoma specific bio-marker α -fetoprotein as well as hepatocarcinogenesis (Shachaf et al. 2004).

5 TGF- β Signaling Pathway

Transforming growth factor β (TGF- β), secreted by nonparenchymal stellate cells, plays an essential role in hepatocyte growth and apoptosis. It limits hepatic regeneration in response to injury through inhibiting DNA synthesis and inducing apoptosis. Active TGF- β directly binds to TGF receptor III (T β RIII) or T β RII, which recruits T β RI forming a heterotetrameric complex. T β RII is able to phosphorylate serine residues of T β RI, and thus activates it. The complex then phosphorylates serine residues of R-Smads, Smad-2, and Smad-3, increasing their affinity for a co-Smad Smad-4. The phosphorylated R-Smad/coSmad complex translocates into the nucleus, where it up-regulates specific genes including integrins and β -catenin. Inhibitory Smads, Smad6 and Smad7, compete with Smad2/Smad3 in binding with TGF receptor, inhibiting their phosphorylation. Meanwhile, the activated T β R complex may also activate Smad independent signaling through MAPK pathways, PI3-K, protein phosphatase 2A/p70 S6 kinase (PP2A/p70S6K), etc.

TGF- β pathway plays a dual role in hepatocarcinogenesis. At early stage of HCC development, TGF- β responses to liver injury and triggers cell-cycle arrest as well as apoptosis. Expression of T β RII in liver tissues is remarkably reduced in patients with HCC compared to those with chronic hepatitis or liver cirrhosis. In addition, ectopic expression of T β RII in the hepatoma cell line induces cell arrest and apoptosis (Baek et al. 2008). At later stage of HCC development, TGF- β facilitates HCC metastasis by up-regulating the expression of metalloproteinases and angiogenesis along with down-regulation of immune system associated molecules. The most significant characteristic of late TGF- β signature is the overexpression of cell-cycle accelerators (cyclins and CDKs) and proteins involved in metastasis or angiogenesis (VEGF, HIF1A) and EMT (MMP1, VIM), which represents a more aggressive phenotype of HCC. Additionally, down-regulation of liver function genes (glucose and lipid metabolism, or detoxication) and genes required in antioxidant response (catalase and superoxide dismutase 1) implicates that HCC defined by the late TGF- β signature are less differentiated and more sensitive to oxidative stress (Coulouarn et al. 2008).

6 IKK/NF- κ B Signaling Pathway

Chronic infection and inflammation is closely associated with carcinogenesis and accounts for nearly 20% of cancers. HCC is widely accepted as the outcome of continuous injury and hepatitis virus-induced chronic inflammation. IKK/NF- κ B

signaling pathway plays a critical role in liver diseases including hepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma.

The majority of NF- κ B transcription factors are retained in the cytoplasm by their specific inhibitor I κ B. In response to TNF- α , ILs in particular IL-6, and viral proteins triggered inflammatory signals, I κ B is phosphorylated by the I κ B kinase (IKK) complex, which results in its ubiquitination and degradation (Naugler et al. 2007). Thus, released NF- κ B translocates into the nucleus and trans-activate the expression of anti-apoptotic genes, such as the caspase-8 inhibitor c-FLIP, the Bcl-2 family members Bcl-xL and A1/Bfl-1, X-linked inhibitor of apoptosis (XIAP), TRAF1, TRAF2, and cellular inhibitors of apoptosis (cIAPs). The sustained activation of NF- κ B in the premalignant stage confers a survival advantage to hepatocytes that have acquired oncogenic mutations, thus facilitating oncogenic transformation.

Hepatocyte multidrug resistance two knockout (*Mdr2*^{-/-}) mice spontaneously developed cholestatic hepatitis and further HCC with prolonged NF- κ B activation in hepatocytes. Production of TNF- α by nonparenchymal cells is required in this process. Inhibition of NF- κ B or blockage of TNF α signaling results in the apoptosis of transformed hepatocytes and suppression of hepatocarcinogenesis. These results implicate that NF- κ B activation in hepatocytes by TNF is involved in the late phases of tumor development via increasing the survival of dysplastic hepatocytes (Pikarsky et al. 2004).

Moreover, it is also reported that mice with hepatocyte and cholangiocyte specific deletions of the *Ikk γ* present massive and fatal hepatocyte apoptosis with concomitant highly enhanced hepatocyte proliferation in response to TNF or lipopolysaccharide. These mice spontaneously develop HCC in 9–12 months accompanied with a persistent activation of JNK, suggesting that persistent activation of JNK mediate hepatocyte apoptosis in these mice as well. Blockage of IKK/NF- κ B pathway in hepatocytes tends to render the liver more susceptible to HCC in the context of a chronic inflammatory disease (Elsharkawy and Mann 2007).

Thus, the role of NF- κ B pathway in HCC development is indeed complicated, which depends on cell context to some extent. A physiological level of NF- κ B in liver displays tumor suppressive effect by antagonizing aberrant JNK activation, ROS production, and epigenetic alterations. Whereas, NF- κ B is usually overactivated in transformed or tumor cells in which NF- κ B exhibits tumor promotion effect. Therefore, it appears to be desirable to develop reagents that remove excessively activated NF- κ B, but maintain a normal level of NF- κ B.

7 PI3-K/Akt Signaling Pathway

PI3-K/Akt/mTOR pathway is another important pathway involved in HCC development. Activated by RTKs or Ras, PI3-K triggers the generation of phosphatidylinositol 3,4,5-triphosphate (PIP3), which is a lipid second messenger and is capable of activating the downstream serine/threonine kinase AKT. AKT then phosphorylates the mammalian target of rapamycin (mTOR) which in turn activates the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and the 40 s ribosomal protein S6

kinase (p70S6K). 4E-BP1 and p70S6K regulate the expression of numerous genes required in cell proliferation and angiogenesis including c-Myc, cyclin D1, and hif-1 α . Tumor-suppressor phosphatase and tensin homologue (PTEN) can catalyze the removal of the D3 phosphate from PtdIns(3,4,5)P3 and inhibit PI-3 K signaling.

During the last 10 years, PI3-K/AKT/mTOR pathway has emerged as an essential contributor to HCC development. It is reported that liver has high level of endogenous PI3-K activity in comparison with most other tissues. Hepatoma also exhibits the highest percentage of pik3ca (p110 catalytic subunit of PI3-K) mutations (36%) (He et al. 2008). Akt mediates cell survival, cell-cycle progression, cell growth, and cell metabolism by phosphorylating a set of molecules such as Chk1, BAD, caspase 9, GSK-3 β , and I κ B kinase. Thus, sustained Akt activation could lead to a potential deleterious effect. Akt can be activated in HCC by a number of upstream stimuli including RTKs, PI3-K, and Ras. In addition, activation of Akt pathway can prohibit TGF- β -elicited apoptosis, which promotes tumor formation at the cirrhosis stage.

Irrespective the high rates of activating mutations in pik3ca, loss-of-function in PTEN is still one of the most common mechanism of PI3-K pathway activation in HCC. PTEN expression is reduced or absent in almost half of the human HCC. Furthermore, it is reported that hepatocyte-specific abrogation of PTEN expression in mice results in the development of HCC as well.

8 JAK/STAT Signaling Pathway

Upon binding to extracellular polypeptides such as hormones, cytokines, and growth factors at cellular membrane, cognate receptor of JAK/STAT signaling pathway dimerizes and activates tyrosine kinases, Janus Kinases (JAKs), to phosphorylate the cytoplasmic counterpart of cytokine receptors, creating phosphotyrosine-binding Src Homology 2 domain (SH2 domain), which recruits the signal transducer and activation of transcription (STAT) proteins. After tyrosine phosphorylated by JAKs, STATs form hetero- or homo-dimers and translocate to the nucleus thus inducing target gene transcription. STATs activation can also be triggered independently of JAKs, by epidermal growth factor receptor and c-src. JAK/STAT pathway is mainly involved in principal cell-fate decision, regulating genes for cell growth and apoptosis, including bcl-xL, cyclin D, mcl-1, and c-Myc (Georg 2007).

Negative regulation of STATs requires different molecules at different stages. Tyrosine phosphatases are capable of deactivate cytokine receptors as well as STATs in cytoplasm. Suppressors of cytokine signaling (SOCSs) inhibit STAT phosphorylation by inactivating JAKs or competing for binding sites on cytokine receptors. On level of transcription regulation, protein inhibitor of activated STATs (PIAS) binds to the DNA sequences recognized by STAT dimers and blocks transcription initiation. In human liver, STAT3 is the primary variant of STAT family. Its activity is relatively higher in HCC than in normal liver cells, considering that suppression of SOCS-1 is commonly seen in HCC due to hypermethylation. Similarly, if socs-3 gene is deleted in liver parenchymal cells, it is likely that hepatitis will develop into HCC with persistently active STAT3.

9 Hedgehog Signaling Pathway

The hedgehog (Hh) signaling pathway is one of the key regulators in embryogenesis and carcinogenesis. It is first discovered in *Drosophila*, and three homologous ligands were then found in mammals, of which Sonic hedgehog (SHH) is the most studied. Hh ligands target the Patched-1 receptor (PTCH1) in mammals, which is able to inactivate Smoothened (SMO), a coreceptor in the pathway, without the presence of Hh ligands. Activated by Hh ligands binding through inhibition of PTCH1, SMO can regulate downstream activation of GLI family of transcription factors, including activators Gli1, Gli2, and the repressor Gli3. Recent studies show that hepatic stellate cells (HSC) and liver epithelial progenitors can respond to Hh ligands upon adult liver damage, whereas normal adult liver shows little Hh activity, suggesting that abnormal activation of the pathway may regulate hepatocarcinogenesis via transformation of adult liver stem cells into hepatic cancer stem cells (CSCs) that eventually develop into tumor.

Hh pathway activation typically enhances cell growth and viability. Constitutive activation of SHH-mediated SMO signaling has been demonstrated in over 60% of HCC (Llovet and Bruix 2008). Increased expression of snail, cyclin D1, and c-Myc were reported to be consistent with Hh signaling pathway activation, which reveals partial role of Hh pathway in metastasis and liver tumor development, as Snail is known to down-regulate E-cadherin and tight junction proteins (TJP-1), leading to loss of cell adhesion, and cyclin D1 is involved in cell-cycle regulation. The overlapping target genes of Hh and TGF- β signaling pathway suggest their possible interaction during tumorigenesis and metastasis. Activation of Hh pathway usually augments the growth and viability of Hh-responsive cells, whereas blockage of Hh signaling in such cells will induce apoptosis. For example, effective block of tumorigenic Smo activation via antagonist leads to marked cell growth inhibition and apoptosis in Hh-activated HCC cells, and also reduces invasiveness and motility of HCC cells. Similar effect has been observed in HCC cells treated with siRNA of Gli1, which knocks down the mRNA level of Gli1 transcriptional activator.

10 Notch Signaling Pathway

Notch is a family of transmembrane receptors including notch-1, notch-2, notch-3, and notch-4 (Georg 2007). A growing amount of evidence has demonstrated that Notch signaling plays important role in cell-fate decisions during embryo development. Specific binding of Notch receptors with their ligands, such as Delta-Like or Jagged, triggers the downstream signaling activation. The liberated NIC (Notch intracellular domain) translocates into the nucleus and interacts with the transcription factor CSL, which releases corepressors (CoR) and recruits coactivators (CoA), resulting in the transcriptional activation of downstream target genes.

Most current studies have elucidated that the Notch signaling pathway is required in the self-renewal and differentiation of stem cell. Epidemiological study has elucidated that Notch signaling is overactivated in numerous epithelial cancers. It is

accepted that activation of Notch signaling is insufficient to cause cancer. The oncogenic effect of Notch signaling is achieved by the interaction with other oncogenic proteins such as Ras and Myc. In numerous epithelial cancers, Notch-dependent transformation is verified to correlate with the activation of ERK and PI-3 K pathways, which not only increases the Notch mRNA stability, but also participates in the transcription of the Notch target genes. Overexpression of Notch1 could inhibit human HCC cell growth by triggering cell-cycle arrest and apoptosis with the down-regulation of Bcl-2, cyclins, CDK2, and phosphorylation of Rb protein, whereas with the up-regulation of p53, p21, and activation of the stress-activated protein kinase/JNK pathway (Qi et al. 2003). Furthermore, Notch1 signaling sensitizes TRAIL-induced apoptosis in HCC cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression. Thus, activation of Notch1 signaling may be a promising approach to improve the therapeutic efficacy of HCC (Wang et al. 2009).

11 Stem-Cell Related Signaling Pathways

In recent years, more and more efforts have been put into the relationship between stem cells and cancer. They share similar signaling pathways to determine the diverse developmental fates of cells. These pathways include Wnt/ β -catenin, Notch, Hedgehog and Bmi1, BMP and TGF β -associated cascades. In HCC patients, a hepatoblast phenotype usually indicates a significantly worse prognosis. Liver CSCs may account for the initiation, progression, and chemoresistance of HCC. Liver progenitor cells (LPCs) differentiate into hepatocytes and cholangiocytes. Notch signaling facilitates biliary epithelial phenotype differentiation, while growth factor promotes hepatocyte differentiation. Regulation of biliary and hepatocyte morphogenesis during development is also mediated by TGF- β /Smad signaling (Mishra et al. 2009).

