REVIEW

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Hepatic Oncology

Mechanisms of hepatocarcinogenesis in chronic hepatitis C

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Practice points

- Incidence of hepatocellular carcinoma (HCC) associated with chronic hepatitis C virus (HCV) infection is likely to increase over the next few decades.
- Risk of cancer in persons with chronic hepatitis C likely depends upon complex interactions between viral, host and environmental factors. Chronic immune-mediated inflammation plays an important role. A robust small animal model of HCV-associated HCC is needed to develop a more complete understanding of the underlying mechanisms.
- HCV may contribute directly to development of HCC in addition to the effects of chronic inflammation. Potential HCV-specific mechanisms of carcinogenesis include elevated oxidative stress and associated DNA damage combined with impaired DNA damage responses to increase rates of mutation.
- Antiviral therapy resulting in sustained virological response can substantially reduce but not completely eliminate risk of cancer in chronic hepatitis C. Novel direct acting antiviral drugs in clinical development will likely result in increased rates of sustained virological response over the coming decades.
- Persons with advanced liver disease and cirrhosis who are at high risk for HCC development are among the least responsive to antiviral therapies. Additional strategies to reduce cancer risk as well as enhanced screening protocols are needed for these populations.
- A greater understanding of mechanisms that promote HCC in chronic hepatitis C could allow development of novel therapeutics to further reduce cancer risk especially for those patients that do not have access to or fail to respond to antiviral treatments.

SUMMARY: Infection with hepatitis C virus (HCV) is a major risk factor for hepatocellular carcinoma. The genetic changes that drive cancer development are heterogeneous and how chronic hepatitis C promotes the initiation of hepatocellular carcinoma is incompletely understood. Cancer typically arises in the setting of advanced fibrosis and/or cirrhosis where chronic immune-mediated inflammation over decades promotes hepatocyte turnover providing selective pressure that favors the malignant phenotype. As well as contributions of unresolved inflammation to carcinogenesis, evidence from transgenic mice with liver-specific expression of viral sequences suggests that some HCV-encoded proteins may directly promote cancer. Numerous *in vitro* studies suggest roles for HCV proteins in subversion of cellular pathways that normally act to suppress tumorigenesis. Here, we review the mechanisms by which persistent HCV infection might promote cancer in addition to the procarcinogenic effects of inflammatory liver disease.

KEYWORDS

• ATM • DNA damage response • p53 • Rb • tumor suppressor

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Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh most common cancer in women with approximately 750,000 new cases diagnosed each year. HCC may develop following exposure to viral or chemical carcinogens and globally, rates of HCC are highest in those parts of Asia and Africa where chronic infection with the hepatitis B virus (HBV) is endemic [1]. However, the incidence of primary liver cancer has been increasing in many countries with low HBV-incidence over the last few decades and this increase correlates with higher rates of hepatitis C virus (HCV) infection [2]. More than 170 million people are estimated to be infected with HCV worldwide with 3–4 million of those in the USA.

Chronic HCV infection increases the risk of cirrhosis and HCC. Following infection with HCV, a majority of people fail to clear the virus and develop a lifelong persistent infection that may be without symptoms for decades. Although the infection may be without symptoms during this time, ineffective immune responses result in progressive tissue damage leading to fibrosis and eventually, in some individuals, cirrhosis and HCC. Disease progression is slow and variable between patients. Only a minority of patients (~20%) with chronic hepatitis C progress to cirrhosis, which puts them at high risk for HCC development. However, the high prevalence of HCV infection, estimated to be 2% of the world population, makes HCV-associated HCC a significant global health burden.

The molecular mechanisms by which chronic HCV infection causes HCC are poorly understood. As for all other cancers, development of HCV-associated HCC is thought to be a multistep process in which the accumulation of genetic and epigenetic changes results in the transformation of normal cells into tumor cells. Hepatocarcinogenesis may be considered a somatic evolutionary process with individual cells acting as reproductive units within a population. In such a model, there must be heritable genetic variation within the population of cells, as well as selective pressure to favor cells with phenotypes that promote growth and survival [3]. Cells with a selective advantage due to a mutation would be able to resist apoptosis, proliferate and acquire further mutations, forming clonal populations of aberrant cells or dysplastic nodules. For example, somatic mutations that activate the *TERT* promoter have been detected at high frequency in HCCs but also in cirrhotic preneoplastic macronodules

[4] suggesting that this is an early genetic event that provides a selective advantage. In the case of chronic HCV infection, persistent immune-mediated inflammation in the liver resulting in high hepatocyte turnover can be thought of as providing selective pressure to favor the malignant phenotype. Repeated rounds of selection, expansion and mutation ultimately result in accumulation of genetic changes that act in concert to define a tumor. What are the changes that define liver cancer? The genomic changes associated with HCC are heterogeneous between patients [5]. Despite this heterogeneity, recent advances in sequencing technologies may allow very broad classification of molecular variants of HCC according to driver genes [6,7]. A recent study using whole exome sequencing to compare 87 hepatocellular carcinomas with matched normal adjacent tissues revealed that specific genes and pathways are recurrently mutated in tumors from different patients [8]. Mutations resulting in the activation of the Wnt/ β -catenin pathway are found most frequently in HCC. Other commonly mutated pathways included genes with functions in cell cycle regulation, chromatin remodeling and antioxidant responses [6–8].

