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Molecular and functional genetics of hepatocellular carcinoma

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1. ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most common cancer and one of the leading causes of cancer death worldwide. Hepatocarcinogenesis is a multistep process developing from normal through chronic hepatitis/cirrhosis and dysplastic nodules to HCC. Although we have insufficient understanding to propose a robust general model, with advances in molecular methods, there is a growing understanding of the molecular mechanisms in the development of HCC. Hepatocarcinogenesis is strongly linked to increases in allelic losses, chromosomal changes, gene mutations, epigenetic alterations, and alterations in molecular cellular pathways. Special emphasis in this review is given to the genetics, epigenetics, and regulation of major signaling pathways involved in HCC such as Wnt/β-catenin, Ras, and PI3K/Akt/mTOR pathways. A detailed understanding of the underlying molecular mechanisms involved in the progression of HCC can improve our prevention and diagnostic tools for HCC and be an important potential source of novel molecular targets for new therapies.

2. INTRODUCTION

HCC is one of the most common malignancies in the world, ranking fifth among all cancers and being the third leading cause of cancer death (1, 2). A striking geographical difference in the incidence of HCC exists. Most (80%) of new cases occur in developing countries. HCC is particularly prevalent in the sub-Saharan Africa, East and Southeast Asia, and is low in incidence in Northern and Western Europe and North America. In Southeast Asia and China, HCC is the second commonest fatal cancer. Its ranking among the common causes of fatal cancers has not changed since 1970s. However, better control of the risk factors has resulted in a recent decline in HCC in some places like Taiwan and China. In Taiwan, the mortality rates due to HCC in children younger than 15 years of age have decreased by up to 70%, due to vaccination against hepatitis B virus (3). On the contrary, there is a recent trend of rising incidences of HCC in developed countries such as Europe and North America. For instance, in the United States, the age-adjusted incidence has doubled over the past 2 decades (4).

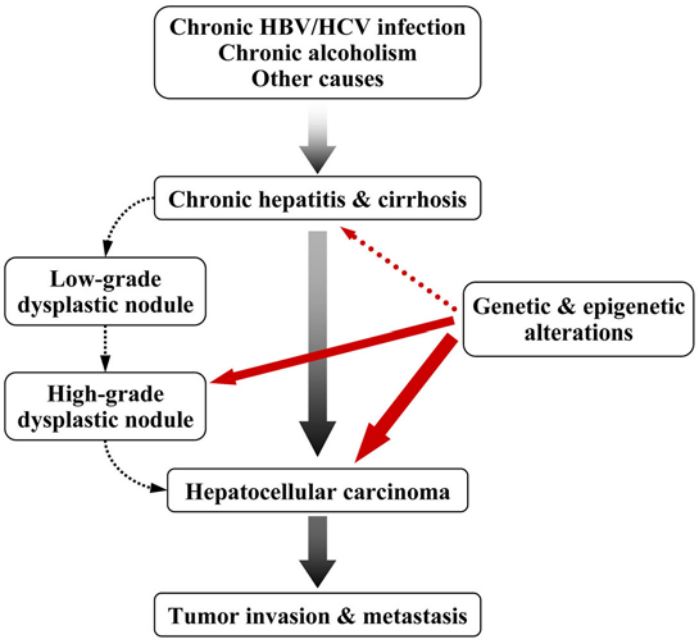

Figure 1. Multistep process of hepatocarcinogenesis. In the majority of the cases, HCC is accompanied with background liver disease of chronic hepatitis or cirrhosis. Dysplastic nodules are precancerous lesions in hepatocarcinogenesis and arise in a cirrhotic background. They are classified into high- and low-grade. Genetic and epigenetic alterations are accumulated in the process of hepatocarcinogenesis.

The risk factors of HCC are well recognized and include chronic hepatitis B (HBV) and C (HCV) viral infections (5, 6), cirrhosis (7), and aflatoxin B1 (8). Chronic HBV and HCV infections with or without cirrhosis are the predominant causes for HCC worldwide. The role of HBV genotypes, core promoter and precore mutants on HCC is still controversial. Recent studies have shown that HCV infection acquired 2-4 decades ago may explain a substantial portion of the rise of increased incidence of HCC in North America. HCC is currently the major cause of liver-related death in patients with compensated cirrhosis (9). Alcohol abuse is related to HCC by promoting liver cirrhosis. However, only severe, but not moderate, alcohol consumption is related to HCC (10, 11). The role of tobacco smoking in the causation of HCC is controversial. Inherited metabolic diseases such as hereditary hemochromatosis (12), alpha-1-antitrypsin deficiency and hereditary tyrosinemia are other risk factors of HCC. Non-alcoholic steatohepatitis, which is also an established risk factor for HCC, are commonly related to obesity and diabetes and can progress to cirrhosis and HCC (13).