Activation of Wnt/ β -catenin in liver progenitor/stem cells seems to enhance their self-renewal activity and carcinogenesis potential (Yang et al. 2008). Wnt/ β -catenin activation confers a growth advantage or self-renewal capacity in a small HCC subpopulation endowed with progenitor-like features. Wnt/ β -catenin pathway is also important in activation and expansion of oval cells in HCCs and therapies targeted to the Wnt/ β -catenin signaling may provide a specific direction to disrupt the resistance mechanism and consequently improve overall tumor control with chemotherapy.

Hepatoma cells expressing stem-cell markers such as STAT3, NANOG, and OCT4 have unexpectedly lost T β RII and ELF compared with normal human progenitor/stem cells. Therefore, these cells are more likely liver CSCs, which have the potential to give rise to HCCs. In heterozygous *Elf*^{+/-} mice model, over 40% spontaneously develop HCC with concomitant activation of the IL6/STAT3, Wnt, and CDK4 pathways as well as disruption of TGF- β signaling, suggesting that dysfunction of TGF- β signaling and activation of IL-6/STAT3 are necessary in normal liver stem cells to cancer progenitor/stem cells transformation. *Elf*^{+/-} mice die with hypoplastic livers similar to *Smad2*^{+/-}/*Smad3*^{+/-} mutant mice, indicating the reason

why TGF- β pathway interruption leads to HCC may lie in the disruption of cellular differentiation of hepatic progenitor/stem cells (Tang et al. 2008). Decreased STAT3 activity by its specific inhibitor NSC 74859 leads to the proliferation suppression of HCC cell with loss of TGF- β irrespective of CD133⁺ status, which indicates STAT3 might be a potential target for antagonizing HCC progenitor/stem cells with inactivation of the TGF- β /ELF pathway (Lin et al. 2009). In addition, recent studies showed that CD133⁺ cells isolated from 16-month-old *Matla*^{-/-} mice possess higher p-ERK levels compared with CD133⁻ cells, indicating activated MAPK pathway may be responsible for resistance of CD133⁺ CSC to TGF- β -induced apoptosis, which has been well-documented in HCC (Ding et al. 2008).

12 Peroration

It has been widely accepted that hepatocarcinogenesis is a multi-factor and multi-stage process. Accumulating study in the past few decades has identified a number of signaling pathways involved in hepatocarcinogenesis, most of which are part of the precisely controlled regenerative and protective physiological process of the liver upon acute tissue injury. Whereas, when the aberrant activation of these signaling pathways persists, they will contribute to the oncogenic transformation and the maintenance of the transformed phenotype of HCC cells. A comprehensive knowledge of the signaling pathways involved in HCC will facilitate the development of more effective targeted therapies of HCC.

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Chapter 19

Molecular Events on Metastasis of Hepatocellular Carcinoma

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Abstract Metastasis is the key issue for conquering cancer; molecular events of metastasis are important for controlling metastasis. Based on 15 years of studies on molecular events of hepatocellular carcinoma (HCC) metastasis, several points have attracted attention: (1) In addition to the traditional “clonal selection” concept, we found that metastatic potential of HCC originated from primary tumor, even in small HCC. (2) Many HCC metastasis-related molecules have been identified, but most of them are similar to that of other cancers. (3) Microenvironment, including endothelium of tumor vessel and surrounding liver parenchyma, plays important role for HCC metastasis. (4) Predictive biomarkers may vary from different phenotypes of HCC, the sensitivity and specificity is therefore limited. (5) HCC metastasis is a highly selective dynamic process; the metastatic potentials as well as metastatic targets will be influenced by microenvironment and host status. (6) Prevention and treatment of HCC metastasis remain a great challenge. We should focus not only on molecular targeting, but also on the clinically available agents that are of interventional value.

Keywords Hepatocellular carcinoma (HCC) · Metastasis · Recurrence · Molecular events · Microenvironment

1 A Synopsis of Studies on Metastasis of Hepatocellular Carcinoma at Authors’ Institute

Primary liver cancer, with the majority of hepatocellular carcinoma (HCC), was the third cancer killer in the world, and 55% of liver cancer deaths were in China (Parkin et al. 2005). Metastasis and recurrence are the major causes of death, even after curative resection, and the 5-year recurrence rate was as high as 61.5%, being 43.5%

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after small HCC resection (Tang 1989). HCC is mainly hypervascular cancer, with high rate of metastases to lung, bone, adrenal gland, and others; on the other hand, lymph node metastasis, particularly in the hepatic hilum area, is also frequently encountered.

In the authors' institute, based on 15 years' studies on molecular events of HCC metastasis, particularly the collaboration with NCI, NIH of the United States, several points have drawn attention: (a) *Metastatic potential of HCC originated from primary tumor*. Besides the previous "clonal selection" concept, we demonstrated that metastatic potential of HCC existed in primary cancer, even in small HCC (Ye et al. 2003). (b) *Identification of HCC metastasis-related molecules remains a key issue, but many of them are similar to that of other cancers*. In order to narrowing the areas for screening of HCC metastasis-related molecules, we have carried out studies particularly in chromosome 8p (Qin et al. 1999, 2001; Yang et al. 2003; Zhang et al. 2003). A good number of HCC metastasis-related molecules have been found, such as osteopontin (Ye et al. 2003), cytokeratin 19 (Ding et al. 2004), HTPAP (Wu et al. 2004), KIAA0008 (Zhao et al. 2004), MSRA (Lei et al. 2007), uPA, uPAR, and PAI-1 (Zheng et al. 2000); molecules found by proteomic analysis (Cui et al. 2004, 2006; Song et al. 2006); and glycoprotein (Dai et al. 2006). Recently, a metastasis-related microRNAs in HCC has also been identified (Budhu et al. 2008). However, most of them are not specific to HCC. (c) *Microenvironment plays critical role for HCC metastasis*. HCC metastasis-related molecules could be identified not only from HCC cells, but also from endothelial cells of tumor vessels (Zhang et al. 2005), and from peritumoral liver tissue (Budhu et al. 2006; Zhu et al. 2008). Basement membrane proteins play an active role in the invasive process of human HCC cells (Tian et al. 2005). (d) *Predictive biomarkers may vary from individuals, and combination of molecules might be superior to single molecule*. Numerous predictive biomarkers have been found of clinical value: VEGF/PD-ECGF (Li et al. 1999; Zhou et al. 2000), osteopontin (Zhang et al. 2006), CK19 fragment CYFRA 21-1 (Li et al. 2006), LOH at D14S62 (Niu et al. 2003), plasma DNA level and its allelic imbalance on chromosome 8p (Ren et al. 2006), LOH at D8S298 for TNM stage I HCC (Pang et al. 2007), and others (Qiu et al. 2008). CK19 expression was prognostic factor for HCC with lymph node metastasis (Zhuang et al. 2008). The combination of molecules seemed superior to that of single molecule, such as CK10+CK19 (Yang et al. 2008b), osteopontin+CD44 (Yang et al. 2008a), and protein expression profiling of VEGF and its receptors (Jia et al. 2008). Unfortunately, none of them could cover the entire HCC population with high predictive value. Due to the complicated factors that may involve, such as geographic variation of etiological factors, genetic background, predictive markers or signatures may satisfy only some of the subtypes of HCC, such as EpCAM, and alpha-fetoprotein expression defines novel prognostic subtypes of HCC (Yamashita et al. 2008). (e) *Metastatic potential of HCC is an alterable event*. In order to carry out studies on HCC metastasis, we have established a patient-like human HCC metastatic model (LCI-D20) in nude mice (Sun et al. 1996) as well as a cell line (MHCC97) with metastatic potential that originated from LCI-D20 tumor (Tian et al. 1999). Metastatic potential is alterable. A stepwise metastatic potential human HCC cell lines (HCCLM3, HCCLM6), with up-regulation of MMP-2,

MMP-9, CK19, and down-regulation of Rb2/p130, could be established through consecutive in vivo selection (Li et al. 2003, 2004). Lung tissue extracts contributed to the alteration of invasion and migration of human HCC cells with metastatic potentials to lung (Ji et al. 2003). These indicated that HCC metastasis is a highly selective dynamic process. *(f) Intervention of HCC metastasis remains challenging.* Experimental interventions in metastatic human HCC nude mice model have been tried with some response using TNP470 (Xia et al. 1997), BB94 (Bu et al. 1998), β -peptide (Sun et al. 2000), antisense H-ras (Liao et al. 2000), and others. Later, we found that interferon- α (IFN- α) inhibits recurrence in our metastatic nude mice model (Wang et al. 2000), and being verified by randomized clinical trial after curative HCC resection (Sun et al. 2006). IFN- α down-regulates VEGF expression through P13 kinase and MAP kinase signaling pathways (Wang et al. 2003b; Wu et al. 2005), and reduction in p48-ISGF levels confers resistance to IFN- α (Wu et al. 2004), and verified clinically (Qian et al. 2006), indicated that IFN- α is only effective to a subset of HCC. High expression of PD-ECGF in tumors contributed to the effectiveness of capecitabine (Zhou et al. 2003). The IFN- α enhanced anti-tumor effect of capecitabine through up-regulated thymidine phosphorylase (Xiao et al. 2004). Antisense approach has also been tried (Li et al. 2006). The immuno-suppressive macrolide sirolimus was found to prevent metastatic progression in LCI-D20 model with suppression of VEGF via downregulating HIF-1 α (Wang et al. 2008a), and being verified in patients with liver transplantation (Zhou et al. 2003). The combination of rapamycin and sorafenib strongly inhibited lung metastasis in LCI-D20 model, although its molecular background remains to be explored (Wang et al. 2008b).

2 Molecular Events During Establishment of Metastatic Human HCC Models

Metastatic human HCC models in nude mice and cell lines with metastatic potential are essential for the studies of HCC metastasis. Since the first human HCC cell line (Chen 1963) and the first human HCC nude mice model (Shimosato et al. 1976) was established, numerous human HCC cell lines and models have been reported, including the famous PLC/PRF/5 cell line which produces HBsAg (Alexander et al. 1977); unfortunately, metastatic potential was rarely mentioned. Since early 1990s (Fu et al. 1991), “metastatic models” constructed in nude mice by orthotopic transplantation of histologically intact patient specimens has been widely used in the establishment of many kinds of metastatic human tumor model, however, metastatic human HCC model has not been demonstrated.

2.1 Molecular Events During Establishment of Metastatic Human HCC Models

Using orthotopic implantation of histologically preserved tumor tissues, a highly metastatic model of human HCC in nude mice (LCI-D20) was established through

in vivo clonal selection by repeated “lung foci to liver” in our institution (Sun et al. 1996). All mice with transplanted LCI-D20 tumors in the liver exhibited various manifestations of tumor behavior in HCC patients, including expression of AFP and HBxAg, regional invasion, peritoneal seeding, intrahepatic metastases, as well as metastases to lungs and lymph nodes. Meanwhile, a low metastatic model of human HCC in nude mice (LCI-D35) was also established. When comparison was made between LCI-D20 and LCI-D35, using CGH, 8p deletion was found to be one of the important alterations in LCI-D20 (Qin et al. 2001); angiogenesis induced by LCI-D20 tumor was stronger than that induced by LCI-D35 tumor (Sun et al. 1999a).

N-acetylglucosaminyltransferase V (GnT V) activity was much higher in LCI-D20 (Shao et al. 1999). Phasic expression of ICAM-1 was observed with the progression of LCI-D20 (Sun et al. 1998). After our publication, some metastatic human HCC models were reported (Genda et al. 1999; Gao et al. 2004); however, their molecular events were rarely mentioned. Human HCC cell line with metastatic potential was rarely reported in the literature except the one with metastasis to lymph nodes (Seki et al. 1999).

2.2 Molecular Events During Establishment of Metastatic Human HCC Cell Lines

In authors' institution, a human HCC cell line with metastatic potential (MHCC97) was established from LCI-D20 tumor using alternating in vivo and in vitro cultivation. The chromosome number was 59–65 with a median range of 60–61, and aberrant chromosomes i(1)(q) and der(4) (pter→q35::) were its chromosome markers. Upon intrahepatic inoculation in nude mice, the latency period of tumor formation was 15–20 d, and metastasized 100% to the lungs and invasion to the liver, diaphragm, and abdominal wall at 5th week (Tian et al. 1999).