HCV: genome & lifecycle

HCV has a single-stranded positive sense RNA genome encoding a single polyprotein that is proteolytically cleaved by cellular and viral proteases to yield the mature viral proteins (Figure 1). The viral structural proteins include the core protein, which forms the nucleocapsid, and the envelope glycoproteins E1 and E2. Nonstructural (NS) proteins required for viral RNA replication and virus assembly include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.

There are no DNA intermediates in the replication cycle of HCV and replication occurs exclusively in the cytoplasm. Thus, unlike HBV, there is no possibility of insertional activation or inactivation of host genes acting to promote tumorigenesis. *In vivo*, hepatocytes are the major site of HCV replication. There is little evidence for HCV replication in nonparenchymal cells of the liver and replication of the viral genome is highly dependent upon the hepatocyte-specific miRNA, miR-122 [9].

Indirect mechanisms of carcinogenesis associated with chronic liver disease

Following initial infection with HCV, the progression to HCC is slow, and indirect,

Figure 1. Hepatitis C virus genome organization. The positive-sense RNA genome of hepatitis C virus (HCV) encodes a single, viral polyprotein that is proteolytically cleaved to yield mature viral proteins. Structural proteins (shown in white) include the viral core (C) nucleocapsid protein and the envelope glycoproteins, E1 and E2. Nonstructural proteins (shown in green) contribute to HCV replication through various mechanisms. The p7 and NS2 proteins are involved in virion assembly and egress. Proteins NS3 through NS5B are required for HCV genome replication. NS3 contains both helicase and protease domains, and NS4A serves as a cofactor that interacts with NS3 to promote its protease activity. NS4B mediates assembly of the membranous web, the cytoplasmic structure that serves as the site of genome replication. NS5A is a phosphoprotein essential for virus replication and assembly, and NS5B is the viral RNA-dependent RNA polymerase that drives HCV RNA synthesis. The 5' and 3' untranslated regions (UTRs) of the HCV genome contain *cis*-acting elements necessary for genome replication and polyprotein expression, including an IRES within the 5' UTR that directs cap-independent translation of the viral polyprotein.

NS: Nonstructural.

immune-mediated mechanisms are thought to drive cancer development. At the time of diagnosis, the majority of HCV-associated liver cancers are found in the setting of cirrhosis underscoring the important role of chronic inflammation in cancer development. The mechanisms by which chronic inflammation leads to activation of signaling pathways and gene expression to promote tumorigenesis are incompletely understood, but NF- κ B, signal transducer and activator of transcription 3 (STAT3) pathway and inflammatory cytokines, such as IL-6, are thought to be central mediators [10,11]. A detailed understanding of the many molecular signaling pathways that cooperate to mediate hepatocyte injury, inflammation and liver regeneration could allow identification of therapeutic targets to prevent cancer development in persons with chronic hepatitis C.

During HCV RNA genome replication, long double-stranded RNA (dsRNA) intermediates are produced, which can be detected by host antiviral sensors including the Toll-like receptor 3 and retinoic acid-inducible gene I, triggering signaling pathways that result in activation of transcription factors IRF3 and NF- κ B. Additionally, NF- κ B may be activated by HCV proteins or endoplasmic reticulum stress [12]. Several studies have shown that HCV is able to antagonize these signaling pathways by multiple mechanisms (reviewed in [13]), potentially allowing HCV to evade host antiviral responses and establish persistent infection. However, it appears

that these pathways can be activated during persistent infection since increased transcription of interferon-stimulated genes can be observed in the livers of patients with chronic hepatitis C [14]. Subsequent adaptive immune responses, in particular cytotoxic T lymphocyte responses, mediate most of the disease in chronic hepatitis C (for a review see [15]). Adaptive immune responses fail to clear the virus and this inefficient response results in repeated cycles of hepatocyte destruction and regeneration. The amount of hepatocyte turnover is difficult to measure in humans with chronic HCV infection but it is likely to be substantial over decades of infection. Immune-mediated liver injury together with hepatocyte regeneration results in hepatocyte DNA synthesis in the presence of inflammation-associated oxidative stress, potentially leading to the accumulation of mutations in tumor suppressors and proto-oncogenes to drive progression to the malignant phenotype. In addition, chronic immune-mediated liver injury leads to activation of hepatic stellate cells and deposition of extracellular matrix proteins. This fibrogenic response likely provides a microenvironment that favors tumor growth.

Chronic inflammatory liver disease is clearly an important risk factor for HCC development, contributing to replication-induced mutations through high hepatocyte turnover in an environment of oxidative stress. However, HCC is occasionally observed in chronic hepatitis C in

the absence of cirrhosis [16] suggesting a possible direct role for the virus in carcinogenesis in addition to the indirect effects of chronic inflammation.

Virus specific mechanisms contributing to HCC?