3. MOLECULAR PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

3.1. Multistep hepatocarcinogenesis

Hepatocarcinogenesis is believed to be a multistep process (Figure 1). In the majority of the cases, HCC is accompanied with background liver disease of either chronic hepatitis or cirrhosis. Despite the strong suggestion of multistep process in hepatocarcinogenesis, a small proportion of HCC patients have normal livers. In autopsy series worldwide, there are about 10-20% of HCC patients having no cirrhosis (14). However, only a small proportion of HCC arise in absolutely normal or normal-looking livers. The majority of non-cirrhotic livers show fibrosis ranging from no fibrosis to septal or bridging fibrosis. There are also other histological changes including acinar necroinflammation, steatosis or liver cell dysplasia.

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 decreasing mortality rates due to cirrhosis but increasing mortality rates due to HCC in United States and European countries (15, 16). Of the risk factors for cirrhosis and 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 (7). 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 2- to 6-fold relative to each infection alone. In alcoholic cirrhotic patients without HBV or HCV infection, the HCC incidence is lower with 5-year cumulative risk at 8%. Alcohol abuse increases the HCC risk in HBV- or HCV-associated cirrhosis by 2- to 4-fold.

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. LCD and SCD can be observed in up to 30% and 25%, respectively, in liver biopsies from patients with chronic liver diseases due to HBV or HCV infection (17, 18). The risk of LCD in developing into HCC is

controversial (17, 18). However, SCD was an important independent risk factor for developing HCC in HCV-associated cirrhosis (17(19). In another study on LCD and SCD adjacent to HCC using microdissection and chromosomal genomic hybridization (CGH), no abnormalities were detected in LCD foci (20); in contrast, the adjacent SCD showed a subset of chromosomal alterations present in HCCs, supporting the preneoplastic status of SCD in human hepatocarcinogenesis. It has also been shown that SCD, but not LCD, foci had the same immunohistochemical phenotype as putative progenitor (oval) cells, suggesting that differentiating putative progenitor cells could give rise to foci of SCD (21). Thus, there seems to be more evidence supporting that SCD is a risk factor for HCC than LCD.

In cirrhotic livers, space-occupying small nodules include a series of hyperplastic (large regenerative) nodules, dysplastic (low- and high-grade dysplastic) nodules, and malignant hepatocellular nodules (well-differentiated HCC). A dysplastic nodule (DN) is a precancerous lesion in hepatocarcinogenesis, arising from a cirrhotic background (22, 23). These nodules are increasingly detected by radiographic techniques in cirrhotic livers or are removed during transplantation procedures. 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 (23). DNs have been increasingly detected clinically, because patients with HBV- or HCV-associated cirrhosis undergo regular surveillance for HCC. The recognition of DNs provides an important suggestion regarding the pathogenesis of progression from DN to early HCC and finally to overt HCC during multistep human hepatocarcinogenesis (24).

Advances in imaging technology have facilitated the detection of small nodular lesions in chronic liver disease and the natural outcome of macronodules in cirrhosis. Seki *et al.* attempted to study the outcome of DNs in liver, using fine needle aspiration (FNA) coupled with ultrasound features (25). Most of the DNs disappeared or remained unchanged, and 12.1% progressed to HCC. In another study using computed tomographic arterial portography, cumulative HCC development rates at the first, third, and fifth year have been reported to be 46.2%, 61.5%, and 80.8% for HGDN; 2.6%, 30.2%, and 36.6% for LGDN; and 3.3%, 9.7%, and 12.4% for regenerative nodules, respectively. The rate of HCC development was significantly higher in the HGDN group than the other types (26). 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 (27). 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 (28).

Early HCC is defined by the Liver Cancer Study Group of Japan as well-differentiated HCC with an obscure tumor margin and no substantial destruction of the underlying hepatic structure (29). They are tiny (≤ 1.2 cm) and well differentiated, and carry a favorable prognosis, with 5- and 10-year survival rates being 85% and 61%, respectively (30). Similar results were also obtained in another Japanese study on single HCC smaller than 2 cm in diameter (31). However, there is a discrepancy in the diagnosis of well differentiated HCC and HGDN. Many of the vaguely nodular, well-differentiated HCC diagnosed by Japanese pathologists tend to be interpreted as HGDN and not HCC by Western pathologists (32). Hopefully this will be settled with a common consensus, particularly with applications of immunohistochemical or other modern tools. Recently, it has been reported that ductular reaction with cytokeratin (CK7) immunostaining may help identify small foci of tumor invasion and distinguish among noninvasive HGDNs, minimally invasive and overtly invasive HCCs (33).