Later, several stepwise metastatic human HCC cell lines with a similar genetic background, including two subclones with high (MHCC97H) and low (MHCC97L) metastatic potential from MHCC97 (Li et al. 2001), and two even higher metastatic potential cell lines (HCCLM3 and HCCLM6) from MHCC97H were established through in vivo clonal selection procedure (Li et al. 2003, 2004). Compared with MHCC97L, MHCC97H had faster growth rate and high expression of CK19. The pulmonary metastatic rate was 100% in MHCC97H vs. 40% in MHCC97L (Li et al. 2001). The serum CK19 level correlated well with lung metastasis of the animal model and the incidence of portal vein tumor embolus in HCC patients (Ding et al. 2004). After orthotopic implantation of HCCLM3 tumor tissue into nude mouse liver for 35 days, widespread loco-regional and distant metastases were found 100% in lungs and abdominal wall, 80% in intra-abdominal cavity, and 70% in diaphragm. Twenty-five differentially expressed genes were identified in HCCLM3 comparing with MHCC97L by cDNA microarray, including decreased Rb2 and increased MAP kinase (Li et al. 2003). When HCCLM6 tumor tissue was orthotopically implanted, widespread loco-regional and pulmonary metastases occurred and 75% regional lymph node metastasis was produced with inoculation HCCLM6 cells into

the footpad of nude mice. And increased expression of matrix metalloproteinase (MMP-2 and MMP-9) and CK19 and decreased expression of Rb2/p130 were also found in HCCLM6 cells (Li et al. 2004).

The establishment of above metastatic human HCC models in nude mice and human HCC cell lines with different metastatic potential provides an important model system for the in vivo and in vitro study of HCC metastasis.

3 Molecules Related to HCC Metastasis

Exploring the molecular mechanisms involved in HCC metastasis could be helpful in the prediction of HCC recurrence and provides new therapeutic targets for HCC metastasis (Qin and Tang 2005). During the past decade, genomic and proteomics approaches have been used to analyze the genetic aberrations, gene and protein expression profiles of the clinical specimens, as well as the metastatic animal models and cell lines of human HCC, to identify the molecules related to HCC metastasis.

3.1 Genomic Approach

To understand the molecular mechanisms of cancer metastasis, it is indispensable to identify the genetic alterations that accumulate during cancer progression as well as those responsible for the acquisition of metastatic potential in cancer cells (Qin 2002).

3.1.1 Chromosome 8p Deletion Was Found to Be One of the Most Important Chromosomal Aberrations that Associate with Metastasis of HCC

We compared the differences of genomic changes between primary HCCs and their matched metastatic lesions by comparative genomic hybridization (CGH). Several genomic alterations including loss of 8p, 4q, 17p, and 19p; gain of 5p; and amplification of 1q12-q22 were detected more frequently in metastatic lesions. The most significant finding was the loss of 8p ($p = 0.03$) (Qin et al. 1999). This finding was further confirmed by the PCR-LOH (loss of heterozygosity) analysis (Fig. 19.1), and by comparison between nude mice models of HCC with different metastatic potentials (Qin et al. 2001) and the HCC cell lines from the same parents but with different metastatic potentials (Yang et al. 2003, 2005). These suggest that deletion of chromosomes 8p may contribute to the development of HCC metastasis. We further analyzed the genome-wide microsatellite imbalances on the candidate regions related to HCC metastasis, and found that 8p23.3 and 8p11.2 were two likely regions harboring metastasis-related genes. These provide some accurate candidate regions for further study to identify putative metastasis suppressor genes for HCC (Zhang et al. 2003).

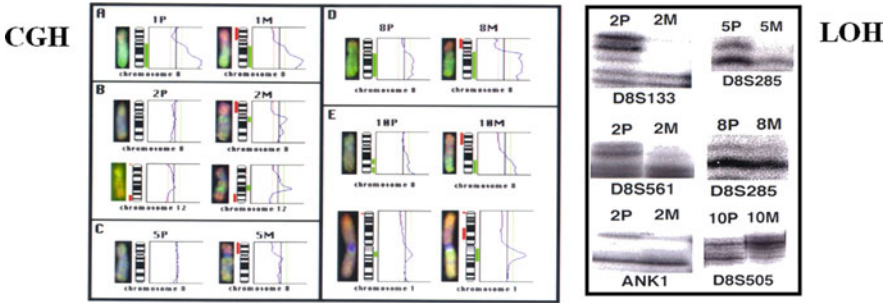


Fig. 19.1 Chromosome alterations in the primary and metastatic HCC detected by CGH and LOH analyses. Loss of 8p in metastatic but not in primary HCC is detected in CGH analyses (*left*), which is further confirmed by PCR-LOH analysis (*right*) (P-primary HCC, M-metastatic lesions) (Qin LX, et al. Cancer Res 1999)

3.1.2 Using “8p-Specific” Microarrays, Two Novel Metastatic Suppressors Were Identified, and Proved to Suppress In Vitro Invasion and In Vivo Metastasis of HCC

Based on the above finding, “8p-specific arrays” was developed using the genes or expressed sequence tags (ESTs) that were found on chromosome 8p, and to compare the difference of expression profiling between the HCC cell lines (MHCC97H and MHCC97L) with different metastatic potentials and similar genetic backgrounds, and also HCC specimens with or without metastasis. One full-length gene, HTPAP (phosphatidic acid phosphatase type 2 domain containing 1B), was identified. Both in vitro and in vivo assays suggested that HTPAP could suppress the invasion and metastasis of HCC. These suggest that HTPAP is a novel metastatic suppressor gene for HCC (Table 19.1) (Wu et al. 2006). Recently, another novel candidate metastatic suppressor, MSRA, was identified on chromosome 8p (Lei et al. 2007).

Table 19.1 The effect of HTPAP gene on the lung metastasis of nude mice bearing human HCC

Group	No.	Lung metastatic rate (%)	No. of metastatic lesions (colonies/mouse, $\bar{X} \pm S$)
PBS control	8	87.5	5.63±2.25
pIRES2-EGFP	7	85.7	4.86±2.27
HTPAP	8	25.0 ^a	0.63±1.19 ^b

^a $P<0.05$ (Pearson’s chi-square test); ^b $P<0.05$ (one-way ANOVA analysis)

The above findings provide not only a holistic view on the molecular cytogenetic bases of HCC metastasis, but also candidate regions for further study to identify metastatic suppressor genes.

3.2 Proteomics Approach

In authors' institute, differential proteomic analysis on two HCC cell strains (MHCC97H and MHCC97L) with different metastatic potentials was conducted. As had mentioned in Section 2.2: CK19 was overexpressed in MHCC97H (Ding et al. 2004), serum CK19 levels in nude mice increased in parallel with tumor progression and rose remarkably when pulmonary metastases occurred. Study on human HCC specimens revealed that more patients in the CK19-positive group had overt intra-hepatic metastases. Besides, S100 calcium-binding protein A4 (S100A4) was also overexpressed in metastatic HCC cell line (Cui et al. 2004), and might contribute to HCC invasion and metastasis through two pathways of MMP9 secretion regulation and strengthened motility and invasion properties (Cui et al. 2006).

Differential proteomic analysis was also performed on human HCC tissues, 16 proteins including HSP27, S100A11, CK18 were annotated, relevant to chaperone function, cell mobility, cytoskeletal architecture, respectively. HSP27 was overexpressed consistently in all metastatic HCC tissues, which suggests that HSP27 may serve as biomarker for early detection and therapeutic targets unique to the metastatic phenotype of HCC (Feng et al. 2005; Song et al. 2006). Recently, calpain small subunit 1 (Capn4), a protein with relevant interactions with many migration-invasion-related proteins, was identified to be related to HCC recurrence after liver transplantation (Bai et al. 2009).

Tumor metastasis might be associated with the expression of glycoproteins and the alteration of their glycan parts. To investigate the aberrantly alpha1,6-fucosylated glycoproteins related to HCC metastasis, a high-throughput glycomic approach was used to compare the lens culinaris agglutinin (LCA) affinity glycoprotein profiles of metastatic HCC cell lines. The alterations of CK8, annexin I, and annexin II in both expression levels and their glycan parts were found to be related to metastatic ability of HCC cells, and might play a role in metastasis of HCC (Dai et al. 2006). With bioinformatic approach, large numbers of proliferation and apoptosis-relative proteins were found to interact with different glycoproteins, and play important roles in metastasis of HCC (Zhou et al. 2007a).

Proteomic study was also used to clarify the mechanism of HCC immune escape concerning dendritic cells (DC) dysfunction, and found that down-regulation of beta-centractin in DCs pulsed with high metastatic potential HCC lysates might associate with DCs dysfunction and HCC invasiveness (Weng et al. 2008).

One new trend is the combination of annotation/protein sequence analysis, transcript profiling, immunohistochemistry, and immunoassay, which provides a more powerful approach for delineating candidate biomarkers of cancer with potential clinical significance. However, no well-linear correlation has been found between gene and protein expression levels due to splice process of mRNA and post-transcriptional regulation. Hence, differential expression profile analysis of a large number of proteins is an essential step in understanding the mechanism of metastasis and in discovering the diagnostic markers and therapeutic targets for HCC.

4 Molecules for Prediction of HCC Metastasis

As shown in Table 19.2, many clinical and pathological factors have been used for prediction of patients’ outcome. Unfortunately, their predictive value was not satisfied particularly for metastasis (Qin and Tang 2002, 2005).

Table 19.2 Risk factors for metastasis and recurrence after surgical resection of hepatocellular carcinoma

Items	Risk factors or predictors
Co-existing liver disease	Inflammation activity: ALT, GGT, viral load, serum HBeAg, genotype C HBV, liver functional reserve
Pathological features of tumor	pTNM stage, size, number, capsule, differentiation
Tumor-associated antigens and detection of circulating cancer cells	Venous invasion; intrahepatic metastasis (IM) Inflammatory cell infiltration (favorable factor) Serum AFP level (protein, mRNA); AFP-L3 Serum MAGE, hTERT mRNA
Invasion and metastasis-related markers	Osteopontin (OPN) (tissue and serum) Intratumor microvessel density (MVD) level VEGF level, p53 gene mutation Reduced expression: p27, E-cadherin Overexpressions: Laminin-5, MMP-2, MMP-9, MT1-MMP
Genomic aberrations and expression profiling	Genomic aberrations 16q; 8p; changed restriction landmark genomic scanning (RLGS) spots Gene expression profiling 90 genes associated with intrahepatic metastasis 153 genes predicting signature for metastases and outcome. 12 genes predictive system 17 genes related to immune response Proteomics analysis CK19, CK10

4.1 Chromosome 8p Deletion Detected in HCC Tissues or Circulating DNA of HCC Patients Can Predict HCC Recurrence and Patient’s Prognosis

We analyzed the LOH on 8p in circulating DNA from 79 patients with HCC before operation. LOH on 8p was found in 60 cases (76.0%) of patients, and more frequently (85.7%) in those with metastatic HCC; and LOH on 8p in plasma DNA is significantly correlated with TNM stage, vascular invasion, and a lower DFS and OS. The 3-year DFS of patients with 8p deletion detected in circulating DNA

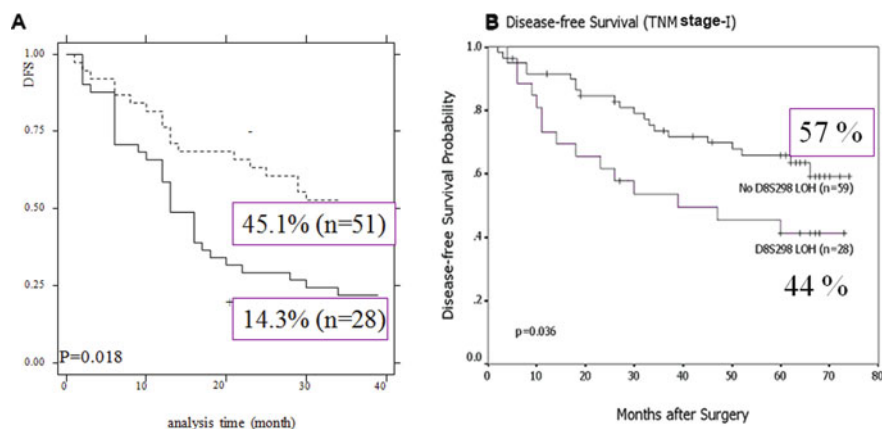


Fig. 19.2 8p deletion detected in HCC tissues and circulating DNA of HCC patients can be used to predict HCC recurrence and patient's prognosis. *Left*: the association of 8p deletion detected in circulating DNA of HCC patients with the disease-free survival (DFS) curves of patients. *Right*: the association of 8p deletion detected in HCC tissues with DFS of patients with TNM stage I of HCC

(14.3%, $n=28$) was significantly lower than that of without 8p deletion (45.1%, $n=51$, $P=0.018$). Therefore, LOH on 8p is an independent predictor for prognosis of HCC patients (Fig. 19.2, left) (Ren et al. 2006). Recently, LOH at D8S298 detected in HCC tissues was found to be associated with a worse 5-year OS and DFS of patients after curative resection, even in those with early stage of HCC (44% vs. 57%, $P=0.036$). In multivariate analyses, LOH at D8S298 was an independent predictor of decreased DFS. Therefore, 8p deletion can serve as a novel prognostic predictor for HCC patients, even in those with early stage of HCC (Fig. 19.2, right) (Pang et al. 2007).