As well as the indirect cancer-promoting effects of immune-mediated inflammation, several lines of evidence suggest the additional contribution of direct virus-specific mechanisms of carcinogenesis in HCV. Among persons with cirrhosis of several etiologies (HCV, HBV, alcohol, hereditary hemochromatosis, autoimmune), chronic HCV infection is associated with the highest risk of HCC [17], suggesting additional virus-specific mechanisms may contribute to HCC risk in chronic hepatitis C. Differences in the genetic changes underlying HCC of different etiologies might be expected to provide information regarding potential virus-specific mechanisms but given the high genetic heterogeneity of HCC, this might prove difficult. More direct evidence for a cancer promoting activity for HCV comes from studies of transgenic mice with liver-specific expression of HCV proteins (discussed in the following section).

Does HCV cause cancer in an animal model?

Animal models for the study of HCV pathogenesis and carcinogenesis are limited. Other than humans, the chimpanzee is the only animal susceptible to infection with HCV. However, ethical concerns have limited the use of chimpanzees as a model. To overcome this problem, humanized mouse models have been successfully developed that support HCV replication. The first chimeric mouse models to be developed used immunodeficient (SCID) mice engineered to overexpress a lethal transgene (urokinase-type plasminogen activator) in hepatocytes. Death of the murine hepatocytes allows engraftment with human hepatocytes to repopulate the mouse liver [18]. Refinements and variations on this model have been developed that use different strategies to ablate the murine hepatocytes or harbor different immune deficiencies [19]. These mice support HCV replication and have been used in preclinical evaluation of candidate antiviral therapies. However, the lack of a functional immune system in these models represents a problem for the study of HCV immunopathogenesis.

Recently, Washburn *et al.* [20] reported progress towards an immunocompetent humanized mouse model of HCV infection. This system is based upon the Balb/C *Rag2^{-/-} γC* null mouse, which lacks functional T, B and NK cells. These mice were engineered to express an inducible liver-specific suicidal transgene. Induction of hepatocyte apoptosis together with the cotransplantation of human hepatic progenitor cells and CD34⁺ hematopoietic stem cells allows repopulation of the liver with human hepatocytes and partial reconstitution of antiviral immune responses [21]. Following inoculation with patient sera, these mice support HCV infection in the liver. HCV-specific T-cell responses, inflammation, hepatitis and fibrosis can be detected in infected mice [20]. Drawbacks of this system include the weak B-cell response that precludes the study of HCV-specific antibody responses. Additionally, virus replication is low and infected mice do not develop detectable viremia.

An alternative approach to develop a mouse model of HCV infection involves expression of specific human factors required to render murine hepatocytes permissive to HCV. Genetically humanized mice expressing human *CD81* and *occludin* support HCV entry and the additional knockout of specific genes involved in innate antiviral immunity (e.g., *STAT1*) allows HCV replication with detectable viremia over several weeks [22]. A potential disadvantage of this system is that HCV is a human virus and even though the engineered murine cells are permissive for virus replication, aspects of pathogenesis may depend upon interactions of viral proteins with human proteins. Although these mice have engineered defects in innate immune signaling pathways to facilitate HCV replication, they are otherwise intact and long term study could allow advances in the understanding of HCV pathogenesis and associated carcinogenesis.

Despite these recent remarkable advances in humanized mouse models of chronic hepatitis C, there is currently no robust small animal model of HCV infection that fully recapitulates the course of disease in humans, in particular carcinogenesis. As an alternative to infection models, several groups have developed transgenic mouse lines with liver-specific expression of HCV proteins. Despite lacking viral RNA replication and viremia, transgenic mouse lines have the potential to illuminate the contributions of individual HCV proteins to specific disease phenotypes. Of

the transgenic mouse lines expressing HCV proteins that have been reported in the literature, only some show increased incidence of HCC. Differences in cancer incidence between studies seem to depend mostly upon genetic background with the C57BL/6 mouse being most susceptible [23], underscoring the complex interplay of host genetic factors and viral factors in HCV-associated carcinogenesis. The first observation of cancer in a mouse model was in transgenic mice lines with high level, liver-specific expression of HCV core [24] and later studies of mice with expression of the core E1–E2 coding region of the HCV genome also demonstrated higher levels of liver cancer development compared with nontransgenic littermates [25]. Another transgenic mouse line with liver-specific expression of the full length polyprotein of a genotype (gt) 1b HCV at low but physiologically relevant levels showed a significantly higher risk of cancer development compared with nontransgenic littermates while a related transgenic mouse line expressing only the core-E1-E2-p7 coding region of the same gt 1b HCV did not show increased cancer risk, suggesting a potential role for non-structural proteins in cancer development [26]. Others have demonstrated a significantly higher frequency of liver cancer in mice expressing the NS5A protein [27]. In summary, both structural and nonstructural proteins of HCV, particularly core and NS5A, are implicated in cancer development in transgenic mouse models of hepatitis C. The observation of cancer in mouse lines expressing HCV proteins has important implications for understanding the mechanism of HCV-associated carcinogenesis. When expressed from a transgene, the HCV proteins are not recognized as foreign by the murine immune system and there is no development of inflammatory liver disease suggesting a direct role for HCV-encoded proteins in promoting cancer that is independent of immune-mediated mechanisms.