4. GENETIC ALTERATIONS IN HEPATOCARCINOGENESIS

4.1. Chromosomal abnormalities

Accumulation of genetic alterations is believed to be the underlying impetus of multi-step carcinogenesis. Genetic alterations can emerge at pre-malignant lesions or early stage of cancer development, accumulate during disease progression and result in acquisition of full blown malignant phenotype. It has been known for a long time that cancer genome is strictly distinct from their corresponding normal tissues. Early studies using flow cytometry to analyze DNA content have clearly revealed aneuploidy as a common feature of primary HCC (34, 35). In the past two decades, attempts have been made to reveal chromosomal abnormalities in HCC. Conventional karyotyping using cytogenetic banding methods has been challenged by technical difficulties in primary tumor cell culture and the complexity of chromosomal aberrations (36, 37). Thus, reports on human HCC are scanty. These technical barriers were relieved by the introduction of molecular biology techniques, such as fluorescence *in situ* hybridization (FISH) and loss of heterozygosity (LOH) analysis. 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 (37-39). 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 (38). LOH analysis using polymorphic DNA microsatellite markers is widely used to determine allelic losses of specific loci. Genome-wide allelotyping studies on HCCs have shown that allelic losses were frequently found in many chromosomal regions, with higher frequencies of LOH observed in loci on chromosomal arms 1p, 4q, 6q, 8p, 9p, 13q, 16q, 17p, and 19q (40-45). Comparative genomic hybridization (CGH), first described by Kallioniemi in 1992, is another major technical advancement of molecular cytogenetic

(46). The first CGH analysis on human HCC was done by Marchio and colleagues in 1997. In their study on advanced HBV-associated HCCs from different geographical origins, they found that chromosomal alterations were detected in 43 (86%) of the 50 cases (47). Thereafter, a number of similar studies have been reported and their findings were generally consistent. Recurrent chromosomal gains were most frequently observed on 1q (46-78%), 8q (41-69%), 17q (30-36%) and 20q (20-37%); losses were commonly detected on chromosome 1p (24-36%), 4q (32-70%), 6q (37-70%), 8p (29-65%), 13q (37-39%), 16q (30-64%), and 17p (31-52%) (47-53).

For these frequently affected chromosomes, studies have been made in an attempt to reveal their clinicopathological and prognostic implications. Tamura and colleagues found that accumulation of allelic losses in HCC was associated with more aggressive tumor behavior and poor prognosis (54). Results from other research teams also found that allelic losses on chromosome 4q, 8p, 13q, and 16q were associated with poorer tumor differentiation, larger tumor size, or more advanced tumor stages (41, 44, 55-61). Chromosomal aberrations in human HCC seem more common in patients associated with HBV infection than other etiological backgrounds (48). The patterns of chromosomal abnormalities also differ between HBV- and HCV-associated HCCs. For instance, gain of chromosome 10q was detected mainly in HCV-positive HCCs, whereas an amplification of 11q13 was often seen in HCC associated with HBV infection (48). There is also an increasing incidence of chromosomal abnormalities along the multi-step process of hepatocarcinogenesis. Chromosomal abnormality is relatively uncommon in cirrhotic livers. However, it was remarkably increased in dysplastic nodules and HCC (62). Interestingly, the frequency and pattern of chromosomal aberration observed in DN was very similar to that of HCC, supporting the notion that chromosomal abnormalities is an early event and could occur at the pre-malignant stage of HCC development (62-64).

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 (65), 4q35 (55), 8p21.3-22 (57, 58), 13q12.3-14.1, 13q32 (61, 66) and 16q24 (67). The higher incidences 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. The next challenging question is whether any of these candidate genes might play a direct role in hepatocarcinogenesis. Two putative tumor suppressors, namely *Deleted in liver cancer 1* (*DLC1*) and its homolog *DLC2* located at the minimal deleted region of 8p21.3-22 and 13q12.3, respectively, have been cloned and characterized in human HCC (68-70). DLC1 and DLC2 share 51% sequence identity and represent a new family of Rho GTPases activating proteins (RhoGAP) (68). DLC1 and DLC2 function to negatively regulate RhoA activity by enhancing its intrinsic GTPase activity, which then converts active GTP-bound RhoA into its inactive GDP-bound form (68, 71). DLC1 and DLC2 are frequently deleted and underexpressed in HCC (68, 71). Ectopic expression of DLC1 and DLC2 in HCC cell lines resulted in remarkable suppression of cell proliferation, motility and invasiveness (72-74). This suppressive effect was abolished when the RhoGAP domains of DLC1 and DLC2 were mutated, indicating that DLC1 and DLC2 counteract RhoA-mediated cell growth and metastasis in HCC (72, 73). Apart from DLC1 and DLC2, other candidates genes such as RUNX3 at chromosome 1q36 (75), and SIHA at chromosome 16q24 (76) have also been characterized recently and the results indicate a linkage between inactivation and downregulation of these genes and HCC development. Recently, with array-based CGH which provides enhanced chromosomal resolution, investigators now can narrow down the aberrant regions into a more defined region (>1Mb) and pinpoint the affected gene(s) directly (77, 78). We anticipate that technological advances will greatly enhance the power of chromosomal analysis and many more target genes will be identified and characterized in the next few years.