4.2 Plasma Level of Osteopontin (OPN) Can Be a Predictor for Recurrence and Prognosis of HCC Patients

Based on the study of metastasis-related signatures, we demonstrated an important role of OPN in HCC metastasis. OPN is overexpressed in metastatic HCC, and an OPN-neutralizing antibody or microRNA against OPN can efficiently block invasion and metastasis of highly metastatic HCC cells both in vitro and in vivo (Ye et al. 2003). Recently, we use lentiviral vectors encoding microRNA (miRNA) against OPN (Lenti.OPNi) to down-regulate the OPN level in HCCLM3 cells which had an overexpression of OPN and a higher metastatic potential, and find that these Lenti OPNi induce a significant decrease in MMP-2 and uPA expression, and a obvious inhibition of both in vitro invasion and in vivo lung metastasis of HCCLM3 cells. This further suggests that OPN plays an important role in metastasis, and can be a hopeful target for the control of HCC (Sun et al. 2008). A higher plasma

OPN level was closely related to a poorer survival for HCC patients. The 2-year disease-free survival rate (DFS) of HCC patients with a higher plasma OPN level (≥ 200 ng/ml) (16.3%) was significantly lower than that of patients with a lower OPN level (<200 ng/ml) (59.0%, $P = 0.0001$). The plasma level of OPN is an independent prognostic factor for both OS and DFS, and can be used as a predictor for HCC recurrence and survival (Zhang et al. 2006) (Table 19.3; Fig. 19.3). OPN combined with CD44 is a promising independent predictor of tumor recurrence and survival in patients with HCC (Yang et al. 2008a).

Table 19.3 The association of preoperative plasma OPN level with recurrence in HCC patients

	N	Distribution by OPN ^a		OPN level (ng/ml) (median [IQR])	p ^b
		<200 (%)	≥ 200 (%)		
Without recurrence	39	32 (82.1)	7 (17.9)	153.70 (101.05, 184.70)	0.0013
With recurrence	54	24 (44.4)	30 (55.6)	213.55 (158.85, 355.09)	

^aCase numbers within different OPN levels
^bMann-Whitney U test

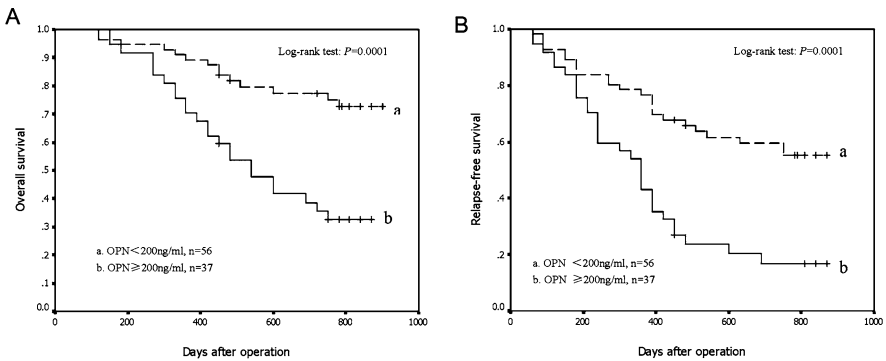


Fig. 19.3 The association of plasma osteopontin (OPN) levels with the disease-free survival (DFS) of patients with HCC after resection. The DFS of HCC patients with a higher plasma OPN level (≥ 200 ng/ml) was significantly lower than that of patients with a lower OPN level (<200 ng/ml, $P = 0.0001$)

4.3 Through Proteomics Analysis, CK19 and CK 10 Are Identified as Predictors for HCC Metastasis

Differential proteomic analysis was conducted to analyze the protein expression profiles of cell lines and clinical specimens of HCC. CK10, CK19, and HSP27 were identified as potential predictive markers for HCC metastasis. Overexpression of CK10 in HCC tissues, and serum CK19 level might reflect the pathological

progression in HCC and useful prognostic markers and therapeutic targets for HCC patients with metastases (Ding et al. 2004; Yang et al. 2008b).

Unfortunately, none of the above-mentioned biomarkers has been accepted as clinical routine, the combination with clinico-pathological features seems to be one of the solutions. Many factors, including other clinical parameters, tumor morphology, and tumor histological features, as well as treatment-related factors, have been reported as risk factors, and as significant predictors of HCC recurrence.

5 Molecular Signature of HCC Metastasis

The development of microarray technology, which allows us to undertake parallel analysis of tens of thousands genes in a genome-wide scale, has led to a new era in medical science (Schena et al. 1995; DeRisi et al. 1996). This approach has been widely used in successful molecular classification and prediction of many kinds of human malignant tumors with respect to their stage, outcome, or response to therapy (Golub et al. 1999; Alizadeh et al. 2000; Van 't Veer et al. 2002; Valk et al. 2004).

5.1 Molecular Signature of HCC Metastasis Derived from Tumor Gene Expression Profiling

In our recent study collaborated with NCI/NIH of the United States, we applied gene expression profiling to investigate the global gene changes associated with HCC metastasis. We discovered that the gene expression profiling of primary HCCs with accompanying metastasis was very similar to that of their matched metastases, but was significantly different from that of metastasis-free primary HCCs (153 significant genes, $p < 0.0001$) regardless of tumor size, encapsulation, liver cirrhosis, and patients' age (Ye et al. 2003). Similar results had also been found in other studies. Weigelt found that the gene expression profiles of primary breast and colorectal tumors were strikingly similar to that of their matched distant metastases (Weigelt et al. 2003). Ramaswamy et al. (2003) uncovered from primary adenocarcinomas a molecular signature that was associated with high metastatic potential and poor clinical outcome.

Our findings, together with others', indicate that metastatic capacity is an inherent feature of cancer and genes favoring metastatic progression are initiated in the bulk cells of primary tumors. These provide rationales for early prediction and prevention of cancer metastasis.

Using the 153 significant genes we generated a molecular signature that could classify HCC patients and predict the metastatic potential and patients' survival with a prediction accuracy of 85% (Ye et al. 2003). The presence of molecular prognostic signature in primary HCCs was confirmed by other studies (Lizuka et al. 2003; Lee et al. 2004; Kurokawa et al. 2004).

Recently, we examined the miRNA expression profiles of 241 HCC specimens using miRNA array and built a unique 20-miRNA metastasis signature that could

significantly predict both survival and relapse in 89 early stage HCCs. However, the other clinical HCC prognostic staging systems (TNM, OKUDA, CLIP, or BCLC) were incapable of predicting the prognosis. These 20 miRNAs provides a simple profiling method in identifying HCC patients who are likely to develop metastases (Budhu et al. 2008).

5.2 Molecular Signature of HCC Metastasis Derived from Gene Expression Profiling of Non-tumor Liver Tissues

Cancer metastasis is an extraordinarily complex process, involved the interactions between cancer cells, microenvironment, and the host. Therefore, besides the above studies on the genomics alterations in HCC cells (“seed”), we also focused on the alterations of local environment (“soil”) to HCC metastatic potential. We analyzed the gene expression profiling of non-cancerous liver tissues from HCC patients with or without metastasis using cDNA microarray and revealed a unique molecular signature comprising 17 genes associated with immune/inflammation response that could significantly discriminated HCC patients with or without metastasis, suggesting that the immune/inflammatory status of the microenvironment may play an important role in promoting HCC metastatic progression (Budhu et al. 2006). Recently, Hoshida et al. uncovered a significant molecular signature highly correlated with patients’ survival from the profiles of the surrounding non-tumoral liver tissue and it was validated in another independent 225 HCC patients from other countries (Hoshida et al. 2008). These results indicated that the conditions of liver parenchyma could significantly contribute to HCC metastatic progression whether they are influenced by liver damage mediated by viruses hepatitis B, C, and aflatoxin B1 or by individual genetic constitution (Budhu et al. 2005).

However, due to the complicated factors such as geographic variation of etiological factors, genetic background, and the molecular heterogeneity of the tumors (Bruix et al. 2004; Llovet, Burroughs and Bruix. 2003), none of them could cover the entire HCC population with high predictive value. It is indicating that HCCs comprise several biologically distinctive subgroups and the signatures with multiple genes may satisfy to only some of the subtypes of HCC, such as EpCAM and alpha-fetoprotein expression defined novel prognostic subtypes of HCC (Yamashita et al. 2008), TGF- β specific classification, and prediction subtypes of HCC (Coulouarn et al. 2008). This will be the basis of molecular targeting therapy and individualized treatment of HCC metastasis.

6 Molecular Events in Microenvironment with Special Reference to Tumor Vasculature

Generally, tumor is composed of two cell compartments, tumor cells and “normal” cells; interaction between these two compartments plays an important role in carcinogenesis and tumor progression. Angiogenesis switch, as a hallmark

of carcinogenesis (Folkman and D'Amore 1996; Hanahan and Weinberg 2000), involves active interaction between tumor cells, endothelial cells, stromal cells, cytokines, and others. Nowadays, anti-angiogenesis treatment becomes an accepted approach to fight cancers (Folkman 2007). Even evolution of cancer stem cells is also regulated by tumor microenvironment (Bissell and Labarge 2005). Furthermore, a number of novel treatments related to tumor microenvironment have been investigated, such as anti-inflammation and immunomodulation.

6.1 Angiogenesis of HCC

Endothelial cells (ECs) and pericytes are two major components of tumor vasculature. Specific markers for ECs in HCC include CD34, CD31, CD105, and VIII factors. Our data showed CD34 staining microvessel density in HCC associated with patients' survival after resection of HCC (Sun et al. 1999b), which was supported by other studies (Poon et al. 2002; Nanashima et al. 2008). CD105 (also known as receptor of transforming growth factor- β , TGF β) has been shown as more specific marker of proliferating ECs (Burrows et al. 1995); staining of CD105 in the adjacent non-tumor tissue was associated with early recurrence (Ho et al. 2005; Yu et al. 2007). Pericytes support vasculature and stabilize ECs. Therefore, targeting both EC and pericytes should be more potent to inhibit in established tumor vessels. In the liver, hepatic stellate cell (HSC) is regarded as pericyte (Lee et al. 2007), which produces vascular endothelial growth factor (VEGF) to activate EC (Theret et al. 2001). Our data showed isolated EC from HCC, also expressed PDGFR α ; inhibition of PDGFR α suppressed angiogenesis, growth, and metastasis of HCC (Zhang et al. 2005).

Together with other report (Poon et al. 2001b), our studies showed VEGF is one of major players in angiogenesis of HCC (Li et al. 1998, 1999). Other angiogenesis stimulators and inhibitors include basic fibroblast growth factor (bFGF) (Poon et al. 2001a), endostatin (Hu et al. 2005). Expression levels of VEGF and its receptors in HCC were similar to that in the adjacent non-tumoral liver, while angiopoietin and Tie receptors expression in HCC is lower than that in non-tumoral adjacent liver (Zeng et al. 2008). Our data showed the expression of VEGF and its receptors in the adjacent non-tumoral liver was even higher than that in tumor, and in which some were prognostic (Jia et al. 2008).

Tumor cells in HCC and hepatocytes in the adjacent non-tumoral liver expressed VEGFR1, 2, 3 (Liu et al. 2005; Jia et al. 2008), which makes prediction of efficacy of VEGF targeting therapy more complicated; one interesting finding is anti-VEGF treatment which may improve survival of tumor bearing mice through off-tumor VEGF target (Xue et al. 2008). So far, there is no proof of principle for VEGF targeting therapy in HCC patients (Zhu 2008).

Interferon- α (IFN- α) is a cytokine with multiple functions. Our studies showed IFN- α treatment suppressed tumor growth and metastasis by inhibiting angiogenesis via down-regulation of VEGF and direct inhibition of ECs (Wang et al. 2000, 2003b). Two randomized control trials evaluating adjuvant IFN- α treatment in

patients after hepatectomy for HCC with hepatitis B virus (HBV) infection background have been reported with a positive result (Sun et al. 2006; Lo et al. 2007). Furthermore, our study showed in P48 positive HCC, IFN- α directly inhibited tumor cell proliferation (Wu et al. 2004), and associated with clinical outcome (Qian et al. 2006).

6.2 Inflammation-Related Microenvironment

Active HBV replication was associated with a higher risk of tumor recurrence after resection of HCC (Nakai et al. 2006). HBX gene enhanced angiogenesis and metastasis by stabilizing hypoxia signaling pathway (Yoo et al. 2008), or up-regulating matrix metalloproteinases (MMPs) (Ou et al. 2007) therefore contribute to progression of HCC.

A gene expression signature in the non-tumor liver tissue is associated with a high risk of multicentric recurrence of HCC, the poor prognosis signature contained gene sets associated with inflammation and poor liver function (Hoshida et al. 2008). As mentioned in Section 5.2, another gene expression signature, containing 17 unique inflammation/immune response-related genes, was found to be associated with a high risk of recurrence, most of which were early recurrence (Budhu et al. 2006).