Molecular mechanisms underlying virus-specific promotion of carcinogenesis in HCV-associated HCC

How might HCV infection further compromise barriers to cancer in addition to procarcinogenic effects of chronic inflammation? HCV-encoded proteins may contribute to the accumulation of genetic and epigenetic changes that result in cancer by interacting with numerous cellular pathways. Much of this evidence comes from *in vitro* studies and should be interpreted with

caution but potential mechanisms include elevated oxidative stress, increased susceptibility to DNA damage, deregulated cell cycle controls, modulation of apoptosis and deregulation of differentiation pathways.

• Increased oxidative stress & oxidative DNA damage

Reactive oxygen species (ROS) can cause oxidative damage to cellular molecules including DNA, potentially resulting in mutagenesis. Evidence for elevated oxidative stress has been detected in livers of patients with chronic hepatitis C [28]. Immune-mediated mechanisms contribute to increased ROS in the chronically infected liver but HCV encoded proteins, in particular core and NS5A, have been shown to induce oxidative stress by modulation of calcium signaling, both *in vitro* and in transgenic mouse models.

In transgenic mice expressing HCV core protein, increased oxidative stress has been observed in the absence of inflammation while *in vitro*, expression of core results in increased mitochondrial ROS production [29]. Studies in transgenic mice expressing the HCV structural proteins have shown that a portion of core protein can localize to mitochondria where it exerts direct effects including oxidation of mitochondrial glutathione pools, inhibition of electron transport and elevated ROS production by mitochondrial electron transport complex I [30]. Additionally, when incubated with mitochondria isolated from normal mouse liver, core protein can associate with the mitochondrial outer membrane and increase calcium (Ca^{2+}) entry to the mitochondria to increase ROS [30].

An alternative mechanism for HCV induction of ROS involves nonstructural proteins, especially NS5A, which localize to the endoplasmic reticulum (ER). Hepatocytes isolated from transgenic mice expressing NS5A exhibit increased oxidative stress (again in the absence of inflammation) compared with nontransgenic littermates [27]. Likewise, hepatocytes of transgenic mice expressing the full-length HCV polyprotein demonstrate increased ROS production and oxidative DNA damage, and these effects have been attributed, at least in part, to NS5A-mediated activation of Akt [31]. *In vitro* studies have shown that NS5A can cause ER stress, increasing Ca^{2+} release from the ER, which modulates transmembrane potential in the mitochondria, ultimately resulting in increased mitochondrial ROS production [12].

• Increased susceptibility to DNA damage

The DNA damage response (DDR) is a complex network of cellular pathways that maintains genome integrity by detecting damaged DNA and coordinating cell-cycle arrest, DNA repair, senescence and apoptosis. Components of this response serve as tumor suppressors by preventing the accumulation and propagation of chromosomal abnormalities [32]. Interestingly, overexpression studies have indicated that HCV proteins can interact with multiple DDR factors and, thereby, modulate this host response. While such overexpression studies must be interpreted with caution, they suggest that HCV proteins may contribute directly to carcinogenesis by disrupting DDR signaling and DNA repair. Deregulation of the DDR, if it occurs during HCV infection, would be expected to compromise host genome stability in the context of virus-induced oxidative stress. Moreover, the liver is the major site for metabolism of environmental toxins. Hepatocytes, the metabolic workhorses of the liver, are responsible for detoxification of xenobiotics and are also the major cell type in which HCV replicates. Thus, HCV infection could potentially alter the ability of hepatocytes to effectively deal with toxins found in the environment that may possess mutagenic or carcinogenic properties. Altogether, these mechanisms could establish an environment of increased DNA damage and mutagenesis, thereby contributing directly to cellular transformation and oncogenesis.

ATM signaling

The DDR is initiated by members of the PIKK family, which includes the ataxia-telangiectasia mutated kinase (ATM). Upon overexpression, several HCV proteins, including core, NS3/4A and NS5B, interact with components of the ATM-driven response and interfere with canonical DDR signaling and DNA repair [33–35]. These interactions have yet to be confirmed during HCV infection. However, knockdown of ATM or its downstream signal transducer, Chk2, reduces viral RNA replication and virus yields in cultured hepatoma cells [35], suggesting that HCV targets these factors to promote its replication. HCV also inhibits phosphorylation of histone 2A.X (H2AX), a well characterized ATM substrate, within infected Huh7.5 cells [36], providing further evidence for manipulation of ATM signaling pathways during HCV infection. Notably, phosphorylated

H2AX (γ -H2AX) plays a critical role in DNA repair through recruitment of additional DDR and DNA repair proteins to the sites of DNA damage [37]. Thus, repression of γ -H2AX could represent an important, direct contribution of HCV to genome instability.

p53

Following DNA damage, the tumor suppressor protein p53 is phosphorylated by DDR kinases, including ATM and Chk2 [38–40]. These phosphorylation events stabilize p53 and stimulate its transcriptional activity. As a result, p53 promotes the expression of cellular genes involved in cell-cycle arrest, senescence and apoptosis, thus restricting the proliferation of damaged cells [41]. Conversely, loss of p53 function plays a pivotal role in tumor progression and is associated with the vast majority of human cancers, including HCC [42].