4.2. Somatic mutational analysis

Gene mutations, including point mutations and interstitial deletions/insertions, have been known to contribute to cancer development since the early part of last century (79). Mutations could arise from exposure to DNA damaging carcinogens or defects in DNA repair systems. In human cancers, mutations have been found in a large number of genes that regulate cell proliferation, cell-cycle progression, apoptosis and metastasis. Of those, *p53* probably is the most frequently mutated genes in primary HCC. In Asian population, *p53* mutation was detected in 13 to 33% of human HCC (80-82) and more frequently seen in poorly differentiated and larger HCCs (83). p53 mutations, in general, are scattered over exons 5-9. However, specific mutation hot spot at codon 249 (exon 7) was found in aflatoxin-prevalent regions (84). *In vitro* models demonstrated that Ser-249 mutant promoted cell proliferation and suppressed wild-type p53-mediated apoptosis (85, 86). Although this p53 Ser-249 mutant was unable to induce cellular transformation in normal hepatocytes directly (86), loss of p53 function has been considered a critical step of cellular transformation induced by chemical carcinogens and oncogenic pathways (87). In addition to p53, other frequently mutated genes include β-catenin (88, 89), Axin (90), Caspase-8 (91), LKB1 (92) and KLF6 (93). Interestingly, except β-catenin, most of the mutations in human HCC are found in tumor suppressor genes. Although no germline mutations have been found, these somatic mutations may combine with other genetic or epigenetic alterations to inactivate tumor suppressor genes.

4.3. Single nucleotide polymorphism

Chronic HBV and HCV infections are the major etiological factors of HCCs, and HBV infection alone contributes to 80% of this cancer worldwide (94). However, the clinical outcome of HBV infection varies among individuals and, only in a small portion of patients, HBV infection persists and progresses to HCC. It has been estimated that the lifetime risk of HCC development among

HBV carriers are 27% for male and 4% for female subjects (95). It has been hypothesized that this intriguing variation in the clinical outcome of HBV infection may, in a larger extent, be determined by host genetic factors. Indeed, family history has been established as a risk factor associated with HBV infection and HCC development (96). It has become clear that millions of single nucleotide polymorphisms (SNP) present in the human genome significantly contribute to the genetic diversities among individuals, and these genetic variations may also play a role in determining the risk of developing HCC among individuals who have HBV infection. SNP, by definition, is stable single nucleotide sequence variant that has a minor allele frequency greater than 1% within a population. It has been estimated that the human genome contains more than 10 million SNPs, at a frequency of 1 per 1,000 base pairs (97, 98). SNPs located in the gene coding region or regulatory regions may have a direct impact on gene activities or expression levels. However, most of SNP are found in non-coding regions and considered as "silent" SNP as they may not affect gene expression or gene functions directly. Nevertheless, these silent SNPs can be used to define disease-linked haplotypes or serve as linkage markers in discovering novel functional SNPs in adjacent genes.

Previous studies have established an association between host immune response and clinical outcome of HBV infection. The human leukocyte antigen (HLA) class I and class II molecules are the key coordinators in immune response, which mediates the viral clearance by presenting foreign antigens to CD8+ and CD4+ T-cells, respectively. In a pioneering study, Thursz and colleagues compared the MHC class I and II genotypes between the groups of HBV carriers and patients who spontaneously recovered from HBV infection in Gambia. Their findings indicated that MHC class II allele DRB1*1302 was less frequent in HBV carriers, suggesting a protection role of DRB1*1302 genotype against persistent HBV infection (99). This association was then confirmed by follow-up studies in different ethnic groups, further strengthening the role of HLA polymorphisms as a host genetic factor determining the HBV viral clearance and clinical outcome of HBV infection (100, 101). Promoter polymorphism of interleukin 10 (IL10) has also been linked to the HBV progression and HCC development. Lower expression ATA haplotype at positions -1082/-819/-592 of IL-10 promoter was more frequently seen in asymptomatic HBV carriers than in HBV patients with chronic progressive liver disease (102). On the other hand, ACC phenotype, which linked to higher IL-10 expression, was associated with a higher risk of HCC development and exhibited an increased susceptibility to cirrhosis and HCC (103). These findings on IL-10 promoter polymorphism exemplify how SNPs on promoter regions can influence gene expression levels and contribute to the development of HCC. In addition to gene related to HBV clearance and disease progression, SNP on estrogen receptor α (ESR1) (104), methylenetetrahydrofolate reductase (MTHFR) (105, 106), thymidylate synthase (TYMS) (106), liver intestine-cadherin (CDH17) (107) and UDP-glucuronosytransferase genes (108) are all found to be associated with increased risk of HCC development.

It should be noted that identification of susceptibility SNP to predict clinical outcome of HBV infection and HCC development is just at a very early stage and the number of genes identified so far is small. All the aforementioned genetic association studies were preformed in candidate gene approach and relied mainly on current knowledge of HBV-related immune response or hepatocarcinogenesis. Since the development of HCC in HBV/HCV infected patients is of complex traits and it is likely that several polymorphic genes, instead of one major gene, will exert effects on the outcome. Own to the development of microarray and other high-throughput analysis platforms, genome-wide association studies with larger sample sizes have become feasible. The beauty of genome-wide association study in predicting cancer susceptibility loci has been elegantly demonstrated in breast cancer (109, 110). However, such genome-wide analysis has not been reported in human HCC and therefore is much awaited.