Our study suggested that tumor-favoring microenvironment could be created by tumor itself, which can be a center of inflammation, or inflammatory reaction of liver parenchyma. Macrophages, distributed in the adjacent non-tumor liver tissue in a centripetal style, suggesting macrophages were attracted by tumor, which is in accordance with other reports (Halin et al. 2009). Furthermore, monocyte colony stimulating factor (CSF-1) expression in the hepatocytes in adjacent non-tumor liver tissue was also found in a centripetal style (Zhu 2008). So, this finding showed tumor is a center of CSF-1 expression and macrophage accumulation, similar to that of chronic infection.

7 Molecular Changes During Intervention of HCC Metastasis

Growing evidence has showed acquired resistance to chemotherapy or molecular targeting therapy in different types of cancers, however, in HCC there were only little data that have been reported.

7.1 Molecular Changes Induced by Anti-angiogenesis Therapies

Anti-angiogenesis treatment induces tumor hypoxia, which decreases tumor metabolism and proliferation and therefore suppressed tumor growth (Bix et al. 2006; Gatenby and Gillies 2008); however, hypoxia-induced biologic changes may also promote tumor metastasis by selecting tumor cells to overcome nutritive deprivation and escape from the hostile primary microenvironment (Chan and Giaccia

2007; Vaupel and Mayer 2007). It has been reported that c-met, uPA, and IL-8 expression regulated by hypoxia facilitated movement of tumor cells into the circulation, thus increasing the number of circulating tumor cells and lung metastasis (Mizukami et al. 2005; Yoon et al. 2006). Likewise, transcatheter arterial chemoembolization (TACE), which is a treatment choice for advanced HCC with intrahepatic metastasis, may induce elevation of serum or plasma VEGF and bFGF as a response to tumor hypoxia, followed by tumor relapse and metastasis (Sergio et al. 2008; Xiong et al. 2004). Hypoxia-induced factor (HIF) 1 is the key factor induced by hypoxia, regulating downstream gene expression (e.g., VEGF, bFGF), blockage of HIF1 α can help to augment the effect of TACE (Sun et al. 2009).

The most popular anti-angiogenesis approaches are targeting VEGF/VEGFR. However, tumor can be resistant to VEGF/VEGFR blocking treatment due to redundancy of angiogenesis factors, or even acquire resistance by reactivation of other angiogenesis signal pathway, for example, bFGF can be up-regulated when tumor undergoes VEGF blocking treatment (Casanovas et al. 2005). A new evidence showed that tumor-associated fibroblasts (TAFs) produced platelet derived growth factor C (PDGF-C), which helped tumor to evade anti-VEGF therapy (Crawford et al. 2009). These changes are usually mediated by tumor hypoxia, which resulted from anti-angiogenesis treatment. HIF1 α is a transcription factor, which up-regulates expression of glycolytic enzymes, VEGF, angiopoietin, and bFGF (Wang et al. 2003a; Casanovas et al. 2005; Zanker 2007), all of which provide a surviving signals to hypoxia cells.

Clinical evidences have shown that resistance to anti-angiogenesis treatment is almost inevitable; tumor may adopt one or some mechanisms to develop resistance to anti-angiogenesis treatment (Bergers and Hanahan 2008). To overcome these, combination treatment is one of the choices. Our study showed that combination of Sorafenib (raf-1 and VEGFR inhibitor) and Sirolimus (mTOR inhibitor) was more effective in treating HCC than single agent (Wang et al. 2009).

7.2 Molecular Changes During Interferon-Alpha Treatment

Together with other studies, our studies showed that IFN- α exerted its inhibitory effect on HCC growth mainly through anti-angiogenesis by down-regulation of VEGF-A (Wang et al. 2000, 2003b; Wada et al. 2007). IFN- α treatment can also increase transmembrane protein 7 (TMEM7) expression in HCC cells, which mediated suppressed cell proliferation, colony formation, cell migration in vitro, and reduced tumor formation in nude mice (Zhou et al. 2007b).

7.3 EMT (Epithelial to Mesenchymal Transition) Associates with Molecular Changes During Intervention

Mesenchymal type of HCC cells is intrinsically resistant to EGFR targeting therapies, while mesenchymal to epithelial transition (MET) render the resistant cell

to be sensitive to EGFR targeting therapies (Fuchs et al. 2008). On the contrary, epithelial to mesenchymal transition (EMT) is one of mechanism of acquired resistance to chemotherapy or molecular targeting therapies due to a higher capacity of resistance to apoptosis, or elevated expression of other surviving signal transduction pathway (Yang et al. 2006; Rho et al. 2009). Moreover, mesenchymal type of tumor cells present a high capacity of invasion, motility, and metastasis (Yilmaz and Christofori 2009).

8 Future Prospects

Much has been done and much remains to be done, particularly in the field of translational medicine. Many molecular markers have been claimed with value of prediction, unfortunately a clinical routine was not yet available. Molecular signature including up to hundred genes has also been found; the predictive value is not yet satisfied based on the complicated subsets of HCC with different phenotypes. Although interferon-alpha was proved of value for the prevention of metastasis, it is only benefited to a subtype of HCC.

In future, the followings deserve to be noted for studies on molecular events of HCC metastasis: (1) More and more evidences have shown that metastatic potential of HCC is a result of interaction among cancer, microenvironment, and host. Searching for HCC, metastasis-related molecules will focus not only on cancer itself but also on microenvironment and particularly the host. Therefore, molecular background of relationship between metastasis and nervous/endocrinal/immunologic systems as well as metabolism will attract more attention. (2) The process from bench to bedside needs to speed up; however, the sensitivity and specificity of predictive biomarkers will not be as high as expected because of the complicated phenotypes. (3) The side effects of the major therapies for HCC have been recognized; however, the molecular background of “opposite effects” that may enhance the metastatic potential of the treated cancer are not well understood. Therefore, the biological concept of major therapies will be another important issue to be studied.

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Chapter 20

Molecular Pathogenesis of Hepatocellular Carcinoma

Chun Ming Wong, Judy Wai Ping Yam, and Irene O.L. Ng

Abstract Hepatocarcinogenesis is a multistep process developing from normal through chronic hepatitis and cirrhosis to HCC. With advances in molecular technology, there is a growing understanding of the molecular mechanisms in the development of HCC. In hepatocarcinogenesis, there is a strong link to increases in allelic losses, chromosomal changes, gene mutations, epigenetic alterations, and alterations in molecular cellular pathways. In this Chapter, special emphasis is given to the multistep process of hepatocarcinogenesis, genetics, epigenetics, and regulation of major signaling pathways involved in hepatocarcinogenesis. A detailed understanding of the molecular pathogenesis involved in the progression of HCC can improve our prevention and diagnostic tools for HCC and may help identify novel molecular targets for new therapies.

Keywords Molecular pathogenesis · Genetic · Epigenetic · MicroRNA · Signaling pathways

1 Multistep Hepatocarcinogenesis

Hepatocarcinogenesis is believed to be a multistep process. In the majority of the cases, HCC arises from a background of either chronic hepatitis or cirrhosis; only a small proportion of HCC patients have normal livers. Cirrhosis is a common risk factor for HCC. Because of better management of patients with cirrhosis resulting in longer patient survival, there are trends of increasing mortality rates due to HCC

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in United States and European countries. Of the risk factors for HCC, cirrhosis due to HCV infection is associated with the highest HCC incidence. The 5-year cumulative incidence in Japan and in the west is 30 and 17%, respectively (Fattovich et al. 2004). In patients with HBV-related cirrhosis, the 5-year cumulative HCC risk is 15% in high endemic areas and 10% in the west. In viral-related cirrhosis, HBV/HCV and HBV/HDV co-infections may increase the HCC risk by two- to sixfold relative to each infection alone. Alcohol abuse increases the HCC risk in HBV- or HCV-associated cirrhosis by two- to fourfold.

Dysplasia is a precancerous lesion of HCC and is classified into large (LCD) and small-cell dysplasia (SCD), both usually found in a background of cirrhosis or chronic hepatitis. SCD is an important independent risk factor for developing HCC in HCV-associated cirrhosis (Libbrecht et al. 2001); however, the risk of LCD in developing into HCC is controversial (Libbrecht et al. 2001; Makino et al. 2000).

A dysplastic nodule (DN) is a precancerous lesion in hepatocarcinogenesis and arising from a cirrhotic background (Ferrell et al. 1993; Roncalli 2004). They are classified into high- and low-grade DNs (HGDN and LGDN), depending on the histological features that include cellular architecture, presence or absence of portal tracts, and cytological features (Ferrell et al. 1993). DNs have been increasingly detected clinically, because patients with HBV- or HCV-associated cirrhosis undergo regular surveillance for HCC. Advances in imaging technology have facilitated the detection of small nodular lesions in chronic liver disease and the natural outcome of macronodules in cirrhosis. The rate of HCC development was significantly higher in the HGDN group than the other types (Kobayashi et al. 2006). Major progress in the classification and understanding of DN has been achieved through image analysis techniques combined with careful histological dissection of explanted native livers. In a study on explanted livers, which allow examining the hepatocellular nodules and confirming their nature, HCC nodules were significantly associated with the presence of HGDNs (Mion et al. 1996). Moreover, LGDNs and macro-regenerative nodules did not show chromosomal imbalances of allelic losses on 8p and of gains of 1q, as in HGDN and HCC (Tornillo et al. 2002).

Small HCC is defined as a tumor measuring less than 2 cm (Ferrell et al. 1993). Small lesions with malignant potential have only subtle differences from the surrounding parenchyma. These have created difficulty in reproducible assessments between western and eastern pathologists. Very recently, there has been consensus among a group of eastern and western liver pathologists in the stratification of small HCC into two clinicopathologic groups of early HCC and progressed HCC (International Consensus Group for Hepatocellular Neoplasia 2009). Early HCC has a vaguely nodular appearance and is well-differentiated, whereas progressed HCC has a distinctly nodular pattern, is mostly moderately differentiated, and often has evidence of microvascular invasion. Significantly, early HCC has a longer disease-free survival rate and 5-year survival rate as compared with progressed HCC.

2 Genetic Alterations in Hepatocarcinogenesis

2.1 Chromosomal Abnormalities

Cancer is a genetic disease; like other solid tumors, liver cancers are characterized by frequent genetic abnormalities. Genetic alterations can emerge at pre-malignant lesions or early stage of cancer development, accumulate during multistep carcinogenesis, and result in acquisition of full-blown malignant phenotype. These genetic abnormalities, from gross chromosomal number to single nucleotide sequence changes, are commonly believed to be the underlying impetus of carcinogenesis. Early studies using flow cytometry to analyze DNA content have clearly revealed aneuploidy as a common feature of primary HCC. Gain of DNA content is likely an early event of carcinogenesis since DNA aneuploidy was found in approximate half of cirrhotic livers and can be found in up to 80% of HCC biopsies samples (Attallah et al. 1999). Attempts have been made to reveal specific chromosomal abnormalities in HCC using conventional karyotyping methods; however, the interpretation has been difficult due to the complexity of chromosomal aberration features in cancer cells (Table 20.1). In the last decade, the introduction of spectral karyotyping (SKY) analysis has significantly facilitated the study of chromosomal changes in HCC. SKY is molecular cytogenetic technique first developed in 1996 (Schrock et al. 1996), in which 24 chromosomes are simultaneously hybridized with

Table 20.1 Chromosomal abnormalities in primary HCCs

	Chromosomal abnormalities	Reference
Gain	1q (58%), 6p (33%), 8q (60%), 17q (33%)	Marchio et al. (1997)
Loss	4q (70%), 6q (37%), 8p (65%), 13q (37%), 16q (64%), 17p (51%)	
Gain	1q (72%), 8q (48%), 17q (30%), 20q (37%).	Wong et al. (1999)
Loss	4q (43%), 8p (37%), 13q (37%), 16q (30%)	
Gain	1q (78%), 8q (66%)	Kusano et al. (1999)
Loss	4q13-22 (32%), 7p (51%), 8p (29%), 10q (17%), 13q13-14 (37%), 16q (46%)	
Gain	1q (66%), 8q (48%), 20q (20%).	Guan et al. (2000)
Loss	1p (36%), 4q (40%), 6q (70%), 8p (32%), 17p (52%), 19p (42%), 22q (28%).	
Gain	1q (46%), 5p (27%), 6q (27%), 8q (69%), 12q (42%), 17q (46%), 20q (31%), Xq (27%)	Zondervan et al. (2000)
Loss	1p (35%), 4p (35%), 4q (42%), 8p (58%), 9p (27%), 9q (27%), 13q (39%), 14q (31%), 16p (35%), 16q (54%), 17p (31%), 18q (35%),	
Gain	1q (46%), 6p (20%), 8q (41%), 11q (27%), 17q (37%)	Tornillo et al. (2000)
Loss	1p (24%), 4q (39%), 6q (41%), 8p (44%), 9p (24%), 11q (24%), 12q (22%), 13q (39%),	

different fluorescence labeled probes that greatly relieved the complicated chromosome recognition during cancer karyotyping analysis. SKY is particular useful in identifying chromosomal translocation and rearrangement. However, the involvement of tedious primary cancer cell culture is the major drawback of SKY analysis, which limited the application of this technique in primary HCC samples hence most reports were done on established cancer cell lines. In a pilot study, Wong and colleagues had successfully preformed SKY analysis in 15 human HCC after a short-term primary culture. In that study, the authors showed that chromosomal rearrangement was very common in HCC and multiple chromosomal rearrangements have been found in all HCC samples. Among these, the most frequent rearrangements were found in the centromeric regions of chromosome 1, 8, 17, and 19, resulting in translocation of the whole chromosome arms. Complex chromosomal rearrangement patterns were also observed in some primary HCCs. Interestingly, most of these rearrangement involved chromosome 8, suggesting that chromosomal rearrangement, led to the activation of proto-oncogenes or the loss of tumor suppressor gene on chromosome 8, may have specific pathologic implication to human HCC (Wong et al. 2000).