Individual HCV proteins, including core, NS3 and NS5A, have been reported to interact with p53 and modulate its activity following overexpression (for a review see [43]). More recently, HCV polyprotein expression was shown to impair p53 activation in both cultured cells and HCV transgenic mice [44], providing the first evidence for repression of p53 amidst the full milieu of HCV-encoded proteins. Nonetheless, the impact of HCV infection on p53 function remains undefined, largely because those cell-lines that are most permissive for HCV (e.g., Huh7 cells and their derivatives) express a transcriptionally inactive form of p53 [45].

Repression of p53 could facilitate HCV replication by countering p53-mediated senescence and apoptosis. Emerging evidence also supports a role for p53 in regulating the expression of cellular immune factors [46]. Thus, p53 repression may represent an additional strategy whereby HCV evades host immunity. In contrast to these potential benefits for HCV, repression of p53 signaling could render cells resistant to cell-cycle arrest, senescence and apoptosis, thereby promoting transformation.

• Increased proliferation

The retinoblastoma tumor suppressor protein (Rb) restricts cellular proliferation via repression of E2F-family transcription factors [47]. During normal cell-cycle progression, Rb is transiently inactivated to allow for the expression of E2F-dependent genes necessary for S-phase entry. In contrast, constitutive inactivation or loss of Rb

promotes cellular hyperproliferation and is associated with a wide variety of human cancers, including HCV-associated tumors [48,49].

In a surprising parallel with DNA tumor viruses, HCV has been shown to negatively regulate Rb and thereby stimulate E2F-responsive promoters [50–52]. In particular, NS5B induces partial relocation of Rb from the nucleus to the cytoplasm and promotes polyubiquitination and proteasomal degradation of Rb in association with the cellular ubiquitin ligase, E6-associated protein (E6AP) [51,52]. These effects are mediated by an LxCxE motif within NS5B that bears sequence homology to the Rb-binding domains of DNA virus oncoproteins [52]. In addition to potentially facilitating HCV replication, inactivation of Rb may directly contribute to HCV-associated oncogenesis. Disruption of Rb deregulates E2F activity and induces cellular hyperproliferation, DNA replication stress, and chromosomal instability [53,54]. These effects are normally countered by the cellular DDR, which detects E2F-induced DNA damage and activates p53 [53,55]. Additionally, E2F upregulates p53-activating factors, including ATM [56] and ARF [57]. These mechanisms provide an important fail-safe to restrict proliferation and maintain genomic stability in the absence of Rb function. However, as discussed above, DDR signaling and p53 activity may also be repressed during HCV infection. Thus, HCV proteins may promote cellular transformation through dual inhibition of Rb and p53 (Figure 2).

If HCV represses both Rb and p53, then why doesn't progression to HCC occur more rapidly and with greater frequency? It follows that additional cellular factors must be capable of constraining hepatocellular proliferation and transformation during HCV infection. Mice lacking *Rb* and *p53* expression in the liver do not exhibit increased rates of spontaneous tumorigenesis [58], further suggesting that other factors can compensate for the loss of these tumor suppressors. Nonetheless, *Rb/p53*-deficient mice are sensitized to tumorigenesis following treatment with a hepatocarcinogen [58]. By analogy, inhibition of Rb and p53 during HCV infection may promote genomic instability in the face of chronic oxidative stress. Such instability would likely be exacerbated by alcohol, which further increases levels of oxidative stress within the liver [59]. Over the course of life-long HCV infection, these mechanisms could lead

to mutation and inactivation of additional tumor suppressors, thus explaining the slow and uncertain path to oncogenesis.

• Deregulation of differentiation pathways

Wnt/ β -catenin

The Wnt signaling pathway is activated upon binding of Wnt ligands to their cognate receptor, Frizzled. In the canonical Wnt pathway, this binding initiates a signaling cascade that ultimately stabilizes the transcription factor β -catenin. Following stabilization, β -catenin enters the nucleus and modulates the expression of genes involved in cell survival and proliferation. Importantly, mutations in Wnt-signaling components correlate with β -catenin stabilization *in vivo* and are frequently observed in liver cancer [60].

NS5A may deregulate Wnt signaling via its interaction with the p85 regulatory subunit of PI3K. This interaction promotes activation of PI3K and the downstream kinase Akt [61]. Akt subsequently induces phosphorylation and inactivation of GSK-3 β , a critical component of the β -catenin degradation complex [62]. Consequently, NS5A expression stabilizes β -catenin and promotes β -catenin-dependent transcription [62,63]. Likewise, overexpression of HCV core protein may also inhibit GSK-3 β , thereby stabilizing β -catenin and stimulating β -catenin-dependent gene expression [64]. Although a functional role for β -catenin in HCV-associated oncogenesis remains undefined, a recent study suggests that overexpression of *c-myc*, a known oncogene and target of β -catenin, may contribute to this process [31]. This work revealed that *c-myc* is overexpressed within the liver of HCV-infected patients. Similarly, *c-myc* was overexpressed in HCV-transgenic mice in an Akt- and β -catenin-dependent manner, and this overexpression contributed to oxidative DNA damage and aberrant cell-cycle arrest.

