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Viral hepatocarcinogenesis

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide. Despite recent advances in the diagnosis and treatment of HCC, its prognosis remains dismal. Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major risk factors for HCC. Although both are hepatotropic viral infections, there are important differences between the oncogenic mechanisms of these two viruses. In addition to the oncogenic potential of its viral proteins, HBV, as a DNA virus, can integrate into host DNA and directly transform hepatocytes. In contrast, HCV, an RNA virus, is unable to integrate into the host genome, and viral protein expression has a more critical function in hepatocarcinogenesis. Both HBV and HCV proteins have been implicated in disrupting cellular signal transduction pathways that lead to unchecked cell growth. Most HCC develops in the cirrhotic liver, but the linkage between cirrhosis and HCC is likely multifactorial. In this review, we summarize current knowledge regarding the pathogenetic mechanisms of viral HCC.

Keywords

hepatocellular carcinoma; hepatitis B virus; hepatitis C virus; genomics; signaling pathways pathogenesis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide (El-Serag and Rudolph, 2007). In the year 1990, it was estimated that there were 4 37 408 cases of HCC worldwide (Parkin *et al.*, 1999), but by the year 2000, new cases of HCC had increased to 5 64 300 (Parkin *et al.*, 2001). More than 80% of these cases occur in developing countries, especially Southeast Asia and sub-Saharan Africa. However, the incidence of HCC has been rising in Western countries in recent years. In fact, HCC has become the fastest growing cause of cancer death in men in the United States, and age-adjusted HCC incidence rates increased more than twofold between 1985 and 2002 (El-Serag and Rudolph, 2007). The incidence and mortality rates of HCC in the United States are also expected to double over the next 10–20 years (El-Serag, 2002). Surgical resection or liver transplantation remains the most effective treatment

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options for HCC, but few patients are deemed suitable for operative intervention. Despite recent advances in the diagnosis and treatment of HCC, its prognosis remains dismal.

Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is the major risk factor for HCC worldwide. Globally, up to 80% of HCC is attributable to HBV or HCV (Perz *et al.*, 2006). The risk of HCC is increased 5- to 15-fold in chronic HBV carriers (El-Serag and Rudolph, 2007) and 11.5- to 17-fold in HCV-infected patients (Donato *et al.*, 1998, 2002). Antiviral therapy is effective in preventing HCC in only a proportion of patients (Sorrell *et al.*, 2009; Tai and Chung, 2009). Moreover, sustained clearance of HBV or HCV may be difficult to accomplish, particularly among cirrhotic patients. Thus, it is important to understand the molecular mechanisms underlying viral hepatocarcinogenesis, so as to maximize options to interrupt these pathways.

Molecular pathways in hepatocarcinogenesis

HCC is a highly heterogeneous tumor. Hepatocarcino-genesis is a complex multistep process involving a number of genetic and epigenetic alterations, the activation of cellular oncogenes and/or the inactivation of tumor suppressor genes, and dysregulation of multiple signal transduction pathways. These pathways include Wnt/β-catenin, p53, pRb, Ras, mitogen-activated protein kinase (MAPK), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K)/Akt, Hedgehog and growth factors such as epidermal growth factor, and transforming growth factor-β (TGF-β) pathways (Branda and Wands 2006; Aravalli *et al.*, 2008; Llovet and Bruix, 2008a).

Fibrosis, cirrhosis and HCC

Of all HCCs, 80–90% develop in a cirrhotic liver (Caldwell and Park, 2009). After 20–30 years of chronic infection, 20–30% of patients develop liver cirrhosis. HCC develops at an annual rate of 1–7% in HCV-infected cirrhotic patients (El-Serag and Rudolph, 2007) and 3–8% in HBV-infected cirrhotic patients (Fattovich, 2003).

During the progression of liver injury, hepatic stellate cells (HSCs) become activated, losing retinoid-containing lipid droplets and transforming into myofibroblast-like cells, which produce extracellular matrix (Okuno *et al.*, 2002), the first step in hepatic fibrosis. Unchecked progression of fibrosis ultimately eventuates in irreversible cirrhosis. The activated HSCs become responsive to both proliferative (platelet-derived growth factor (PDGF)]) (Friedman and Arthur, 1989; Pinzani *et al.*, 1996) and fibrogenic (TGF-β) (Matsuzaki, 2009) cytokines, which are upregulated in fibrogenesis and modulate inflammatory signaling from infiltrating immune cells (Parsons *et al.*, 2007). PDGF can activate both MAPK and PI3K/Akt signaling cascades (Parsons *et al.*, 2007). In PDGF-C transgenic mice, activation and proliferation of HSCs precedes development of fibrosis, which in turn is followed by the occurrence of HCC. This progression is analogous to that seen in human HCC (Campbell *et al.*, 2005). The cirrhotic liver is also associated with telomere shortening, which may in turn lead to chromosomal instability and deletion of checkpoints (Satyanarayana *et al.*, 2004). The decreased liver reserve in cirrhosis may also increase accumulation of toxic metabolites, which could also possibly increase the risk of HCC. Increased survival factors that prevented apoptosis of DNA-damaged hepatocytes and activated stellate cells (for example, $Gas6^{215}$) and reduced tumor surveillance function due to decreased natural killer cell function are all possible factors related to HCC development in cirrhosis (Friedman, 2008). Recent studies have found that stellate cells express stem cell markers such as CD133, nestin, c-kit and p75 neurotrophin receptor (Fujio *et al.*, 1994; Niki *et al.*, 1999; Cassiman *et al.*, 2001; Kordes *et al.*, 2007), and activated stellate cells appear to contribute to the stem cell niche (Roskams 2006; Gaudio *et al.*, 2009). Hedgehog and Wnt signaling pathways involved in stem cell differentiation and cancer formation are also found

in stellate cells (Myung *et al.*, 2007; Yang *et al.*, 2008). These lines of evidences suggest that stellate cells may harbor the potential to transdifferentiate into progenitor cells and possibly be linked to the development of HCC (Friedman, 2008).