To get rid of the technical challenge of primary cancer cell culture encountered in conventional karyotyping and SKY analysis, various molecular techniques such as fluorescence in situ hybridization (FISH), loss of heterozygosity (LOH) assay, and comparative genomic hybridization (CGH) were developed to study the chromosomal abnormalities in human cancers. FISH is the technique of choice for showing chromosome gains. Using a cloned DNA probes that can recognize chromosome-specific repeat sequences, such as α -satellite centromeric DNAs, chromosome aneusomy can easily be detected in interface nuclei in frozen sections (Hamon-Benais et al. 1996; Huang et al. 1999; Zimmermann et al. 1997). With this technology, Zimmermann and colleagues showed that gain of chromosome 1 was one of the most common chromosomal aberrations in HCC, and polysomy was found in 72% of primary HCCs (Zimmermann et al. 1997). FISH can also be applied to formalin fixed paraffin embedded tissues and copulate with tissue microarray to allow high-throughput analysis of chromosomal or gene specific amplifications (Chan et al. 2004). LOH analysis using polymorphic DNA microsatellite markers perhaps is the most widely used molecular technique to determine allelic losses of specific loci. The simple PCR-based procedure and widely distributed polymorphic markers throughout the genome, by the first time, allow researchers to map the global chromosomal deletion in cancer samples. Genome-wide allelotyping studies on HCCs have shown that allelic losses were frequently detected in many chromosomal regions, including 1p, 4q, 6q, 8p, 9p, 13q, 16q, 17p, and 19q (Kawai et al. 2000; Kuroki et al. 1995; Li et al. 2001; Nagai et al. 1997; Okabe et al. 2000; Piao et al. 1998a). Allelic losses on chromosome 4q, 8p, 13q, and 16q were found to be significantly associated with poorer tumor differentiation, larger tumor size, or more advanced tumor stages (Bando et al. 1999; Bluteau et al. 2002; Chan et al. 2002; Emi et al. 1993; Kuroki et al. 1995; Okabe et al. 2000; Piao et al. 1998b; Pineau et al.

1999; Wong et al. 2002). There is also an increasing incidence of allelic loss along the multistep process of hepatocarcinogenesis from chronic hepatitis, cirrhosis, and DN to HCC (Lee et al. 2008). Accumulation of allelic losses in HCC was reported to be associated with more aggressive tumor behavior and poor prognosis of HCC patients (Tamura et al. 1997). More recent studies further indicated that although relatively uncommon, allelic loss started to be detected in cirrhotic livers. Along the disease progression, allelic losses accumulated and exhibited a stepwise increase from cirrhotic liver to dysplastic nodules and finally to HCC (Raidl et al. 2004). Interestingly, the frequency and pattern of chromosomal aberration observed in high-grade DN was very similar to that of HCC, supporting the notion that allelic loss is an early event and could occur at the pre-malignant stage of HCC development (Kahng et al. 2003; Raidl et al. 2004; Sun et al. 2001). Allelic losses seem to be more common in HBV-related HCCs than those HCV-associated tumors. It has been shown that HBV-associated HCCs had more frequent (40% on average) losses at 4q, 16q, and 17p (including the p53 region) than in non-viral HCC samples, suggesting that these abnormalities are much associated with HBV infection (Zondervan et al. 2000). In addition, HCCs that developed from different etiological background may have different patterns of chromosomal aberration. For instance, gain of chromosome 10q was detected exclusively in cases with HCV infection, whereas an amplification of 11q13 was often seen in HCC associated with HBV infection (Zondervan et al. 2000).

After defining the common chromosomal abnormalities in primary HCC, several “minimal deleted regions” have further been defined with chromosome-specific high-density allelotyping. Examples include 1p36 (Fang et al. 2000), 4q35 (Bando et al. 1999), 8p21.3-22 (Chan et al. 2002; Emi et al. 1993), 13q12.3-14.1, 13q32 (Lin et al. 1999; Wong et al. 2002), and 16q24 (Bando et al. 2000). The higher incidence and recurrent nature of chromosomal deletions at these particular loci strongly imply that these regions may harbor putative tumor suppressor genes. Nevertheless, these minimal deletion regions often span several Mb in length and may encompass many candidate genes. For example, in the regions of the recurrent deletion region at chromosome 8p21.3-22, several genes, including *DLC1*, *MTUS1*, *FGLI*, and *TUSC3*, have been identified as candidate tumor suppressors in this region. The next challenging question is whether any of these candidate genes might play a direct role in hepatocarcinogenesis. Recently, with array-based CGH which provides enhanced chromosomal resolution, investigators now can narrow down the aberrant regions into more defined fragment (>1 Mb) and pinpoint the affected gene(s) directly (Albertson et al. 2000; Pinkel et al. 1998). In a recent study, Xue et al., using array-based CGH elegantly demonstrated that deletion of *Deleted in liver cancer 1* (*DLC1*) was observed in ~70% of primary HCC samples and was more frequently than those observed for the well-established tumor suppressors such as *INK4a/ARF*, *PTEN*, and *TP53*. This finding echoed with the previous LOH and conventional CGH studies and confirmed that *DLC1* is the major target of deletion in the 8p21.3-22 region in primary HCC samples.

2.2 Somatic Mutational Analysis

In human cancers, gene mutations, including point mutations and interstitial deletions/insertions, have been found in a large number of genes that regulate cell proliferation, cell-cycle progression, apoptosis, and metastasis. Gene mutations are known to contribute to cancer development since the early part of last century (Ruddon 1995). Frequently mutated genes in human HCC include p53, β -catenin (de La Coste et al. 1998; Wong et al. 2001), Axin (Satoh et al. 2000), Caspase-8 (Soung et al. 2005), LKB1 (Kim et al. 2004), and KLF6 (Kremer-Tal et al. 2004). However, gene mutations in HCC are relative uncommon compared with other solid tumors like colon and breast cancers. Of these genes, p53 and β -catenin probably are the most frequently mutated genes in HCC. p53 gene is the most well-characterized tumor suppressor gene known to be mutated at very high frequency in tumors of different cellular origins (Greenblatt et al. 1994). The frequency of p53 mutation in Asian population has been detected in a range from 13 to 33% of human HCCs (Ng 1998; Ng et al. 1994a,b). p53 mutations, in general, are randomly scattered over the exons 5-9 of the gene. Mutant p53 has a much longer half-life than the wild-type protein and is therefore closely associated with p53 protein overexpression. Pathologically, p53 overexpression was more frequently seen in tumors with poor cellular differentiation and of larger tumor size, suggestive of a late event in development (Ng et al. 1995). Interestingly, in HBV prevalent regions like southern China and sub-Saharan Africa, G to T mutation are the predominant form, which is in contrast to the HCV prevalent regions that exhibit no specific mutation pattern. More strikingly, a specific G to T mutation hot spot on the third nucleotide of codon 249 was found in aflatoxin prevalent region, Qidong country in Jiangsu Province of mainland China (Hsu et al. 1991). This mutation results in an arginine to serine substitution that accounts for 30% of total p53 mutations in Qidong regions. It has also been reported that Ser-249 mutant was sufficient to promote cell proliferation and suppressed wild-type p53-mediated apoptosis in in vitro models (Dumenco et al. 1995; Ponchel et al. 1994). Although this p53 Ser-249 mutant was unable to induce cellular transformation in normal hepatocytes directly (Dumenco et al. 1995), loss of p53 function has been considered a critical step of cellular transformation induced by chemical carcinogens and oncogenic pathways (Eferl et al. 2003). β -catenin, on the other hand, represents the most commonly mutated proto-oncogene in human HCC. β -catenin is the key modulator of the Wnt-signaling pathway. In the absence of Wnt signals, cytoplasmic β -catenin is subject to degradation via ubiquitin-proteasome system that requires the phosphorylation of β -catenin by APC/GSK-3 β complex. Deregulation of Wnt-signaling pathways is a hallmark of human carcinogenesis. This notion has been well-illustrated by the frequent mutations on APC, Axin, and β -catenin genes, which mimic the activation of Wnt-signaling pathway and results in β -catenin accumulation and nuclear localization in hereditary as well as sporadic cancers. In HCC, mutation of β -catenin was detected at a frequency of 18–34%. It is noteworthy that β -catenin mutation and nuclear localization were more common in HCV-associated HCCs. Several independent studies had estimated that β -catenin mutation in HCV-associated HCC was

found in approximately 50% of tumors, which was strikingly higher than HBV-associated HCCs, suggesting that HCV infection may play an even more significant role in the activation of Wnt-signaling pathway than HBV infection. Mostly of β -catenin mutations, if not all, were located at codon 32–37, 41, and 45, encoded by the exon 3 of the genes. Among these, codons 32 and 45 were found to be the most frequently mutated sites that account for more than half of β -catenin mutations in human HCC. These mutations may either alter the GSK-3 β -mediated phosphorylation by replacing the phosphorylatable serine/threonine residues (codons 33, 37, 41, and 45) or affecting the degradation via the ubiquitin-proteasome pathway by inducing structural changes (codons 32 and 37). Consequently, these mutations impaired the free cytoplasmic β -catenin degradation and led to β -catenin nuclear localization, where it functions as a transcriptional activator to promote the expression of Wnt-signaling target genes, such as c-myc and cyclin D1.

3 Epigenetic Alterations in Hepatocarcinogenesis

The term “epigenetic” generally refers to heritable changes in DNA methylation and histone modifications that stably modify gene transcription, but do not involve changes of the DNA sequences (Bird 2007). Epigenetic gene regulation is physiologically essential in controlling gene expression pattern during cell differentiation and embryonic development (Li et al. 1992). However, the epigenetic information, if deregulated, may confer growth advantage to the cell, leading to malignant transformation and cancer development.

DNA methylation is the most well-characterized epigenetic events. DNA methylation refers to a covalent addition of methyl group ($-\text{CH}_3$) to the 5-position of cytosine residues of CpG dinucleotides. Methylated cytosine is more susceptible for deamination that results in frequent C to T transition and as the consequence, the observed frequency of CpG dinucleotide in mammalian genome is much lower than expected, a phenomenon known as CG suppression. CpG dinucleotides are not evenly distributed across the genome. The vast majority of CpG dinucleotides (~70%) reside within the repetitive elements. CpG dinucleotides in the repetitive elements are heavily methylated, which is important for heterochromatin formation and maintaining chromosomal stability. In addition to repetitive regions, there is another class of CG-rich regions, namely CpG islands, which usually lie within the promoter or 5' end of protein-coding genes. It has been estimated that there are approximately 45,000 CpG islands per haploid genome in human and are associated with 50% of all genes (Antequera and Bird 1993). These promoter-associated CpG islands are usually free from methylation, however, once being methylated, it almost always accompany with transcriptional silencing of the associated gene. This DNA methylation-mediated gene silencing has been shown to be essential for normal development (Li et al. 1992), X-chromosome inactivation (Panning and Jaenisch 1998), and imprinting (Li et al. 1993). In the past decade, researchers start to realize the importance of DNA methylation in human carcinogenesis. Cancer cells often

exhibit a paradoxically aberrant DNA methylation pattern. On the one hand, the overall DNA methylation level is reduced, known as global DNA hypomethylation. On the other hand, increase of DNA methylation is found in promoter-associated CpG islands, known as promoter DNA hypermethylation.

Global DNA hypomethylation is a characteristic of HCC, which is mainly attributed by the loss of DNA methylation of CpG dinucleotides in repetitive elements (Lin et al. 2001; Saito et al. 2001). DNA hypomethylation of repetitive elements may lead to structural decondensation of heterochromatin and consequently facilitate aberrant chromosomal rearrangement. For example, DNA hypomethylation of the centromeric Sat-2 sequence on chromosome 1 has been linked to chromosome 1q gain and translocation in HCC (Wong et al. 2001). Besides, there are some evidence suggesting that DNA hypomethylation can also occur in normally methylated promoter CpG islands, and results in inappropriate activation of imprinted genes and proto-oncogene genes (Nambu et al. 1987; Tang et al. 2006).