5. EPIGENETIC ALTERATIONS IN HEPATOCARCINOGENESIS

Traditionally, mutation and gene deletion are considered as the major mechanisms in the "two hit" inactivation of tumor suppressor genes in cancer development. Nowadays, the concept of epigenetic alteration has increasingly been appreciated as an alternative to the conventional two hit hypothesis. The Greek prefix 'epi' refers to an additional regulatory layer on top of genetic information stored in DNA sequence (111). Epigenetic modifications consist of DNA methylation, histone modifications and chromatin remodeling machinery. They work closely to regulate gene expression and are essential for embryonic development (112), X-chromosome inactivation (113) and gene imprinting (114). In differentiated somatic cells, epigenetic information, stored in the genome as DNA methylation or post-translocation modification mark on the N-terminal tail of histone proteins, is persistent within cell cycle and inheritable after replication. Mounting evidence has revealed that aberrant change of epigenetic information may confer growth advantage to the cell, leading to malignant transformation and cancer development.

DNA methylation refers to a covalent addition of methyl group ($-CH_3$) to the 5-position of cytosine, catalyzed by DNA methyltransferases (DNMTs). In mammalian genome, DNA methylation is mainly found in the cytosine residues of CpG dinucleotides within the repetitive elements or promoter-related CpG islands. DNA methylation is related to heterochromatin formation and is associated with transcriptional silencing when present in the promoter region of genes. DNA methylation is essential for development and differentiation, but aberrant DNA methylation can result in chromosomal instability and dysregulation of gene expression (115). Cancer cells often simultaneously exhibit global DNA hypomethylation and gene specific promoter DNA hypermethylation. In human cancers, promoter hypermethylation of tumor suppressor genes perhaps is the most common and well characterized epigenetic alteration.

Several independent studies have revealed that DNA hypermethylation of tumor suppressor genes is an early event in hepatocarcinogenesis and can be detected in pre-malignant lesions such as cirrhotic liver and dysplastic nodules (116-118). Moreover, the frequencies of promoter hypermethylation exhibit an increasing trend along the multi-steps of hepatocarcinogenesis (119, 120). Rb1 gene is the first classical tumor suppressor gene reported to be inactivated in human cancers by promoter hypermethylation (121). 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. These hypermethylated tumor suppressor genes include p16/INK4A (122, 123), E-cadherin (124), RASSF1A (125), GTSP1 (126), SOSC-1 (127), SFRP1 (128), DLC1 (71) and PTEN (129). Nowadays, cancer-specific promoter hypermethylation has been recognized as one of criteria in tumor suppressor gene identification. With advances in high-throughput methylation analysis platforms, the number of hypermethylated genes in human HCC will further increase in future.

6. MOUSE MODEL OF HEPATOCELLULAR CARCINOMA

Mouse models have been widely employed in the study of the molecular mechanisms of hepatocarcinogenesis. Genetically engineered mouse models provide information about the normal functions of targeted genes. Phenotypic alterations in transgenic mouse models further give insight into the progressive steps of pathogenesis of diseases. Mouse models can also be used for testing therapeutic agents and global gene expression profiling.

6.1. Mdr2 knockout mouse model

Mdr2 gene encodes a p-glycoprotein that is abundantly expressed in the bile canaliculi of hepatocytes. The Mdr2 protein plays an essential role for phosphatidylcholine transport across the canalicular membrane (130). Homozygous disruption of Mdr2 gene caused failure to secrete phospholipids into bile and eventually resulted in obliterative cholangitis (131). Liver inflammation and toxic injury of the biliary system at an early age in Mdr2 knockout mouse lead to the development preneoplastic lesions in the liver and eventually metastatic liver cancer. The Mdr2 knockout mouse provides a vital model for the study of inflammation-associated cancer model, which is characterized by the upregulation of NF-κB, a hallmark of inflammatory responses, in hepatocytes. Inhibition of NF-κB in later stage of tumor development resulted in apoptosis of hepatocytes and prevention of HCC formation (132). In addition, Mdr2 knockout mouse provides an excellent model for the study of different stages of HCC (133). Analysis of the liver tissues of Mdr2 knockout mice of early and late precancerous stages revealed induction of anti-inflammatory and antioxidant genes in early stage (14). Dysregulation of genes responsible for the regulation of lipid and phospholipid metabolism was associated with the appearance of multiple DNs in the late precancerous stage. Elevated levels of oncogenes, such as choline kinase A, cyclin D1, Jun and some ras homologues were detected. In a continual study of different stages of inflammation-associated HCC in this model (134), gene expression profiling of aged mice shared similar features with those data previously published in human and murine HCC models (15, 16). This study also showed that the longest genomic region of downregulated genes in murine is syntenic to human chromosomal regions known to be frequently deleted in HCC where multiple tumor suppressor genes are located (135, 136). Analysis of gene expression revealed that nuclear cyclin D1 was increased in dysplastic hepatocytes that do not form nodules, but decreased in most DNs and in liver tumors. This finding has implicated a role of cyclin D1 at early cancerous stages in the Mdr2 knockout HCC model. Cyclin D1 has been well known to be amplified and overexpressed in various stages of HCC (137-140). Apart from the expression level, the nuclear and cytoplasmic localizations of cyclin D1 also determine its differential functional roles. Redistribution of cyclin D1 from the cytoplasm to the nucleus was associated with the proliferative neoplastic hepatocytes (24). In another study, only cytoplasmic cyclin D1 staining was detected in patients with HCC and was significantly associated with aggressive tumor behavior (141). Cyclin D1 overexpression correlates well with the activation of Wnt pathway in human and murine HCC models (142). In Mdr2 knockout mouse, most of liver tumors were characterized by the absence of activation of the classical Wnt pathway. Thus, the Mdr2 knockout mouse can serve as a model for subgroup of HCC characterized by the absence of β-catenin activation and low nuclear cyclin D1 level. Mdr2 knockout model can also be applied to test the effect of therapeutic agents against HCC. Administration of tannic acid and selenomethionine showed partial chemopreventive effect on HCC development as revealed by reduced incidence of tumor nodules formed (143).