HBV hepatocarcinogenesis

Virology

HBV belongs to the Hepadnavirus family. The HBV virion, which is 42 nm in diameter, is composed of an outer envelope formed by hepatitis B surface antigen. This envelope surrounds an inner nucleocapsid, the hepatitis B core antigen, that packages the viral genome and polymerase. The HBV genome is a partially double-stranded relaxed circular DNA molecule about 3200 nt in length. The minus strand contains four partially overlapping open-reading frames encoding the envelope (pre-S/S), core (pre-C/C), polymerase (P) and X proteins (X). After attachment to the hepatocyte, the HBV genome moves to the nucleus and assumes a highly stable conformation, called covalently closed circular DNA. Covalently closed circular DNA serves as a template for transcription of viral RNA, which is translated into viral proteins. The HBV polymerase, which reverse transcribes and replicates HBV DNA, lacks proofreading ability, and is therefore prone to generate mutations. Common mutations include the precore (G1896A) mutation, basal core promoter mutations (A1762T/G1764A) and deletion mutations of *pre-S*/*S* genes (Locarnini *et al.*, 2003). On the basis of genomic sequence divergence, HBV can be classified into eight genotypes, A to H (Schaefer, 2005).

HBV DNA integration

HBV genomic integration is present in over 85–90% of HBV-related HCCs and usually precedes the development of HCC. The integration of HBV DNA is not restricted to HCC but also is found in non-tumor tissue of patients with chronic HBV (Brechot *et al.*, 1980; Shafritz *et al.*, 1981). Exposure to oxidative stress or agents that cause DNA damage increases the frequency of HBV integration (Dandri *et al.*, 2002). During chronic inflammation, enhanced DNA replication and DNA damage also promote the process of viral integration (Guerrero and Roberts, 2005).

HBV integration induces a wide range of genetic alterations within the host genome, including chromosomal deletions, translocations, production of fusion transcripts, amplification of cellular DNA and generalized genomic instability (Guerrero and Roberts, 2005; Feitelson and Lee, 2007). Many integrated events occur near or within fragile sites or other cancer-associated regions of the human genome that are prone to instability in tumor development and progression. Genetic instability associated with integration may alter the expression of oncogenes, tumor suppressor genes and microRNAs (Feitelson and Lee, 2007). A recent large-scale analysis of HBV DNA integration sites in cellular DNA found a preference at sites regulating cell signaling, proliferation and viability (Murakami *et al.*, 2005). Common gene targets of integration include the human cyclin A2 gene (Wang *et al.*, 1992), the retinoic acid receptor β gene (Yaginuma *et al.*, 1987), human telomerase reverse transcriptase (Horikawa and Barrett, 2001), PDGF receptor, calcium signaling-related genes, mixed lineage leukemia and 60S ribosomal protein genes (Murakami *et al.*, 2005). A large proportion of HCCs have integrated HBV sequences encoding HBV X (HBx) and/or truncated envelope pre-S2/S proteins, which both contribute to hepatocarcinogenesis.

HBV proteins

HBx protein

HBx is a protein of 154 amino acids that increases virus gene expression and replication by transactivating cellular promoter and enhancers important for persistent viral infection (Keasler *et al*., 2007; Feitelson *et al*., 2009). Integration of the HBx sequence into host DNA is a common event in HCC. After integration, HBx promotes genetic instability through a variety of mechanisms. HBx compromises nucleotide excision repair by inactivating the UV-damaged DNA binding protein, inactivating p53 and disrupting the association of p53 to excision repair cross complementing factor 3 and the transcription factor IIH transcription factors, XPB and XPD (Wang *et al*., 1994; Jia *et al*., 1999).

HBx transactivates several cytoplasmic signaling pathways, including protein kinase C (PKC), JAK/STAT, PI3K, stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK), ras-raf-MAPK, activator protein-1 (AP-1), AP-2, nuclear factor-κB (NF-κB), Smad and Wnt, and by binding to nuclear transcription factors, including cAMP response element binding (CREB), activating transcription factor 2 (ATF-2), Oct-1 and TATA box binding protein (TBP) (Feitelson and Lee, 2007; Feitelson *et al*., 2009) (Figure 1).

HBx expression also increases cytosolic calcium levels and then activates the Ca^{2+}-dependent proline-rich tyrosine kinase 2 (Bouchard *et al*., 2001; Oh *et al*., 2003), which in turn activates Src kinase (Lev *et al*., 1995). Src increases association of Shc-Grb2-Sos, then activates Ras and the Ras-Raf-ERK and Ras-MEKK-JNK cascades and induces transcription of genes of the AP-1 family, such as c-fos and c-jun (Benn and Schneider, 1994; Oh *et al*., 2003). HBx also activates NF-κBina Ras-dependent manner (Su and Schneider, 1996).

HBx also decreases proteasomal degradation of β-catenin; there is a significant correlation between HBx expression and β-catenin accumulation in HCC tissue (Ding *et al*., 2005). HBx also increases the downstream targets of β-catenin, c-myc and cyclin D-1. Pin1, a Wnt signal regulator, can interact with a specific serine-proline motif of HBx protein and overexpression of Pin1 increases the protein stability of HBx. HBx-mediated transactivation is also enhanced by co-expression of Pin1 (Pang *et al*., 2007).

HBx causes transcriptional repression of the *p53* gene (Lee and Rho, 2000) and interacts with p53 to inactivate several important p53-dependent activities, including apoptosis, cell-cycle regulation, DNA repair and tumor suppression (Kremsdorf *et al*., 2006).

TGF-β is an important mediator of fibrosis and apoptosis. TGF-β expression is upregulated by HBx in HCC tissue (Yoo *et al*., 1996). TGF-β signals through phosphorylation of Smad3 at its middle linker and/or C-terminal regions. Reversible shifting of Smad3-dependent signaling between tumor suppression and oncogenesis indicates that Smad3 phosphorylated at the C-terminal region (pSmad3C) transmits a tumor-suppressive TGF-β signal, whereas oncogenic activities such as cell proliferation and invasion are promoted by Smad3 phosphorylated at the linker region (pSmad3L) (Matsuzaki, 2009). HBx shifts hepatocytic TGF-β signaling from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway in the early carcinogenic process (Murata *et al*., 2009). A recent study found that the HBx protein induces paracrine activation of human HSCs and anti-TGF-β blocks these paracrine effects (Martıń-VıÍchez *et al*., 2008).