Studies on promoter DNA hypermethylation in cancer cells have revolutionized the classical “two hit hypothesis” in the recent years. Traditionally, mutation and gene deletion are considered as the major mechanisms for tumor suppressor gene inactivation as illustrated by the *RB1* gene in inherited retinoblastoma. Nevertheless, increasing evidence has clearly demonstrated that epigenetic gene silencing is also playing an even more important role in tumor suppressor gene inactivation. *Rb1* gene is the first classical tumor suppressor gene reported to be inactivated in human cancers by promoter hypermethylation (Sakai et al. 1991). In human HCC, although *Rb1* hypermethylation is uncommon, DNA hypermethylation has been detected in a number of tumor suppressor genes that regulate various cellular pathways. Since the past decade, the list of DNA methylation silenced tumor suppressor genes has been growing and new members have been added continuously. Currently, well-characterized hypermethylated tumor suppressor genes in human HCC include p16/INK4A (Liew et al. 1999; Pang et al. 2003), E-cadherin (Kanai et al. 1997), RASSF1A (Schagdarsurengin et al. 2003), GTSP1 (Zhong et al. 2002), SOSC-1 (Yoshikawa et al. 2001), SFRP1 (Shih et al. 2006), DLC1 (Wong et al. 2003), and PTEN (Wang et al. 2007). With support from the above findings, cancer-specific promoter hypermethylation has now been recognized as one of the criteria in tumor suppressor gene identification (Table 20.2).

Aberrant DNA methylation changes are believed to be an early event in multistep hepatocarcinogenesis. It has been reported that DNA hypomethylation on c-myc gene and satellite regions can be observed in precancerous conditions (Nambu et al. 1987; Saito et al. 2001). In addition, the degree of DNA hypomethylation in human HCC was closely associated with larger tumor size and late histopathology grades (Lin et al. 2001). Likewise, several independent studies have revealed that promoter DNA hypermethylation of tumor suppressor genes can also be found in non-cancerous liver tissues and stepwise increase along with the multistep hepatocarcinogenesis (Choi et al. 2003; Di Gioia et al. 2006; Kondo et al. 2007). For instant, the p16 hypermethylation has been detected in approximately 60% of cirrhotic livers and gradually increases through DNAs, to HCCs (Shim et al. 2003). Similarly, two recent studies have further demonstrated this stepwise accumulation

Table 20.2 Promoter hypermethylation of tumor suppressor gene in human HCC

Tumor suppressor gene	Gene function	Percentage of promoter hypermethylation
14-4-3 sigma	G2/M check point control	89
APC	β -Catenin regulator	53
Cyclooxygenase-2	Prostaglandin-endoperoxide synthase	18
DLC1	RhoGTPase regulator	24
E-cadherin	Calcium dependent cell–cell adhesion	13–67
GSTP1	Prevent oxidative DNA damage	54–65
MGMT	Repair of DNA guanosine methyl adducts	39–61
p14/ARF	Regulator of p53 level	15–46
p15/INK4B	Cyclin-dependent kinase inhibitor	49–64
p16/INK4A	Cyclin-dependent kinase inhibitor	16–82
PTEN	Phosphoinositide 3-kinase signaling regulator	17
RASSF-1A	Effector in Ras signaling pathway	85–93
SOCS-1	JAK/STAT pathway regulator	65
TIMP3	Metallo-protease inhibitor	25

of promoter DNA hypermethylation with a panel of well-characterized tumor suppressor genes (Lee et al. 2003; Oh et al. 2007). All above findings suggest that aberrant DNA methylation is an early event and may accumulate along the progression of cancer development from normal liver, chronic hepatitis, cirrhosis, dysplastic nodule to HCC.

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) using S-adenosylmethionine (SAM) as methyl donor. It is commonly believed that DNA methylation pattern is established during embryonic development via the action of de novo DNA methyltransferases, DNMT3A and DNMT3B. DNA methylation pattern in somatic cells is then preserved during DNA replication by semi-conserved manner mediated by maintenance DNA methyltransferase, DNMT1. Methylated CpG serves as a molecular signature that can be recognized by a group of methyl-DNA binding proteins (MBDs). MBDs bound to methylated DNA functions as scaffold proteins by recruiting various transcriptional repressors and histone modifiers to the region to directly suppress the gene transcription or establish a transcriptional incompement chromatin status via nucleosome remodeling. It has been hypothesized that deregulation of DNMTs and MBDs may contributing to the frequently observed aberrant DNA methylation in human cancers. Several independent studies have revealed significant overexpression of DNMT1, DNMT3A, and DNMT3B in human HCC (Lin et al. 2001; Saito et al. 2003). Although the exact mechanisms of how DNMTs deregulation leading to DNA methylation abnormalities in cancer genome remains elusive, it is commonly believed that aberrant expression of DNMTs can give rise to methylation errors or to de novo methylation events at normally unmethylated CpG sites (Jones 1996). In support of this hypothesis, in vitro studies have demonstrated that overexpression of exogenous DNMT1 can induce DNA hypermethylation and result in transformation phenotype

in NIH3T3 cells (Vertino et al. 1996; Wu et al. 1993). Consistently, inactivation of DNMT1 by RNA interference (RNAi) or anti-sense oligonucleotide results in demethylation and re-expression of silenced tumor suppressor gene in cancer cells (Leu et al. 2003; Robert et al. 2003). In contrast to DNMTs, the mRNA level of MBDs, *MBD4*, *MBD2*, and *MeCP2* were found to be reduced in human HCC (Saito et al. 2001) and other cancers (Kanai et al. 1999; Muller-Tidow et al. 2001), suggesting that reduced expression of methyl-CpG binding proteins may play a role at certain steps of multistage carcinogenesis. One of the possible extrapolations is that reduced expression of MBDs may be implicated in the global hypomethylation of cancer genomes.

4 MicroRNA and HCC

MicroRNAs (miRNA) are endogenous single-stranded non-coding small RNAs, which function as post-transcriptional regulators in eukaryotic cells. The primary transcript of an miRNA (pri-miRNA) is generated by RNA polymerase II, which is then cleaved by microRNA processor, Drosha, to form an ~90 nt hairpin structured miRNA precursor (pre-miRNA). Pre-miRNA is then exported into the cytoplasm, where it is further processed by Dicer to become a mature miRNA of 18–22 nt. A mature miRNA exerts its function via binding to the imperfect complementary sequence on the 3'UTR of protein-coding mRNA targets and subsequently results in mRNA degradation or translational blockage through a mechanism currently not fully elucidated (Slack et al. 2000). Since the discovery of the first miRNA *lin-4* in *Caenorhabditis elegans* in 1993 (Lee et al. 1993), thousands of miRNAs have been identified in organisms ranging from plants to humans. Although the biological functions of individual miRNAs remain to be elusive, previous studies on *C. elegans* and *Drosophila* models have revealed that miRNAs are involved in almost all major physiological processes including cell proliferation, differentiation, apoptosis, hormonal secretion, and viral infections (Alvarez-Garcia and Miska 2005). Mounting evidence has demonstrated that miRNAs are implicated in the initiation, development, and progression of human malignancies (Calin et al. 2005, 2004; Murakami et al. 2006; Takamizawa et al. 2004; Volinia et al. 2006; Yanaihara et al. 2006). Some miRNAs have been shown to promote or suppress cancer cell proliferation and survival, in which they function as oncogenes or tumor suppressor genes (He et al. 2005). Following the first report demonstrating the down-regulation of miR-15 and miR-16 in B-cell chronic lymphocytic leukemia (Calin et al. 2002), many studies have revealed that deregulation of miRNA expression is a common characteristics of almost all human cancers. In fact, it has been reported that miRNA expression profiles could be used to distinguish tumors from normal tissues and to classify human cancer types in a way more accurate than the conventional protein-coding mRNA expression profiling (Lu et al. 2005).

Several independent miRNA profiling studies on human HCC samples have been reported recently. Their findings all suggest that miRNA expression profiles in HCC are significantly different from their corresponding non-tumorous livers (Gramantieri et al. 2007; Meng et al. 2007; Murakami et al. 2006). Moreover, miRNA expression alterations in primary HCC have been linked to various clinico-pathologic features, including background liver disease, viral infection status, tumor stage, and patient survival (Budhu et al. 2008; Jiang et al. 2008; Murakami et al. 2006). From these studies, liver specific miR-122 was found to be downregulated in most of HCCs. In contrast, miR-21 and miR-221 were consistently overexpressed in primary HCCs. It has been reported that increased expression of oncogenic miR-21 modulated HCC cell proliferation and invasion by negatively regulating PTEN, a well-characterized tumor suppressor gene (Meng et al. 2007). Nevertheless, it is worthy to note that a single miRNA can regulate many mRNA targets while the expression of one mRNA can be regulated by multiple miRNAs simultaneously. We are just about to understand the implications of miRNA in liver carcinogenesis and for most miRNAs, their cellular functions and mRNA targets are still awaited to be investigated.

5 Signaling Pathways in HCC

Genetic and epigenetic profiling analyses of HCC samples have provided compelling evidence in the alterations of genes during HCC development. Accumulation of gene alterations during multistep hepatocarcinogenesis is believed to result in the full acquisition of malignant behavior. Various signaling pathways have been revealed to be critically involved in hepatocarcinogenesis (El-Serag, Rudolph 2007; Villanueva et al. 2007), thus providing potential molecular targets for diagnosis and intervention of therapeutic agents. Here, we briefly review some of the signaling pathways implicated in human HCC.

5.1 *Wnt/β-Catenin Pathway*

Wnt-signaling pathway plays essential role in embryonic development, cell fate determination, planar cell polarity, and tissue homeostasis. Signaling cascades of Wnt pathways are classified into the canonical and non-canonical pathways. β-catenin is the key player of the canonical pathway and activates genes in the nucleus (Fig. 20.1). The non-canonical pathways are transduced by small GTPases, jun N-terminal kinase (JNK), and intracellular Ca^{2+} signaling (Huelsenken and Behrens 2002). Dysregulation of players along the canonical Wnt/β-catenin cascade has been implicated in human cancers (Clevers 2006), while the involvement of the non-canonical pathways remains uncertain (Clevers 2006; Fodde and Brabletz 2007).

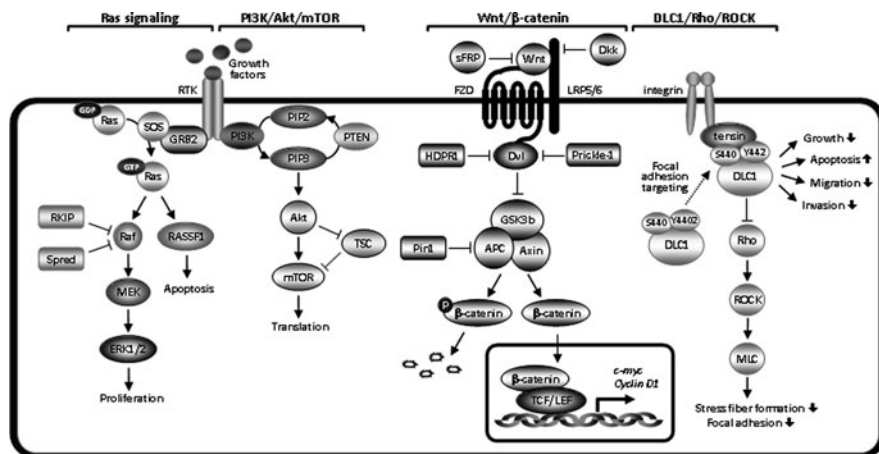


Fig. 20.1 Signaling pathways in HCC. Ras signaling is initiated by the activation of RTK by growth factors. Ras serves as a molecular switch whose activity is activated by SOS (son of sevenless protein). Raf-1 is a direct effector of Ras, which further transduces signals to MEK and ERK1/2. RKIP and Spred are inhibitors of Raf-1. RASSF1 is another Ras effector that mediates apoptosis. PI3K signaling is activated by RTK while its activity is negatively regulated by PTEN. Akt is a critical target of PI3K and transduces signal to mTOR. TSC2 acts as brakes to attenuate mTOR signaling. In the canonical Wnt pathway, binding of Wnt to FZD and LRP5/6 co-receptor activates Dvl, leading to the disassociation of the destruction complex and prevention of β -catenin degradation. Accumulation of stabilized β -catenin facilitates the translocation of β -catenin into the nucleus where it associates with TCF/LEF transcription factors and initiates transcription of target genes, such as c-myc and cyclin D1. Antagonists, such as sFRP, Dkk, HDPRI, Pin1, and Prickle1, are shown. At focal adhesions, DLC1 interacts with tensin and their interaction has been shown to be critical for the growth suppression activity of DLC1. Residues Y442 and S440 are responsible for the focal adhesion targeting of DLC1. Introduction of DLC1 into HCC cells has also been shown to induce apoptosis, suppress migration and invasion, and inhibit stress fiber formation and focal adhesions. In addition, DLC1 negatively regulates the Rho/ROCK/MLC-mediated formation of stress fibers and focal adhesions

In the absence of Wnt, β -catenin forms complex with adenomatous polyposis coli (APC), axin, and glycogen synthase kinase-3 beta (GSK-3 β). Phosphorylation of β -catenin by GSK-3 β targets β -catenin to degradation. Binding of Wnt to the transmembrane frizzled receptor (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor activates the Wnt/ β -catenin pathway. Dishevelled (Dvl) is then activated by phosphorylation and eventually leads to the stabilization of β -catenin (He et al. 1998). Accumulation of stabilized β -catenin facilitates the translocation of β -catenin into the nucleus where it associates with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and initiates transcription of target genes, such as c-myc (He et al. 1998) and cyclin D1 (Tetsu and McCormick 1999).