Hedgehog pathway

Perturbations in the Hedgehog (Hh) signaling pathway may also be involved in the development of HCV-associated HCC. Levels of Hh ligands are elevated in patients with chronic hepatitis C, as well as in Huh7 cells infected with HCV. Increased production of Hh ligands correlates with cirrhosis and HCC and may influence Hh target cells, including myofibroblasts and epithelial progenitors that expand during fibrosis and cirrhosis [65].

Figure 2. Model for direct contributions of hepatitis C virus to oncogenesis. The HCV RNA-dependent RNA polymerase NS5B inactivates the retinoblastoma tumor suppressor protein (Rb). Inactivation of Rb deregulates E2F activity, resulting in cellular hyperproliferation and host DNA replication stress. This replication stress damages the cellular genome in combination with HCV-induced oxidative stress, which is enhanced by alcohol. Oxidative stress and DNA damage activate ATM and its downstream effector, p53. In turn, p53 restricts proliferation and maintains genomic stability by initiating cell-cycle arrest, DNA repair, senescence, and apoptosis. However, HCV proteins, including core, NS3, and NS5A, may inhibit p53 to block these antiviral processes. Dual inhibition of Rb and p53 promotes genomic instability. Over the course of life-long HCV infection, this genomic instability may result in mutations within other tumor suppressors and oncogenes, ultimately leading to cellular transformation and the development of HCV-associated liver cancer. HCV: Hepatitis C virus; NS: Nonstructural.

• Modulation of apoptosis

Apoptosis is a form of programmed cell death critical for maintaining tissue homeostasis and removing diseased or damaged cells. Beyond these roles, apoptosis can also function as a potent antiviral response, and viruses have therefore evolved a variety of strategies to circumvent this host defense [66]. HCV replication is known to promote oxidative stress and ER stress. As a virus with the capacity to establish life-long, persistent infection, HCV might be expected to block apoptosis or render cells less sensitive to proapoptotic signaling in order to prevent clearance of infected cells. Early *in vitro* studies suggested that multiple HCV-encoded proteins are capable of modulating apoptosis (see reference [43] for an extensive review of these studies). Although informative, these studies were largely limited to overexpression of individual viral proteins in the absence of viral replication.

Recent studies using cell culture infectious virus systems have hinted at a potentially complex relationship between HCV and apoptosis. Multiple studies have demonstrated increased levels of apoptosis in Huh7 cells infected with cell-cultured derived strains of HCV [67–70]. Moreover, HCV was shown to trigger apoptosis in transplanted human hepatocytes within the chimeric *SCID/Alb-uPA* mouse model in a replication-dependent manner [71]. Thus, HCV infection may be inherently proapoptotic. These results may initially seem difficult to reconcile with chronic HCV infection and virus-associated oncogenesis, as apoptosis is typically considered to be antiviral and anticarcinogenic. However, repeated cycles of HCV-induced apoptosis and regenerative cell proliferation could contribute to chromosomal instability, mutagenesis and transformation, particularly in combination with ongoing oxidative stress. An important caveat to cell-culture studies that have demonstrated HCV-induced apoptosis is that many rely on the highly replicative genotype 2 JFH1 strain of HCV or derivatives thereof, which accumulate high levels of intracellular viral antigens to generate ER stress. Similarly, apoptosis observed in immunodeficient mouse models may be a consequence of unrestrained viral replication in the absence of immune control. Cell culture infectious viruses that replicate to lower levels, such as the genotype 1a H77S.3, do not strongly induce apoptosis [69], possibly reflecting more accurately the situation in liver during persistent infection where viral antigen

levels are low [72]. Other reports indicate that HCV may repress apoptosis via NS5A-mediated inhibition of the stress-activated kinases MLK-3 and p38-MAPK [73,74].

• Increased cell motility

It is not clear whether HCV-associated HCC has a greater metastatic potential compared with HCC of other etiologies but *in vitro* studies suggest HCV infection may promote hepatoma migration and invasion. Expression of the HCV glycoproteins E1 and E2 in polarized HepG2 cells resulted in decreased cell polarity and tight junction integrity [75] and HepG2 cells expressing E1/E2 show an increased migratory capacity [75].

HCV might also increase cell motility by triggering epithelial-to-mesenchymal transition (EMT), a process which contributes to the development of metastatic carcinomas [76]. Evidence suggests that HCV promotes EMT both *in vitro* and in the liver of chronically infected patients [77]. Core protein expression is sufficient to drive phenotypic changes characteristic of EMT in primary human hepatocytes, as well as in hepatocytes derived from core-transgenic mice, likely via modulation of TGF- β signaling pathways [78]. NS5A expression also induces EMT in cooperation with TGF- β *in vitro* [79]. Nonetheless, the contributions of core and NS5A to EMT *in vivo* and the role of EMT in HCV-associated oncogenesis remain undefined.

• Angiogenesis

Chronic HCV infection is associated with increased liver angiogenesis compared with chronic HBV, suggesting that this process may play a particularly important role in HCV-associated hepatocarcinogenesis [80]. HCV promotes expression and secretion of a proangiogenic factor, VEGF, in cultured Huh7 cells [81]. Consistent with this observation, levels of VEGF and other angiogenic markers are also elevated within the liver of HCV-infected patients [82].