Steatosis is also an important cofactor for chronic liver disease and HCC. A recent study found that HBx induces hepatic steatosis by transcriptional activation of SREBP1 and

PPAR-γ (Kim *et al*., 2007). Liver X receptor was found to mediate HBx protein-induced lipogenesis in HCC (Kim *et al*., 2008; Na *et al*., 2009).

Other potential mechanisms of HBx hepatocarcinogenesis include inhibition of Fas-mediated apoptosis (Diao *et al*., 2001), induction of genetic instability by binding to damaged DNA binding protein 1 (Martin-Lluesma *et al*., 2008), modulation of the degradation of cellular and viral proteins through inhibition of serine protease inhibitors and proteasome complexes (Sirma *et al*., 1998), induction of oxidative stress and mitochondrial translocation of Raf-1 kinase (Chen and Siddiqui, 2007), overexpression of insulin receptor substrate-1 (Longato *et al*., 2009), upregulation of beclin 1 and a corresponding increase in starvation induced autophagy (Tang *et al*., 2006, 2009), interaction with DNA methyltransferase (DNMT) with aberrant epigenetic modifications (Park *et al*., 2007; Zheng *et al*., 2009) and survival benefit under oxidative stress (Severi *et al*., 2007).

Pre-S/S protein

The truncated form of the *pre-S2/S* gene is commonly found from deleted integrated viral genomes (Tai *et al*., 2002). The pre-S2/S product also has transactivational abilities. These proteins transactivate cellular genes, including c-myc, c-fos and c-Haras (Schluter *et al*., 1994; Luber *et al*., 1996). A recent study found that pre-S2 may increase malignant transformation of human HCC cell lines by upregulating human telomerase reverse transcriptase and inducing telomerase activation (Liu *et al*., 2007). The pre-S2 activator activates PKC and causes phosphorylation of the pre-S domain, then activates the c-Raf-1/MEK/ERK signal transduction cascades (Hildt *et al*., 2002).

The pre-S mutant large surface antigens can activate endoplasmic reticulum (ER) stress to induce oxidative DNA damage and genomic instability (Wang *et al*., 2006). The pre-S mutant also can upregulate cyclooxygenase-2 and cyclin A to induce cell-cycle progression and proliferation of hepatocytes (Wang *et al*., 2006). A recent study found that vascular endothelial growth factor-A (VEGF-A) is upregulated by pre-S mutants and that pre-S mutant-expressed Huh-7 cells exhibited activation of Akt/mTOR (mammalian target of rapamycin) signaling and increased growth advantage, which could be inhibited by VEGF-A neutralization (Yang *et al*., 2009).

HBV genotype, mutations and viral load

HBV genotypes have distinct geographical and ethnic distributions (Kidd-Ljunggren *et al*., 2002; Liu *et al*., 2005). Genotype C infection is associated with delayed HBe seroconversion, more severe liver damage and liver cirrhosis than genotype B (Kao *et al*., 2000; Chu *et al*., 2002). Longitudinal and cross-sectional studies have shown that genotype C is significantly more common than genotype B in HCCs (Fujie *et al*., 2001; Chan *et al*., 2004; Yu *et al*., 2005; Yang *et al*., 2008). A recent prospective study disclosed that HBV genotype C, particularly subgenotype Ce, increased the risk of HCC in chronic hepatitis B (Chan *et al*., 2008). In a study from India, the prevalence of genotype D was higher in patients with HCC younger than 40 years of age (Thakur *et al*., 2002). In Alaska, genotype F is associated with the development of HCC (Livingston *et al*., 2007).

HBV basal core promoter mutations (A1762T/G1764A) are found to be associated with the occurrence of HCC in several cross-sectional (Baptista *et al*., 1999; Kao *et al*., 2003) and longitudinal studies (Chou *et al*., 2008; Fang *et al*., 2008; Wu *et al*., 2008; Yuan *et al*., 2009). The REVEAL-HBV study from Taiwan found that the multivariable-adjusted hazard ratio of developing HCC was 1.73 for basal core promoter mutations and that the risk was highest among participants infected with genotype C HBV and who harbored the precore 1896 variant and mutations for the basal core promoter (Yang *et al*., 2008).

Several recent studies suggested that HBV viral load is associated with HCC (Yu *et al*., 2005; Chen *et al*., 2006, 2009; Chan *et al*., 2008; Wu *et al*., 2008). The REVEAL-HBV study found that after adjusting for age, sex, smoking, alcohol use, HBeAg status, ALT level and cirrhosis, the risk of HCC was approximately six times higher for persons with viral load above 10^5 copies per ml compared with persons with undetectable viral load (Chen *et al*., 2006). HBV viral load is also closely related to the development of cirrhosis (Iloeje *et al*., 2006).

HBV animal models

The eastern woodchuck harbors the woodchuck hepatitis virus, which is similar to HBV in structure and life cycle. Woodchuck hepatitis virus can infect the liver, cause acute or chronic hepatitis and lead to HCC development after 2–4 years (Tennant *et al*., 2004). However, when ducks are infected with duck hepatitis B virus, the risk of HCC development is low. This may be because duck hepatitis B virus does not have an *HBx* gene, supporting a function for HBx in hepatocarcinogenesis.

In HBV transgenic mouse models, only the large envelope and HBx proteins are closely related to HCC development (Leenders *et al*., 2008). Chisari *et al*. (1989) developed a transgenic mice model carrying the integrated HBV large envelope polypeptides on a C57BL/6 genetic background. Toxic quantities of hepatitis B surface antigen accumulate within the hepatocyte and develop hepatocellular injury characterized by inflammation, regenerative hyperplasia, transcriptional deregulation and aneuploidy, which eventually leads to HCC development. Kim *et al*. (1991) developed an HBx transgenic mouse model using microinjection of HBV DNA containing the *HBx* gene into single-cell embryos from CD1 mice. On autopsy, 80–91% of male and 60–67% of female mice developed HCC. However, outcomes in other HBx transgenic mice have been variable. This may be attributable to differences in mouse strain, transgene sequences and integration site (Leenders *et al*., 2008) (Table 1).

HCV hepatocarcinogenesis

Virology

HCV belongs to the Flaviviridae family. Its genome is a 9.6-kb uncapped linear positive-stranded RNA. It contains 5′ and 3′ untranslated regions including control elements required for translation and replication. The untranslated regions flank an uninterrupted open-reading frame encoding a single polyprotein of 3010 or 3011 amino acids, which is processed into structural (core, envelope glycoproteins E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins by host and viral proteases (Chisari 2005; Lindenbach and Rice, 2005).