6.2. Transgenic mice of hepatitis viruses

HBV is well recognized as the causative factor for HCC (5, 6), but the underlying mechanism of HBV in HCC remains to be elucidated. HBV DNA integration is a random event and it is frequently truncated after integration. Analysis of human HCC samples has shown that integrated HBV DNA usually encodes the hepatitis B x (HBx) and hepatitis B surface (HBs) antigens (144, 145). HBx protein has been extensively studied and suggested to be the major player in HBV-related hepatocarcinogenesis. The functional roles of HBx and HBs in hepatocarcinogenesis through direct or indirect mechanisms are still controversial. Some studies reported the induction of liver cancer in HBx transgenic mice (146-148), but not in the other (149). Although it failed to induce HCC in some cases, HBx enhanced the susceptibility of HBx transgenic mice to diethylnitrosamine (DEN). DEN-treated HBx transgenic mice had higher incidence of cancer development than control group (149). Transgenic mice carrying the entire HBV genome also showed that HBV alone was inefficient to induce HCC but could enhance hepatocarcinogenesis initiated by DEN (150). Dysregulation of cellular genes by HBx has been implicated in facilitating proliferation of hepatocytes during

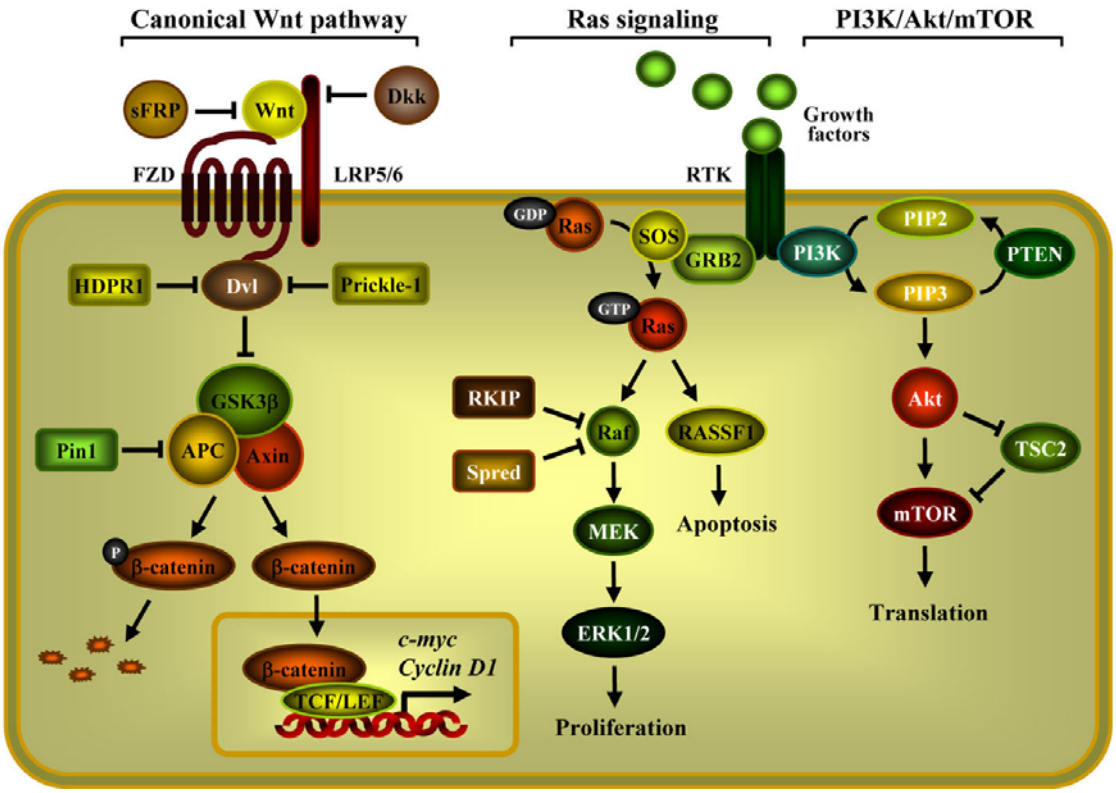

Figure 2. Signaling pathways in HCC. In the canonical Wnt pathway, binding of Wnt to FZD and LRP5/6 co-receptor activates Dvl, leading to the disassociation of the destruction complex and prevention of β-catenin degradation. Accumulation of stabilized β-catenin facilitates the translocation of β-catenin 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, HDPR1, Pin1 and Prickle1, are shown. 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.