Mutations of β -catenin have been observed in a range of 13–34% of HCCs in various studies (de La Coste et al. 1998; Hsu et al. 2000; Huang et al. 1999; Taniguchi

et al. 2002; Wong et al. 2001). Mutations in β -catenin are more frequent in human HCV-associated HCCs (Huang et al. 1999). These mutations locate predominantly at codons 32–37, 41, and 45 and prevent β -catenin from phosphorylation and subsequent degradation (de La Coste et al. 1998; Hsu et al. 2000; Taniguchi et al. 2002; Terris et al. 1999; Wong et al. 2001). Dysregulation of the destruction complex also leads to the stabilization of β -catenin. GSK-3 β is a key player in the regulation of β -catenin stability, yet, mutation of GSK-3 β has not been detected in HCC (Cui et al. 2003). High incidence of APC mutations has been detected in colon cancer (Cottrell et al. 1992), however, APC mutation is uncommon in HCC. Aberrant promoter methylation is responsible for the inactivation of APC (Lee et al. 2003; Yang et al. 2003). Inactivation of APC in liver of transgenic mouse resulted in hepatocyte hyperplasia and accumulation of nuclear and cytoplasmic β -catenin (Colnot et al. 2004). Overexpression of PIN1 also stabilizes β -catenin by inhibiting its interaction with APC (Ryo et al. 2001). Interestingly, overexpression of PIN1 and mutation of β -catenin appear to be mutually exclusive events in Wnt-signaling activation in HCC (Pang et al. 2004). Axin is another component of the destruction complex. Mutations of Axin1 have been found in 5–10% of HCCs (Sato et al. 2000; Taniguchi et al. 2002). Dvl is the immediate effector of Wnt activation. Overexpressions of Dvl-1 and Dvl-3 have been reported in HCC (Chan et al. 2006). In other cancers, Dvl overexpression is found to be associated with β -catenin accumulation and Wnt/ β -catenin signaling activation (Uematsu et al. 2003a,b). Two inhibitors of Dvl, HDPR1, and Prickle-1 have reported to be underexpressed in HCC (Chan et al. 2006; Yau et al. 2005).

Wnt-signaling pathway is initiated by binding of Wnts to receptors. Wnt ligands, Wnt3, 4, and 5a have been found to be upregulated in HCC and peritumoral livers (Bengochea et al. 2008; Kim et al. 2008). Secretory frizzled-related protein (sFRP) and Dickkopf (Dkk), are extracellular antagonists of Wnt pathway and have been studied in human cancers (Kawano and Kypta 2003). Two sFRP genes, sFRP1 and sFRP5, have been shown to be downregulated in HCC (Bengochea et al. 2008). Secretory FRP1 has been found to be epigenetically silenced in human cancers including HCC (Shih et al. 2006; Suzuki et al. 2002). Frequent methylation of other SFRP family genes has been shown in HCC cell lines and primary HCCs (Takagi et al. 2008). Detection of sFRP methylation in HBV or HCV-associated chronic hepatitis and liver cirrhosis suggested that methylation of sFRP family occurs in early liver carcinogenesis. Another extracellular antagonist Dkk-1 interacts with LRP6 and blocks LRP6-mediated Wnt pathway (Mao et al. 2001). Overexpression of Dkk-1 antagonizes the Wnt/ β -catenin pathway and suppresses proliferation and migration of HCC cells (Qin et al. 2007). At present, three receptors FZD3, 6, and 7 have been reported to be upregulated in HCC (Bengochea et al. 2008; Merle et al. 2004). Up-regulation of FZD7 was also detected in HCC of various transgenic mouse models (Merle et al. 2005). In HCC cell lines, expression level of FZD7 is associated with both enhanced nuclear localization of β -catenin and cell migration (Merle et al. 2004). The LRP5/6 co-receptors have been shown to regulate Wnt pathway, but their roles in tumorigenesis need to be elucidated (Tamai et al. 2000; Tolwinski et al. 2003).

5.2 *Ras-Signaling Pathway*

Activation of receptor tyrosine kinases (RTK) by growth factors transduces signals through small GTPase Ras to regulate cell growth, differentiation, apoptosis, and migration (Downward 1998; Satoh and Kaziro 1992; Vojtek and Der 1998). Ras activates downstream Raf-1 serine/threonine kinase (Avruch et al. 1994), which further transduces signals to MEK and ERK1/2 (Fig. 20.1). In addition to the Raf/MEK/ERK pathway, activated Ras also stimulates other downstream effectors including phosphatidylinositol 3-kinase (PI3K), RalGDS, PLC-epsilon, and Tiam1 (Avruch et al. 1994). In HCC, Ras mutation is rare (Ogata et al. 1991), except in patients exposed to vinyl chloride (Weihrach et al. 2001). Overexpression of Ras has been reported in liver cirrhosis and HCC (Nonomura et al. 1987). In vitro studies demonstrated that expression of Ras in cells induces transformation of immortalized hepatic cells and enhancement of metastatic phenotype in human HCC cell lines (Jacob and Tennant 1996; Wang et al. 2001). Downstream effector of Ras, ERK has been shown to be overexpressed and correlated with tumor progression in HCC (Osada et al. 2005). Conversely, underexpression of the physiological inhibitors of Ras/Raf/MEK/ERK pathway has been reported in HCC. In HCC cell lines and tissues, RKIP is downregulated and correlated with enhanced ERK activation. Expression of Raf-1 kinase inhibitory protein (RKIP) in cells resulted in decreased nuclear accumulation of activated ERK (Lee et al. 2006; Schuierer et al. 2006). Sprad (Sprouty-related protein with Ena/vasodilator-stimulated phosphoprotein homology-1 domain), inhibitor of Ras/ERK pathways is frequently downregulated in HCC tissues and its expression is inversely correlated with the incidence of tumor invasion and metastasis. Functionally, Sprad inhibited growth and migration of HCC cells (Yoshida et al. 2006). RASSF1 tumor suppressor has been shown to be a Ras effector that mediates the apoptotic effects of oncogenic Ras (Vos et al. 2000). Frequent epigenetic silencing of RASSF1A in HCC has been reported (Calvisi et al. 2006; Schagdarsurengin et al. 2003; Zhong et al. 2003). Loss of RASSF1 expression in HCC may confer a growth-promoting activity of Ras.

5.3 *PI3K/Akt/mTOR Pathway*

PI3K plays a pivotal role in human cancers. High incidence of PI3K mutation is observed in various human cancers (Samuels et al. 2004), while contradictory results have been obtained in HCC (Lee et al. 2005; Tanaka et al. 2006). PI3K is activated by receptor tyrosine kinase, while negatively regulated by PTEN tumor suppressor (Fig. 20.1). Loss of functions of PTEN results in activation of the PI3K signaling pathways. In HCC, underexpression and epigenetic silencing of PTEN have been reported (Hu et al. 2003; Wang et al. 2007). PTEN is located on chromosome 10q, in which LOH is frequently detected in HCC (Fujiwara et al. 2000). Akt is a critical downstream effector of PI3K. Akt is a serine/threonine kinase that phosphorylates a number of substrates, which involved in various biological processes such as cell

survival, cell growth, apoptosis, differentiation, and metabolism (Bhaskar and Hay 2007). Dysregulation of Akt has been implicated in various human cancers (Blanco-Aparicio et al. 2007), yet, the role of Akt in HCC remains uncertain. Enhanced Akt phosphorylation is shown to be associated with aggressive behavior of HCC (Nakanishi et al. 2005). Enhanced expression of Akt-2 but not Akt-1 has been detected in HCC tissues (Xu et al. 2004). Mammalian target of rapamycin (mTOR) is a central mediator of the PI3K/Akt pathway. Tumor suppressors TSC1 and TSC2 attenuate mTOR signaling (Hay 2005). Phosphorylation of mTOR is reported to be correlated with increased S6K expression level in 45% of HCC. Rapamycin, an inhibitor of mTOR, is demonstrated to reduce S6K phosphorylation and inhibit HCC cell proliferation (Sahin et al. 2004). Another mTOR inhibitor, sirolimus, inhibits HCC cell growth in animal model and exerts antiangiogenic effect on HCC cells (Semela et al. 2007). These studies have provided compelling evidence that mTOR is a promising therapeutic target.

5.4 *DLC1/Rho/ROCK Pathway*

Deleted in Liver Cancer 1 (DLC1), which locates on chromosome 8p21.3-22 is first isolated as a candidate tumor suppressor gene from human HCC (Yuan et al. 1998). It is widely expressed in normal human tissues, but frequent underexpression of DLC1 is detected in HCC cell lines and primary tissues. Underexpression of DLC1 has been attributed to genomic deletion and promoter hypermethylation (Ng et al. 2000; Wong et al. 2003). DLC1 is a Rho GTPase-activating protein (RhoGAP), which colocalizes with vinculin at focal adhesions (Yam et al. 2006a) (Fig. 20.1). In vitro assay first demonstrated the RhoGAP activity of DLC1 against RhoA and Cdc42 (Wong et al. 2003). DLC1 negatively regulates the Rho/ROCK/MLC pathway. Expression of DLC1 abrogates the formation of stress fibers and focal adhesions via RhoGAP activity (Kim et al. 2007; Wong et al. 2008, 2005; Zhou et al. 2008). The importance of RhoGAP activity in the growth-suppressive activity of DLC1 has been demonstrated by the loss of growth-inhibitory activity in RhoGAP mutants (Wong et al. 2005). Introduction of DLC1 in HCC cells inhibits cell growth, migration, and invasion (Kim et al. 2007; Ng et al. 2000; Wong et al. 2005; Zhou et al. 2008). DLC1 also dephosphorylates other focal adhesion proteins, such as FAK, Crk-associated substrate (p130Cas), and paxillin in HCC cells (Kim et al. 2007). Moreover, restoration of DLC1 expression induces apoptosis in HCC cells (Zhou et al. 2004). Functional significance of DLC1 is first demonstrated in a murine model. It is shown that DLC1 silencing cooperates with Myc in promoting hepatocarcinogenesis in mouse (Xue et al. 2008). Focal adhesion localization of DLC1 is crucial to its growth-suppressive ability. Tensin family members have been identified as the interacting partners of DLC1 and shown to mediate the focal adhesion localization and regulate the biological activities of DLC1. Mutations of focal adhesion targeting residues S440 and Y442 in DLC1 result in the loss of focal adhesion localization and consequent ability to reduce cancer cell growth (Liao et al.

2007; Qian et al. 2007). Somatic mutation in the focal adhesion targeting region of DLC1 is first detected in prostate cancers (Liao et al. 2008). Mutations in the focal adhesion targeting region of DLC1 result in reduced growth-suppressive and RhoGAP activities. Somatic mutations of DLC1 in HCC and other cancers seem to be rare, but this awaits further investigation (Wong et al. 2003; Zheng et al. 2003).

DLC1 together with other two members, DLC2 and DLC3, comprise a family of tumor suppressors. All family members share common characteristics in their structural organization, growth-suppressive and RhoGAP activities, and focal adhesions localization (Durkin et al. 2007; Kawai et al. 2007; Leung et al. 2005; Yam et al. 2006a). Apart from localizing at focal adhesions, DLC2 also targets to mitochondria in HCC cells by its START domain (Yam et al. 2006b). DLC2 is localized at chromosome 13q12.3, a region where high frequency of allelic losses has been found (Ching et al. 2003; Wong et al. 2002). Introduction of DLC2 suppresses cytoskeleton reorganization, cell growth, cell migration, and transformation of HCC cells (Leung et al. 2005). Although the tumor suppressive role of DLC3 has not been addressed in HCC, its expression is downregulated in various other human cancer cell lines and tissues (Durkin et al. 2007).

6 Perspective

Chromosomal aberrations, genetic alterations, and epigenetic modifications are molecular perturbations accumulating along the multistep hepatocarcinogenesis. These perturbations lead to dysregulation of important signaling pathways in HCC. Delineation of the underlying molecular mechanisms in the progression of HCC provides information of potential therapeutic targets and insight into development of treatment regimes for HCC.

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