Enveloped virus particles interact with specific surface receptors and are probably internalized. Fusion of the viral and cellular membranes leads to the release of a single-stranded, positive-sense RNA genome into the cytoplasm. This genome serves as a messenger RNA for translation of the viral proteins, as a template for RNA replication, and as a nascent genome packaged into new virus particles (Lindenbach and Rice, 2005; Moradpour *et al.*, 2007).

HCV proteins

HCV is an RNA virus unable to reverse transcribe its genome and thus to integrate it into the host genome. Instead, viral proteins and their evoked host responses contribute mostly to the viral oncogenic processes.

Core protein

HCV core protein appears to have a diverse range of functions and interacts with many cellular proteins. HCV core protein has been proposed to be involved in apoptosis, signal transduction, reactive oxygen species (ROS) formation, lipid metabolism, transcriptional activation, transformation and immune modulation (Anzola, 2004; Irshad and Dhar, 2006) (Figure 2).

HCV core protein binds to several tumor suppressor proteins, including p53, p73 and pRb (Ray *et al.*, 1997; Cho *et al.*, 2001). HCV core interacts with p73, causes nuclear translocation of core protein and prevents p73-α-dependent cell growth arrest in a p53-dependent manner (Alisi *et al.*, 2003; Benard *et al.*, 2003). HCV core can also modulate the expression of the cyclin-dependent inhibitor $p21^{WAF1}$, which is a major target of p53 and regulates the activities of cyclin/cyclin-dependent kinase complexes involved in cell-cycle control and tumor formation (Yamanaka *et al.*, 2002; Kwun and Jang, 2003).

A microarray analysis study revealed threefold or more transcriptional changes in 372 of 12 500 known human genes in core-expressing Huh-7 cells, with most genes involved in cell growth or oncogenic signaling (Fukutomi *et al.*, 2005). Of particular interest is the marked upregulation of both Wnt-1 and its downstream target gene *WISP-2*. Small-interfering RNA against Wnt-1 blunted growth stimulation by HCV core and conditioned medium from Wnt-1 transfected cells accelerated cell growth. HCV core downregulates E-cadherin expression at the transcriptional level. This effect is strongly correlated with hypermethylation of CpG islands of the E-cadherin promoter through concerted action of both DNMT1 and 3b and is abolished by a specific inhibitor of DNMT (Arora *et al.*, 2008).

HCV core also upregulates the expression of TGF-β (Taniguchi *et al.*, 2004; Shin *et al.*, 2005). As HCV-infected livers progress from chronic hepatitis through cirrhosis to HCC, hepatocytic pSmad3L/PAI-1 increases with fibrotic stage and necroinflammatory grade, and pSmad3C/$p21^{WAF1}$ decreases. These results indicate that chronic inflammation associated with HCV infection shifts hepatocytic TGF-β signaling from tumor suppression to fibrogenesis, accelerating liver fibrosis and increasing the risk of HCC (Matsuzaki *et al.*, 2007). Another study showed that different thresholds of Smad3 activation dictate TGF-β responses in hepatocytes and that liver cancer-derived HCV core protein, by decreasing Smad3 activation, switches TGF-β growth inhibitory effects to tumor-promoting responses (Battaglia *et al.*, 2009). A recent study found that HCV core triggers the production of both TGF-β2 and VEGF proteins through multiple pathways, including PKC, RB/E2F1, ASK1-JNK/p38 and ERK (Hassan *et al.*, 2009). HCV core protein also behaves as a positive regulator in androgen receptor signaling and enhances the expression of VEGF in hepatocytes (Kanda *et al.*, 2008).

HCV core protein can activate the Raf/MAPK signal pathway (Hayashi *et al.*, 2000; Erhardt *et al.*, 2002), has immunosuppressive activities through its interaction with the complement receptor C1qR on T cells (Kittlesen *et al.*, 2000) and can interact with numerous transcription factors, including heterogeneous nuclear ribonucleoprotein K, leucine zipper transcription factor, 14-3-3 protein and RNA helicase CAP-Rf (Hsieh *et al.*, 1998; You *et al.*, 1999; Aoki *et al.*, 2000; Jin *et al.*, 2000). HCV core proteins also contribute to oxidative and ER stress and steatosis (see below).

HCV envelope protein

The HCV envelope protein E2 exerts an inhibitory effect on natural killer cells through engagement of CD81 (Crotta *et al.*, 2002). E2 also activates the MAPK/ERK pathway, including the downstream transcription factor ATF-2, and maintains survival and growth of target cells (Zhao *et al.*, 2005).

HCV nonstructural proteins

HCV NS3 protein

HCV NS3 protein may exert its hepatocarcinogenic effect on host cells in early stages (Sakamuro *et al.*, 1995; Zemel *et al.*, 2001). NS3 inhibits the activity of the $p21^{WAF1}$ promoter in a dose-dependent manner and is synergistic with core in this regard (Kwun *et al.*, 2001). NS3 inhibits the function of p53 in an NS3 sequence in an NS3 sequence-dependent manner (Deng *et al.*, 2006).

The expression of NS3 enhances cell growth, JNK activation and DNA-binding activities of the transcription factors AP-1 and ATF-2 (Hassan *et al.*, 2005). NS3 also induces TNF-α production by activation of AP-1 and NF-κB (Hassan *et al.*, 2007).

HCV NS5A protein

NS5A is involved in a large number of cellular functions, including apoptosis, signal transduction, transcription, transformation and ROS production. NS5A binds directly to p53, inhibits transcriptional transactivation by p53 in a dose-dependent manner and inhibits gene transcription of $p21^{WAF1}$ (Majumder *et al.*, 2001; Lan *et al.*, 2002). NS5A interacts with and partially sequesters p53 and hTAF(II)32, a component of TFIID and an essential coactivator of p53, and suppresses p53-mediated transcriptional transactivation and apoptosis during HCV infection (Lan *et al.*, 2002). NS5A protein forms complexes with the TBP and p53 and inhibits the binding of both p53 and TBP to their DNA consensus binding sequences *in vitro*. It also inhibits the p53-TBP and p53-excision repair cross complementing factor 3 protein–protein complex formation (Qadri *et al.*, 2002). NS5A also interacts with Bax as a Bcl-2 homologue and prevents apoptosis in a p53-independent manner (Chung *et al.*, 2003).