hepatocarcinogenesis. HBx/c-myc transgenic mouse model illustrated the collaborative influence of HBx and c-myc in disturbing the cellular growth and apoptosis that eventually led to the development of HCC (151, 152). Another protein encoded by HBV large surface antigen, with deletions at the pre-S regions, has been identified in patients with chronic HBV infection (153). Association of pre-S mutant has been reported to be associated with the development of HCC (154). Transgenic mice model of pre-S mutant supported the role of large surface antigen in inducing hepatocyte hyperplasia (155) and tumor development (156).

In addition to HBV, HCV is a major cause of chronic liver disease and HCC development. Transgenic mouse models of HCV genome have been widely used to study the *in vivo* functions of HCV proteins in hepatocarcinogenesis. Different transgenic mice have been established to express various HCV viral proteins (157, 158). In spite of the contradictory and controversial resulting phenotypes, the findings of independent studies implicate the oncogenic potential of HCV in the pathogenesis of HCV-associated HCC.

7. SIGNALING PATHWAYS IN HEPATOCELLULAR CARCINOMA

Gene expression profiles of HCC samples from genetic and epigenetic analyses have provided information on alterations of genes involved in the development of HCC. Accumulating data have identified various dysregulated signaling pathways involved in hepatocarcinogenesis (159, 160), thus providing potential molecular targets for the intervention of therapeutic agents. We briefly discuss here some of the molecular signaling pathways in human HCC, namely, Wnt/β-catenin, Ras and PI3K/Akt/mTOR pathways.

7.1. Wnt/β-catenin pathway

Wnt signaling pathway is important for embryonic development and the control of cell movement and planar cell polarity. Signaling cascades of Wnt pathways are classified into the canonical pathway in which β-catenin is the key player and the non-canonical pathways in which signals are transduced by small GTPases, jun N-terminal kinase (JNK) and intracellular Ca^{2+} signaling (161) (Figure 2). In the absence of Wnt signaling, β-catenin

is in complex with APC, Axin and glycogen synthase kinase-3 beta (GSK-3β). β-catenin is phosphorylated by GSK-3β and targeted for degradation by ubiquitination. 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 this eventually leads to the disassociation of the destruction complex and prevents β-catenin from degradation (162). 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 (162) and cyclin D1 (163). Various secretory proteins, including the secreted Frizzled-related protein (sFRP) and Dickkopf (Dkk), are extracellular antagonists of Wnt pathway (164).

Dysregulation of players along the canonical Wnt/β-catenin cascade has been implicated in human cancers (165), while the involvement of the non-canonical pathways remains uncertain (165, 166). Although activation of Wnt pathway is commonly dysregulated in HCC, the role of Wnt ligands has not been clearly addressed in HCC. Secretory antagonist, sFRP1, has been found to be epigenetically silenced in human cancers including HCC (128, 167). At present, FZD7 is the only frizzled receptor reported in HCC. Remarkable overexpression of FZD7 was detected in 90% of HCC tissues (168). *In vitro* studies revealed that FZD7 was associated with stabilization of β-catenin and enhancement in cell migration. Upregulation of FZD7 was also detected in HCC of various transgenic mouse models (169). The LRP5/6 co-receptors have been shown to regulate Wnt pathway but their roles in tumorigenesis need to be elucidated (170, 171). Dickkopf-1 (Dkk-1) antagonizes Wnt pathway by blocking the access to LRP co-receptor (172). Functionally, Dkk-1 antagonizes the Wnt/β-catenin pathway and suppresses growth and migration of HCC cells (173). Dvl is the immediate effector of Wnt activation. Overexpression of Dvl-1 and Dvl-3 has been reported in HCC (174). Overexpression of Dvl is associated with β-catenin accumulation and Wnt/β-catenin signaling activation (175, 176). HDPR1 and Prickle-1, both inhibitors of Dvl, were reported to be underexpressed in HCC (174, 177).