Which variables determine whether a chronic hepatitis C patient will develop HCC?

Among persons chronically infected with HCV, disease progression is variable. Although difficult to quantify accurately, only a minority (20%) will progress to cirrhosis [83]. Of this minority, approximately 20% will develop HCC [83]. Risk factors include male sex, age over 40 years at time

of infection, alcohol use and coinfection with HBV and possibly HIV [1]. Although the precise molecular determinants of HCC development are not known, carcinogenesis likely depends upon the complex interplay of viral, host and environmental factors, the relative contributions of which may vary between patients.

• Viral factors

HCV is highly genetically diverse and isolates are currently classified into seven genotypes (gts), which differ by greater than 30% at the nucleotide level with gts being further divided into subtypes, which differ by 20–25%. In addition, the error-prone nature of the HCV RNA-dependent RNA polymerase results in high genetic heterogeneity within infected individuals where the virus circulates as a quasispecies distribution of closely related, variant RNA genomes. Some analyses have suggested that patients infected with gt 1b HCV have almost twice the risk of developing HCC compared with those with other gts [84]. Other analyses have focused on specific polymorphisms within the viral genome. In particular changes resulting in amino acid substitutions in the core protein have been associated with an increased risk for HCC [85]. Furthermore, amino acid changes have been observed in the core proteins of HCV variants isolated from tumor tissue compared with paired nontumor samples, which alter the ability of core to modulate the TGF- β pathway [86] – an important cell signaling pathway implicated in tumor development.

• Host genetic & metabolic factors

Currently there is little information concerning genetic determinants of HCC development in chronic hepatitis C. Some studies focusing on specific genes already known to play a role in carcinogenesis have identified single nucleotide polymorphisms (SNPs) that are associated with HCC development. However, such candidate-gene approaches are restricted in scope and have failed to identify robust predictors of cancer development (for a critical review see [87]). Genome-wide association studies represent a more powerful and unbiased method for identification of genetic variants that confer high risk of cancer development. This method examines the frequencies of SNPs across the genome in patients with a specific disease compared with control patients, to identify genetic loci associated with that disease state. One genome-wide

association study identified a SNP associated with slightly increased risk for HCC in chronic hepatitis C patients at a locus upstream of the MHC class I polypeptide-related sequence A (*MICA*) gene [88]. The mechanism is unclear but the polymorphism appears to affect *MICA* expression levels. Since this study compared HCV-associated HCC cases with cases of chronic HCV without HCC without controlling for fibrosis stage or presence of cirrhosis, it is possible that this SNP is associated with an increased risk for cirrhosis and contributes indirectly to cancer risk. Another study analyzed more than 467,538 SNPs in 212 patients with chronic hepatitis C-associated HCC compared with a control group with chronic hepatitis C without HCC, and identified a different SNP within the *DEPDC5* locus associated with an almost twofold increased risk for HCC [89]. The mechanism underlying this association is unclear. Both studies were performed in Japanese populations and await confirmation in other ethnic groups. Further studies that integrate more clinical observations of disease stage are required to determine the exact role of these variants and whether they influence carcinogenesis, development of cirrhosis, fibrogenesis or simply viral control. Understanding which host factors predispose persons with chronic hepatitis C to develop HCC could allow identification of those high risk patients who might benefit from enhanced screening or preventative measures.

Although genetic factors have been challenging to identify, there are strong links between host metabolic factors and HCV-associated hepatocarcinogenesis. Namely obesity and diabetes are strongly associated with HCC development in chronic hepatitis C [90].

• Environmental factors

Epidemiological studies of environmental factors associated with increased rate of progression to cancer in chronic hepatitis C include alcohol use [90] and coinfection with HBV [17,91]. In addition, studies of transgenic mice with liver-specific expression of part or all of the HCV polyprotein coding sequence suggest that HCV may act synergistically with a number of environmental factors to promote carcinogenesis. These include alcohol [92], aflatoxin [93], diethylnitrosamine [94], iron overload [95], HBV X protein [96], *Helicobacter hepaticus* colonization of the gut [97] and hepatocyte injury mediated by carbon tetrachloride [98].

Of the factors identified as co-carcinogens, alcohol use is perhaps most strongly associated with faster progression to HCC among chronic hepatitis C patients. Chronic HCV infection and alcohol use act synergistically to increase risk of HCC [90] and several mechanisms have been proposed to explain this phenomenon. HCV replication is known to result in oxidative stress and ethanol metabolism may synergistically enhance ROS production to accelerate disease progression. *In vitro* studies suggest ethanol metabolism may enhance HCV replication to contribute to more severe injury [99].

Chronic alcohol consumption may also enhance disease severity by impairing cellular immune responses to HCV through effects on dendritic cell function [100].