NS5A prevents TNF-α-mediated apoptotic cell death by blocking the activation of caspase-3 and inhibiting proteolytic cleavage of the death substrate, poly (ADP-ribose) polymerase (Ghosh *et al.*, 2000). NS5A can also activate NF-κB and lead to anti-apoptotic activity (Bonte *et al.*, 2004).

NS5A interacts with growth-factor-receptor-bound protein 2 to inhibit mitogenic signaling while simultaneously promoting the PI3K/Akt cell survival pathway by interaction with p85 PI3K (He *et al.*, 2002). NS5A expression in the context of HCV polyprotein inhibits the Akt substrate Forkhead transcription factor and stimulates the phosphorylation of glycogen synthase kinase-3β, leading to stabilization of cellular β-catenin and stimulation of β-catenin-responsive transcription (Street *et al.*, 2005).

NS5A can also modulate TGF-β signaling through interaction with TGF-β receptor I (Choi and Hwang, 2006). Another recent study found that NS5A protein downregulates the expression of the mitotic spindle protein ASPM through the PKR-p38 signaling pathway and induces aberrant mitoses, chromosome instability and HCC (Wu *et al.*, 2008).

HCV, ER and oxidative stress

Chronic HCV infection is characterized by increased oxidative stress. Lipid peroxidation and oxidative DNA damage are enhanced in serum and liver specimens of patients with HCV infection (Sumida *et al.*, 2000; Mahmood *et al.*, 2004). Mitochondrial dysfunction, ER stress and immune-cell-mediated oxidative bursts contribute to HCV-associated oxidative stress (Wang and Weinman, 2006). The increased oxidative stress may be accounted for by NAD(P)H oxidase, especially Nox-2 in PMNs and Kupffer cells in the liver (Takeya and Sumimoto, 2006). Both structural and nonstructural proteins of HCV can contribute to the generation of ROS. HCV NS5A protein induces ER stress and Ca^{2+} release, resulting in

increased Ca^{2+} uptake and ROS generation by mitochondria, which has been linked to the activation of NF-κB and STAT-3 (Gong *et al.*, 2001; Tardif *et al.*, 2002). Cellular responses to oxidative stress in NS5A-expressing cells are regulated by both p38 MAPK and JNK by AP-1 (Qadri *et al.*, 2004). NS3 has been shown to activate Nox2, which generates ROS (Bureau *et al.*, 2001). Expression of HCV core uniformly increases ROS and also increases lipid peroxidation products and induces antioxidant gene expression (Okuda *et al.*, 2002). HCV core gene expression decreases intracellular/mitochondrial GSH levels and mitochondrial NADPH levels, which are accompanied by increased Ca^{2+} uptake as well as ROS generation at complex I in mitochondria (Korenaga *et al.*, 2005). Core protein has also been shown to induce ER stress and modulate the production of cytokines and host enzymes that can increase ROS, such as inducible nitric oxide synthase and cyclooxygenase-2 (de Lucas *et al.*, 2003; Nunez *et al.*, 2004; Benali-Furet *et al.*, 2005). A recent study found that HCV triggers the mitochondrial permeability transition with production of ROS, leading to induction of double-stranded DNA breaks and STAT-3 activation (Machida *et al.*, 2006). Autophagy may also function as a survival mechanism in liver cancer cells (Longo *et al.*, 2008), and HCV also induces an incomplete autophagic response. Autophagy also appears to be important in HCV pathogenesis (Sir *et al.*, 2008).

By altering or participating in different signaling pathways, ROS may modulate gene expression, cell adhesion, cell metabolism, cell cycle and cell death and induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation (Choi and Ou, 2006). ROS may also activate cellular signal pathways including the MAPK, NF-κB, PI3K, p53, β-catenin/Wnt and angiogenesis signaling pathways (Martindale and Holbrook, 2002; Tien Kuo and Savaraj, 2006; Czaja, 2007). We have found that HCV increases ROS production and TGF-β1 expression in the JFH1 HCV infection model and that HCV-mediated TGF-β1 enhancement occurs through an ROS-regulated and p38 MAPK-, JNK-, MEK- and NF-κB-dependent pathway (Lin *et al.*, unpublished data).

HCV and steatosis

The prevalence of steatosis in patients with chronic HCV infection is 30–70% (Lonardo *et al*., 2004). Steatosis is associated with worsening fibrosis in chronic HCV infection (Hourigan *et al*., 1999; Adinolfi *et al*., 2001). In patients with HCV-related cirrhosis, steatosis is independently associated with the development of HCC (Ohata *et al*., 2003; Pekow *et al*., 2007).

In HCV transgenic mice that develop HCC, carcinogenesis is preceded by development of steatosis (Moriya *et al*., 1998). In addition, steatosis may induce chronic hepatic inflammation, ROS and DNA damage in animal models (Moriya *et al*., 2001; Okuda *et al*., 2002; Arkan *et al*., 2005; Cai *et al*., 2005; Koike, 2007). Core protein has been found to interact with retinoid X receptor-α (Tsutsumi *et al*., 2002). A dominant-negative retinoic acid receptor causes steatohepatitis and liver tumors in transgenic mice (Yanagitani *et al*., 2004). HCV core protein induces spontaneous and persistent activation of PPAR-αa in transgenic mice (Tanaka *et al*., 2008a). A recent transgenic mouse study using PPAR-α-homozygous, heterozygous and null mice with liver-specific transgenic expression of the core protein gene found that severe steatosis was observed only in PPAR +/+:HCVcoreTg mice and HCC developed in 35% of 24-month-old PPAR +/+:HCVcoreTg mice, but not in the other genotypes (Tanaka *et al*., 2008b). Long-term treatment of these mice with clofibrate, a PPAR-α activator, induced HCC with mitochondrial abnormalities and hepatic steatosis. The core protein induces steatosis by several pathways, leading to `fatty acid spiral' in the presence of the mitochondrial complex 1 dysfunction and PPAR-α activation. These intracellular alterations collectively contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell growth signal activation (Koike, 2009).