In HCC, gene mutation and nuclear accumulation of β-catenin ranged from 13-34% and 11-43%, respectively (88, 89, 178-180). Mutations in β-catenin are more frequent in human HCV-associated HCCs (178). Most of the β-catenin mutations occur predominantly at codons 32-37, 41 and 45. These mutations protect the protein from degradation and hence stabilize the protein (88, 89, 179-181). Dysregulation of the destruction complex also leads to the stabilization of β-catenin. Transgenic mouse with inactivated APC in liver resulted in hepatocyte hyperplasia and accumulation of nuclear and cytoplasmic β-catenin (182). Although high frequency of APC mutations has been detected in colon cancer (183), APC mutation is rarely found in human HCC. Promoter methylation is responsible for the inactivation of APC (120, 184). Overexpression of PIN1 stabilizes β-catenin by inhibiting its interaction with APC (185). Interestingly, overexpression of PIN1 and mutation of β-catenin appear to be mutually exclusive events in Wnt signaling activation in HCC (186). Axin is another protein of the destruction complex. Mutation of Axin1 was found in 5-10% of human HCCs. Point mutations and deletion of Axin1 frequently occurred at the N-terminal half of the protein and this is suggested to stabilize β-catenin by impeding the formation of APC/GSK-3β/β-catenin complex (90, 180). GSK-3β plays a central role in the regulation of β-catenin stability. However, mutation of GSK-3β has not been observed in HCC (187).

7.2. Ras signaling pathway

Activation of receptor tyrosine kinases (RTK) by growth factors transduces extracellular signals to the cytoplasm through small GTPase Ras to control diverse cellular processes, such as cell growth, differentiation, apoptosis and migration (188, 189). Ras serves as a molecular switch, the activation of which is governed by guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP) (Figure 2). Raf-1 serine/threonine is the critical direct effector of Ras (190), which further transduces signals to MEK and ERK1/2. In addition to the Raf/MEK/ERK pathway, activated Ras also stimulates other downstream effectors including phosphatidylinositol 3-kinase (PI3K), RalGDS, PLC-ε and Tiam1 (190).

Point mutations confer to the constitutive activation of Ras. Unlike in other solid tumors, Ras mutations in HCC are uncommon (191), except in patients exposed to vinyl chloride (192). Overexpression of Ras has been reported in human HCC and cirrhotic livers (193). *In vitro* studies demonstrated the induction of transformation of immortalized hepatic cells and enhancement of metastatic phenotype in human HCC cell lines (194, 195). Overexpression of ERK was observed and correlated with tumor progression in HCC (196). Conversely, underexpression of physiological inhibitors of Ras/Raf/MEK/ERK pathway has been reported in human HCC. Raf-1 kinase inhibitory protein (RKIP) has been shown to be downregulated in HCC cell lines and tissues. Expression of RKIP resulted in decreased activity of ERK1/2 (197, 198). Another inhibitor, Spred (Sprouty-related protein with Ena/vasodilator-stimulated phosphoprotein homology-1 domain) was found to be frequently downregulated in HCC tissues and its expression inversely correlated with the incidence of tumor invasion and metastasis. Functionally, Spred inhibited growth and migration of HCC cells (199). Apart from growth promoting, Ras also induces senescence and apoptosis (200, 201). RASSF1 tumor suppressor has been shown to be a Ras effector that mediates the apoptotic effects of oncogenic Ras (202). In human HCC as well as other solid tumors, RASSF1A is frequently epigenetically silenced (125, 203, 204). Loss of RASSF1 expression in HCC may confer a growth-promoting activity of Ras.

7.3. PI3K/Akt/mTOR

PI3K is frequently mutated in human cancers (205), while contradictory results have been obtained in

HCC (206, 207). PI3K is activated both by receptor tyrosine kinase and Ras (Figure 2), while its activity is negatively regulated by PTEN tumor suppressor. Mutations and deletions of PTEN result in activation of the PI3K signaling pathways. LOH in chromosome 10q, in which PTEN is located, is frequently observed in HCC (208). Underexpression and epigenetic silencing of PTEN have also been reported in HCC (129, 209). Akt is a critical target of PI3K. Akt is a serine/threonine kinase that controls cell survival, cell growth, apoptosis, differentiation and metabolism by phosphorylation of a number of substrates (210). Dysregulation of Akt has been implicated in various human cancers (211). However, the role of Akt in HCC remains uncertain. In one study, phosphorylated Akt was associated with aggressive behavior of HCC (212). High expression of Akt-2 but not Akt-1 has been detected in HCC tissues (213). Mammalian target of rapamycin (mTOR) is a mediator of the PI3K/Akt pathway and has been shown to control cell growth. Tumor suppressors TSC1 and TSC2 act as brakes to attenuate mTOR signaling (214). Study has shown that phosphorylation of mTOR correlated with increased S6K level in 45% of HCC. Treatment with rapamycin, an inhibitor of mTOR, reduced S6K phosphorylation and inhibited HCC cell proliferation (215). Another mTOR inhibitor, sirolimus, inhibited HCC cell growth in animal model and exerted antiangiogenic effect on HCC cells (216). These studies have provided evidence that mTOR is a promising therapeutic target.

8. PERSPECTIVE

Molecular perturbations, including chromosomal aberrations, genetic alterations and epigenetic modifications accumulate along the multistep hepatocarcinogenesis. These changes lead to the dysregulation of important signaling pathways which are evident in HCC. Delineation of the molecular mechanisms involved in the progression of HCC provides information of potential therapeutic targets and insights into development of treatment regimes for HCC.

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