NS5A may be a mediator of the synergism between chronic hepatitis C and alcohol in carcinogenesis. A transgenic mouse line with hepatocyte-specific expression of HCV NS5A can be induced to develop tumors following long-term alcohol feeding [92]. Tumors were not observed in the transgenic mice in the absence of alcohol feeding or in nontransgenic littermates [92,101]. In this system, a model was proposed for the synergistic effects of HCV and alcohol. Gut barrier dysfunction caused by alcohol feeding resulted in endotoxemia. At the same time, NS5A expression in hepatocytes was shown to induce TLR4 expression. The authors argue that activation of (NS5A-induced) TLR4 by endotoxins mediates synergistic liver damage and tumorigenesis, through activation of the stem cell marker Nanog.

A recent study has suggested HCV and alcohol might synergize to promote HCC by altering post-translational modifications (PTMs) of the transcription factor and tumor suppressor FOXO3, a component of the hepatic antioxidant response [102]. Individually, HCV and alcohol induce different PTM patterns that activate FOXO3. However, FOXO3 molecules containing HCV-induced PTMs are destabilized in the presence of PTMs induced by alcohol. Thus, the combination of HCV and ethanol has quite different effects on FOXO3 at the molecular level than either agent alone, potentially contributing to reduced antioxidant responses, increased oxidative damage to DNA and accelerated carcinogenesis.

Future perspective

HCV viremia is a major risk factor for HCC, and therapeutic eradication of viral RNA or sustained

virological response (SVR) can substantially reduce, but not completely eliminate, the risk of cancer [103]. Until 2011, the standard of care (SoC) for chronic hepatitis C was dual antiviral therapy with PEG-IFN and ribavirin (RBV). PEG-IFN/RBV therapy is poorly tolerated and only leads to SVR in 50% of patients infected with gt 1a HCV, the most commonly found gt in the USA. Over the past 10 years, efforts to identify new therapies have resulted in the development of several direct acting antivirals (DAAs) that target viral proteins essential for virus replication. In 2011, the first of these new DAAs, boceprevir (marketed as Victrelis® by Merck) and telaprevir (marketed as Incivek® by Vertex), both of which inhibit the viral NS3/4A protease, were approved by the US FDA for treatment of HCV [104]. Addition of protease inhibitors (PIs) to the SoC has improved rates of SVR: triple therapy with a PI, PEG-IFN and RBV increased response rates to 60–90% in treatment-naïve patients but some populations remain difficult to treat (e.g., prior null responders and patients with cirrhosis) and PI-resistant HCV variants are a problem [105]. Very recently, two new DAAs were licensed in the USA for treatment of chronic hepatitis C: the next generation protease inhibitor simeprevir (Johnson and Johnson) and sofosbuvir (Gilead Sciences). Additionally, a number of other DAAs have been identified and currently several of these are in clinical development [106]. Novel therapies currently in clinical development have the potential to increase SVR rates and reduce HCC risk in persons with chronic hepatitis C. However, widespread access to these drugs will likely be limited by their high cost, particularly in poorer, developing countries where the prevalence of chronic HCV infection is high.

Many people with chronic hepatitis C may be unaware of their infection. Increased surveillance of high risk populations may alleviate this problem. For example, since a majority of people in the USA with chronic hepatitis C were born between 1945 and 1965, the CDC recommended in 2012 that this group receive a one-time test for hepatitis C. Even with increased surveillance and detection, for various reasons, some may not have access to or may not respond to new antiviral therapies. A detailed understanding of the molecular mechanisms by which chronic HCV infection promotes HCC could allow enhanced screening of high risk patients with chronic hepatitis C and development of novel therapies to reduce the risk of cancer in this population.

Deeper understanding of the HCV life cycle and pathogenesis would be facilitated by improved animal models of HCV infection, which are able to integrate models of HCV-associated liver disease with genetic studies that make use of advances in sequencing technologies. In this area, humanized mouse models show great promise [22] but further optimization and refinement will be necessary in order that they more accurately reflect the course of disease in humans, especially with respect to cancer development. In the livers of patients with chronic hepatitis C, not all of the cells are infected. Using sensitive techniques to visualize HCV antigen, it has been estimated that 7–20% of hepatocytes are infected in patients with plasma viral RNA loads of 10^5 or greater [72]. To understand molecular mechanisms underlying HCV-associated carcinogenesis, it will be informative to dissect out events that occur in infected versus uninfected bystander hepatocytes using techniques that allow analyses at the single cell level. Such technologies would also be invaluable for study of the complex interactions between hepatocytes and nonparenchymal cells of the liver that contribute to disease during chronic infection.

Finally, for those high-risk individuals that either do not have access to, or do not respond

to, antiviral therapy, early detection is essential for successful intervention and improved survival in HCV-associated liver cancer. Studies from Japan show that intensive screening allows early diagnosis and potentially curative interventions with 5-year survival rates of 57% for radiofrequency ablation and >80% for those meeting criteria for surgical resection [107]. However, the routine use of imaging-based screening procedures is limited by the high cost. Levels of the serum biomarkers α -fetoprotein and des- γ -carboxy prothrombin correlate with HCC, but are not recommended by themselves as screening tools because of poor predictive value [108]. Identification of novel biomarkers could allow development of a routine low cost, noninvasive assay to screen high risk chronic hepatitis C patients for HCC.

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