HCV and iron

Elevated serum iron and increased hepatic iron deposition are relatively common in patients with chronic HCV (Kato *et al*., 2007). However, recent studies have shown that excess hepatic iron accumulation in patients with HCV contributes to liver injury and fibrosis progression (Bassett *et al*., 1999; Kato *et al*., 2007; Missiha *et al*., 2008). Free iron increases oxidative stress and leads to lipid peroxidation. Cytotoxic by-products of lipid peroxidation can impair cellular function, protein synthesis and cause DNA damage (Kew, 2009). Transgenic mice expressing the HCV polyprotein fed an excess-iron diet had greater hepatic content of lipid peroxidation products and 8-hydroxy-2′-deoxyguanosine and increased the risk of HCC development (Furutani *et al*., 2006). A recent study found that retinoids can suppress iron-induced oxidative stress of the liver by regulating hemojuvelin expression (Tsuchiya *et al*., 2009).

HCV animal models

Various HCV gene products have been expressed either alone or together in transgenic mice using different liver-specific promoters (Barth *et al*., 2008; Newell *et al*., 2008) (Table 1). Among these lines, only the transgenic mice expressing the core gene developed HCC (Moriya *et al*., 1998). Moriya *et al*. described a transgenic mouse strain expressing HCV core protein under control of the HBV promoter. These mice developed steatosis without inflammation after 3 months and HCC in 26–31% of male transgenic mice after 16–19 months (Moriya *et al*., 1998). The same study found no carcinoma in transgenic mice overexpressing HCV envelope genes. Our recent study disclosed that transgenic mice expressing core, E1, E2 proteins treated with diethylnitrosamine were found to have an accelerated tumor growth than mice expressing core protein only and the accelerated tumor phenotype is attributable to suppression of apoptosis rather than enhanced proliferation (Kamegaya *et al*., 2005). The transgenic mice expressing the complete HCV polyprotein were found to have an increased risk of liver tumor development than mice expressing structural proteins only (Lerat *et al*., 2002). However, the transgenic mice expressing HCV nonstructural proteins only do not cause any spontaneous liver pathology (Majumder *et al*., 2003). Core protein continues to have the most important function in the hepatocarcinogenesis of HCV.

HBV, HCV and host genomics

Several studies have reported genomic alterations in HBV-related HCC (Villanueva *et al*., 2007). Gain of 11q and 2q and loss of 1p, 16p, 16q and 17p were found to be associated with HBV-related HCC (Lin *et al*., 1999; Zondervan *et al*., 2000; Kusano *et al*., 2002; Katoh *et al*., 2005) (Table 2). A summary of 428 different comparative genomic hybridization analyses of human HCCs found that losses at 4q (43.4 vs 19.6%), 16q (41.8 vs 18.5%), 13q (31.1 vs 19.6%) and 8p (40.6 vs 29.3%) were correlated with HBV etiology (Moinzadeh *et al*., 2005). Another comparative genomic hybridization study of 158 HBV-related HCCs found that gains of 1q21–23 and 8q22–24 were associated with the early development of HCC; gain of 3q22–24 was associated with tumor recurrence and poor survival (Poon *et al*., 2006). A recent genome-wide analysis of gene expression in human HCC found that expression of *UGT1A1*, *UGT2B10* and *GPX2* was preferentially repressed in HBV-HCCs, but were unchanged in most other HCVHCCs (Okabe *et al*., 2001) (Table 3). Viral factors are also involved in the methylation of some genes. A recent study disclosed that E-cadherin and GSTP1 were preferentially methylated in HBV-HCC compared with HCV-HCC (Su *et al*., 2007). TNF-α-863A genotype, IL-1B-511C allele, C-A haplotype of IL-10 gene and XRCC1 Gln allele at codon 399, −509C→T polymorphism in the TGF-β1 gene promoter are all associated with HCC in chronic HBV carriers (Shin *et al*., 2003; Yu *et al*., 2003; Hirankarn *et al*., 2006; Kummee *et al*., 2007; Qi *et al*., 2009).

A previous study found that gain of 10q was detected exclusively in cases of HCC with HCV infection (Kusano *et al*., 1999). A review of 338 different comparative genomic hybridization analyses of human HCCs, of which 100 were HCV positive, found that only losses of 8p were more frequent in HCV-negative cases (Moinzadeh *et al*., 2005) (Table 2). A genome-wide analysis study of gene expression found increased expression of genes encoding phase I enzymes CYP2E, AKR1C4, EPHX1 and FMO3 exclusively in HCV-positive HCCs (Okabe *et al*., 2001) (Table 3). Another study found that genes involved in the regulation of transcription and DNA repair were upregulated, whereas coagulation factors and apoptosis genes were downregulated in HCV-HCC samples when compared with cirrhotic tissues from patients with HCV-HCC (Mas *et al*., 2009). When comparing cirrhotic tissues with and without HCC, the top canonical pathways associated with HCV cirrhosis in patients with HCC included p53 signaling, acute-phase response signaling, xenobiotic metabolism signaling, IL-6 signaling and NFR2-mediated oxidative stress response. From normal liver to HCV cirrhosis to HCV-HCC, a positive trend was found in MHC class-I receptor activity, DNA damage checkpoint cell division and ubiquitin cycle genes (Mas *et al*., 2009). Recently, a three-gene set signature (GPC3, LYVE1 and survivin) that can distinguish dysplastic nodules from early HCC in HCV cirrhosis with a discriminative accuracy of 94% was discovered (Llovet *et al*., 2006). Another recent study that compared the gene expression profiles of HCV tissue samples representing the stepwise carcinogenic process from preneoplastic lesions (cirrhosis and dysplasia) to HCC disclosed the dysregulation of pathways, including the Notch and Toll-like receptor pathways in cirrhosis, followed by dysregulation of several components of the JAK/STAT pathway in early carcinogenesis, then upregulation of genes involved in DNA replication and repair and cell cycle in late cancerous stages (Wurmbach *et al*., 2007). A microarray study found that inflammatory and anti-apoptotic phenotypes were predominant in HCV but pro-apoptotic and DNA repair response were predominant in HBV with p53 and 14-3-3 interacting genes having an important function (Honda *et al*., 2006). Another study found that imprinted genes (*H19* and *IGF2*) and the genes relating to signal transduction, transcription and metastasis are upregulated in HBV-HCC and the genes responsible for detoxification and immune response are upregulated in HCV-HCC (Iizuka *et al*., 2002). Another study revealed differential microRNA expression between HBV and HCV: the pathways related to cell death, DNA damage, recombination and signal transduction were activated in HBV-HCC and those related to immune response, antigen presentation, cell cycle, proteasome and lipid metabolism were activated in HCV-HCC (Ura *et al*., 2009). These differences suggested different oncogenic factors in HBV and HCV. Another microRNA gene expression study disclosed that miR-122, miR-100 and miR-10a were overexpressed whereas miR-198 and miR-145 were up to fivefold downregulated in HCV-HCC tumor tissues compared to normal liver tissues (Varnholt *et al*., 2008). Aberrant DNA methylation is an important epigenetic alteration in HCC. This process was found to be associated with viral infection and is more prominent in HCV than HBV in a recent study (Nishida *et al*., 2008). Taken together, although there are suggestions, there is no clear `signature' for HBV- or HCV-related hepatocarcinogenesis.

Chemical carcinogens, hormones, micronutrients and viral hepatitis

The combination of HBV and aflatoxin B1 exposure may underlie the high frequency of TP53 249ser mutations in HCC (Ming *et al*., 2002). Aflatoxin exposure may also be associated with advanced liver disease in patients with chronic hepatitis C (Chen *et al*., 2007).

An intronic polymorphism in the corticotropin-releasing hormone receptor 2 gene increases susceptibility to HBV-related HCC (Gu *et al*., 2009). Pathways involving androgen signaling may affect the risk of HBV-related HCC among men (Yu *et al*., 2001). In addition,

HCV core protein behaves as a positive regulator in androgen receptor signaling (Kanda *et al.*, 2008).

In HBV carriers, a high serum level of retinol is associated with a decreased risk of HCC (Yuan *et al.*, 2006). In patients with chronic HCV, zinc concentration is lower in patients with HCC (Moriyama *et al.*, 2006) and hepatic copper also may contribute to the development of HCC (Ebara *et al.*, 2003). In patients with chronic viral hepatitis, selenium levels were significantly lower in patients with HCC (Yu *et al.*, 1999).

Conclusions

HCC is a highly complex and heterogenous tumor. Hepatocarcinogenesis is a multistep process and involves multiple cellular signaling pathways. Although HBV and HCV are the major risk factors leading to the development of HCC, the precise pathogenetic mechanisms linking viral infection and HCC remain uncertain. A number of transgenic mouse model studies have found that the expression of various viral proteins either alone or in combination lead to the development of HCC. Viral proteins also have been implicated in disrupting several cellular signal transduction pathways that affect cell survival, proliferation, migration and transformation (Figure 3). HBV and HCV also appear to have distinct paths to cancer (Table 4). Current advances in the understanding of gene expression profile with high-throughput technologies and selective messenger RNA analysis have revolutionized our approach to the pathogenesis of HCC. Recently a multikinase inhibitor, sorafenib, has been shown to provide survival benefit in patients with advanced HCC (Llovet *et al.*, 2008b; Cheng *et al.*, 2009). This represents a breakthrough in the understanding of the mechanisms and treatment of HCC and proves that molecular therapies can be effective in HCC. However, this is only the first step toward cure. Because there are still likely several complex pathogenetic mechanisms that require targeting, it will therefore be of great importance to fully understand the molecular mechanisms underlying viral hepatocarcinogenesis so as to maximize our options to interrupt these pathways.

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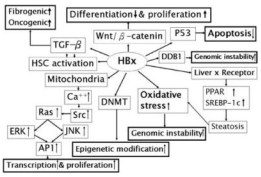

Figure 1.

Cellular signaling pathways implicated in hepatitis B virus (HBV) X protein-related hepatocarcinogenesis. Bolded boxes indicate key driving forces for carcinogenesis.

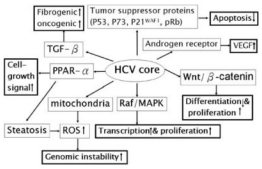

Figure 2.

Cellular signaling pathways implicated in hepatitis C virus (HCV) core protein-related hepatocarcinogenesis. Bolded boxes indicate key driving forces for carcinogenesis.

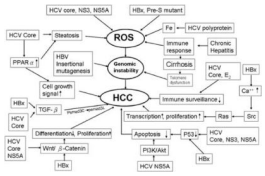

Figure 3.
A unified model of viral hepatocarcinogenesis.

Table 1

Common viral transgenic mouse models of hepatocellular carcinoma

Viral protein	Promoter/mouse strain	Phenotype	References
HBV large envelope protein	Albumin/C57BL/6	Inflammation, regenerative hyperplasia, transcriptional deregulation, aneuploidy, HCC in 72% male and 31% female, 72% of male HCC occurred at 16–21 months	Chisari *et al.*, 1989
HBV X protein	HBV X promoter/CD1	No obvious cell death or regeneration, DNA synthesis induced, HCC in 84% at mean age of 16.7 months	Koike *et al.*, 1994
HBV X protein	HBV X promoter/C57BL/6	No inflammation, no cirrhotic change, HCC in 64% at 11–18 months	Yu *et al.*, 1999
Core	HBV/C57BL/6	Steatosis, oxidative stress, HCC in 25.9–38% at 16–19 months	Moriya *et al.*, 1998, 2001; Tanaka *et al.*, 2008a,b
Core-E1–E2	Albumin/C57BL/6	Steatosis, HCC in 2.3% at age > 13 months	Lerat *et al.*, 2002
Polyprotein	Albumin/C57BL/6	Steatosis, HCC in 13.2% at age > 13 months	Lerat *et al.*, 2002
Core-E1–E2	Albumin/FVB × C57BL/6	Anti-apoptotic, DEN treated: 100% HCC at 32 weeks; HCV core-E1–E2 with largest tumors	Kamegaya *et al.*, 2005
Core-E1–E2	CMV/C57BL/6	Steatosis, HCC in 3.7% after 1 year of age	Naas *et al.*, 2005

Abbreviations: HBV, hepatitis B virus; HBV X, hepatitis B virus X; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine.

Table 2

Chromosomal aberrations in viral-hepatocellular carcinoma

	Gain	Loss	References
HBV vs non-HBV		16q (57 vs 8.3%)	Lin *et al.*, 1999
		4p (P = 0.007), 16q (P = 0.04), 17p (P = 0.04), 18q (P = 0.03), 1P (50 vs 9.5%)	Zondervan *et al.*, 2000
	2q (50 vs 9.5%)	16p (45 vs 4.8%), 16q (75 vs 23.8%), 17p (70 vs 23.8%), 4q (43.4 vs 19.6%)	Katoh *et al.*, 2005
		16q (41.8 vs 18.5%), 13q (31.1 vs 19.6%), 8p (40.6 vs 29.3%)	Moinzadeh *et al.*, 2005
		LOH on 13q and 16q (p <.001)	Okabe *et al.*, 2000
HBV vs HCV	11q13 (36 vs 3%)	10q (26 vs 0%)	Kusano *et al.*, 1999
HBV (early vs advanced tumors)	1q21–q22 (61.6 vs 77.8%), 3q22–q28 (6.8 vs 29.6%), 7q21–q22 (26.0 vs 48.1%), 7q34–q36 (16.4 vs 40.7%)	NR	Sy *et al.*, 2005
HBV (early development of HCC)	1q21–23 (P <0.001), 8q22–24 (P <0.001)	NR	Poon *et al.*, 2006
HBV (tumor recurrence and poor survival)	3q22–24(P = 0.039)	NR	Poon *et al.*, 2006
Non-HCV vs HCV	NR	8p (36 vs 20%)	Moinzadeh *et al.*, 2005

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NR, not reported.

Table 3

Gene expression analysis in HBV- and HCV-related hepatocellular carcinoma

Gene expression analysis	HBV	HCV	References
UGT1A1, UGT2B10 and GPX2	Repressed	No change	Okabe *et al.*, 2001
Genes encoding phase I enzymes CYP2E, AKR1C4, EPHX1 and FMO3	NR	Exclusively in HCV	Okabe *et al.*, 2001
Imprinted genes (*H19* and *IGF-2*)	Upregulated compared to HCV		Iizuka *et al.*, 2002
Genes related to signal transduction, transcription and metastasis	Upregulated compared to HCV		Iizuka *et al.*, 2002
Genes for detoxification and immune response		Upregulated compared to HBV	Iizuka *et al.*, 2002
Inflammatory and anti-apoptotic phenotypes		Predominant compared to HBV	Honda *et al.*, 2006
Pro-apoptotic and DNA repair response	Predominant compared to HCV		Honda *et al.*, 2006
Notch and Toll-like receptor pathways in cirrhosis	NR	Deregulation in cirrhosis	Wurmbach *et al.*, 2007
JAK/STAT pathway	NR	Deregulation in early carcinogenesis	Wurmbach *et al.*, 2007
Genes involved in DNA replication and repair and cell cycle	NR	Upregulation in late carcinogenesis	Wurmbach *et al.*, 2007
miR-122, miR-100, miR-10a	NR	Upregulated	Varnholt *et al.*, 2008
miR-198, niR-145	NR	Downregulated	Varnholt *et al.*, 2008
Pathways related to cell death, DNA damage, recombination and signal transduction	Activated	Not activated	Ura *et al.*, 2009
Pathways related to immune response, antigen presentation, cell cycle, proteasome and lipid metabolism	Not activated	Activated	Ura *et al.*, 2009
Genes involved in the regulation of transcription and DNA repair	NR	Upregulated	Mas *et al.*, 2009
Coagulation factors and apoptosis genes	NR	Downregulated	Mas *et al.*, 2009

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; NR, not reported.

Table 4

Comparison of the pathogenetic mechanisms between HBV and HCV hepatocarcinogenesis

	HBV	HCV	References
Similar pathways			
Inflammation/fibrosis/cirrhosis	Chronic hepatitis/immune response	Chronic hepatitis/immune response	El-Serag and Rudolph, 2007
Ca^{2+} signaling	HBx, HBV integration	HCV core, NS5A	Chami *et al.*, 2006; Feitelson and Lee, 2007
Raf/MAPK pathway	HBx	HCV core	Benn and Schneider, 1994; Hayashi *et al.*, 2000; Erhardt *et al.*, 2002; Oh *et al.*, 2003
TGF-β pathway	HBx	HCV core	Matsuzaki *et al.*, 2007; Matsuzaki, 2009
Oxidative stress	HBx, pre-S mutant	HCV core, NS3, NS5A	Bureau *et al.*, 2001; Gong *et al.*, 2001; Okuda *et al.*, 2002; Wang *et al.*, 2006; Chen and Siddiqui, 2007
Endoplasmic reticulum stress	HBx, pre-S mutant	HCV core, NS5A	Tardif *et al.*, 2002; Benali-Furet *et al.*, 2005; Wang *et al.*, 2006; Li *et al.*, 2007
p53 pathway	HBx	HCV core, NS3, NS5A	Ray *et al.*, 1997; Lee and Rho, 2000; Lan *et al.*, 2002; Kremsdorf *et al.*, 2006; Deng *et al.*, 2006
Androgen signaling pathway	HBx	HCV core	Chiu *et al.*, 2007; Kanda *et al.*, 2008
Wnt/β-catenin pathway	HBx	HCV core, NS5A	Ding *et al.*, 2005; Wong and Ng, 2008
Different pathways			
Genomic instability	Insertional mutagenesis	HCV core, NS3, NS5A	Gong *et al.*, 2001; Okuda *et al.*, 2002; Guerrero and Roberts, 2005; Machida *et al.*, 2006; Feitelson and Lee, 2007
Oncogene activation	Insertional mutagenesis	HCV proteins	Fukutomi *et al.*, 2005; Guerrero and Roberts, 2005; Feitelson and Lee, 2007
Iron deposition	Less common	HCV polyprotein	Furutani *et al.*, 2006
Chromosomal aberrations	monLess common	Less common	Zondervan *et al.*, 2000
Methylation	Less common	More common	Nishida *et al.*, 2008

Abbreviations: HBV, hepatitis B virus; HBx, HBV X; HCV, hepatitis C virus; TGF-β, transforming growth factor-β.