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Pathogenesis and prevention of hepatitis C virus-induced hepatocellular carcinoma

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Clinical Course

Summary

Hepatitis C virus (HCV) is one of the major aetiologic agents that causes hepatocellular carcinoma (HCC) by generating an inflammatory, fibrogenic, and carcinogenic tissue microenvironment in the liver. HCV-induced HCC is a rational target for cancer preventive intervention because of the clear-cut high-risk condition, cirrhosis, associated with high cancer incidence (1% to 7% per year). Studies have elucidated direct and indirect carcinogenic effects of HCV, which have in turn led to the identification of candidate HCC chemoprevention targets. Selective molecular targeted agents may enable personalized strategies for HCC chemoprevention. In addition, multiple experimental and epidemiological studies suggest the potential value of generic drugs or dietary supplements targeting inflammation, oxidant stress, or metabolic derangements as possible HCC chemopreventive agents. While the successful use of highly effective direct-acting antiviral agents will make important inroads into reducing long-term HCC risk, there will remain an important role for HCC chemoprevention even after viral cure, given the persistence of HCC risk in persons with advanced HCV fibrosis, as shown in recent studies. The successful development of cancer preventive therapies will be more challenging compared to cancer therapeutics because of the requirement for larger and longer clinical trials and the need for a safer toxicity profile given its use as a preventive agent. Molecular biomarkers to selectively identify high-risk population could help mitigate these challenges. Genome-wide, unbiased molecular characterization, high-throughput drug/gene screening, experimental model-based functional analysis, and systems-level *in silico* modelling are expected to complement each other to facilitate discovery of new HCC chemoprevention targets and therapies.

Introduction

Liver cancer, predominantly hepatocellular carcinoma (HCC), is the second most deadly cancer worldwide (GLOBOCAN 2012, http://globocan.iarc.fr). HCC is the most rapidly increasing cause of cancer-related mortality in the U.S. In contrast to developing countries in the Asia-Pacific regions and sub-Saharan Africa, where hepatitis B virus (HBV) is the major risk factor for HCC, chronic infection with hepatitis C virus (HCV) has been responsible for the increasing HCC incidence in developed countries [1]. It is estimated that approximately 3% of the world population is chronically infected with HCV (WHO, www.who.int). More than one million individuals, representing the "baby boomer" population, are estimated to develop HCV-related cirrhosis, hepatic decompensation, or HCC by 2020, and estimated costs for management of the patients reach $8.6 billion (non-pharmacological cost only) by 2015 in the U.S. [2]. In Canada, total health care costs associated with HCV are expected to increase by 60% until they peak in 2032 [3]. Given the extremely frequent tumour recurrence even after aggressive treatment (70% after 5 years of surgical resection) and limited treatment options available for advanced-stage liver disease, including liver transplantation, a costly proposition, prevention of HCC development in patients with advanced liver fibrosis may be the most effective strategy to substantially impact patient survival [4]. Prevention of exposure to the risk factors (primary prevention) with vaccination has shown to be an effective measure in reducing HBV-related HCC, although no analogous vaccine is available for HCV [5]. Efforts have been made to prevent HCC in individuals who have already acquired the risk factors (secondary prevention) with no substantial success as of yet. Prevention of HCC recurrence

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after curative therapies (tertiary prevention) has also been explored because the patients are still at risk for new HCC [4].

In patients with chronic HCV infection, the risk of HCC gradually increases as liver fibrosis progresses. Once cirrhosis is established, the annual incidence of HCC is extremely high (1–7% per year), although HCC rarely develops in less fibrotic livers [6,7]. The emergence of highly effective direct-acting antivirals (DAAs) for HCV is expected to reduce HCV-related HCC [8]. However, HCV eradication does not eliminate the risk of HCC, especially when the patients already have advanced liver fibrosis [9]. Although molecular mechanisms of HCV-induced HCC development have not been fully elucidated, these epidemiological observations suggest that the major role of HCV in carcinogenesis is to create a cirrhotic tissue microenvironment that serves as a carcinogenic milieu. In addition, direct carcinogenic effects of HCV proteins have been suggested in a variety of experimental models as additional drivers of HCV-induced HCC development [10]. These findings may lead to the discovery of targets for secondary/tertiary HCC prevention strategies. Targets in the mechanisms of fibrosis/cirrhosis-driven carcinogenesis may also be relevant to other aetiologies, including HBV, alcohol, and non-alcoholic fatty liver diseases (NAFLD).

In this article, we review the current knowledge regarding molecular mechanisms of HCV-induced hepatocarcinogenesis that potentially provide clues about preventive therapies, and discuss strategies to translate the knowledge into clinical practice to ultimately prevent the poor prognosis of HCV-related HCC.

Key Points

- HCV-induced HCC is a model of chronic inflammation-driven cancer, where complex interactions between multiple cell types form a carcinogenic tissue microenvironment that fosters and promotes progression of neoplastic clones
- Recent clinical data suggest that HCV eradication does not eliminate the risk of HCC development, especially when the patients have more advanced fibrosis, indicating the necessity to develop HCC prevention therapies to improve patient prognosis
- Direct and indirect oncogenic effects of HCV have been identified as potential targets to prevent disease progression to HCC development by using various, mostly cell culture-based, experimental systems
- Better *in vitro*, *in vivo*, and *ex vivo* experimental models of HCV infection are needed to study molecular mechanisms of HCV-induced hepatocarcinogenesis under more physiological conditions
- Molecular biomarkers of HCC risk will help clinical translation of molecular targeted chemoprevention therapies for HCV-induced HCC

Molecular targets in HCV-induced hepatocarcinogenesis

As HCV is an RNA virus with little potential for integration of its genetic material into the host genome, it is generally assumed that HCV contributes to HCC development in an indirect way, through induction of chronic inflammation, and directly, by means of viral factors. HCV-induced HCC development is a multi-step process that involves establishment of chronic HCV infection, persistent chronic hepatic inflammation, progressive liver fibrogenesis, initiation of neoplastic clones accompanied by irreversible somatic genetic/epigenetic alterations, and progression of the malignant clones in a carcinogenic tissue microenvironment. This process could take 20–40 years (Fig. 1), and each step in the process could be a target for prevention of HCC.

A major obstacle for the understanding of the mechanisms linking HCV infection, inflammation and carcinogenesis is the lack of efficient and convenient model systems to study disease biology. While tremendous progress has been made in recent years regarding the establishment of novel cell culture models to study HCV-host interactions, there are limited *in vitro* models to study virus-induced liver disease. Moreover, the very narrow host range of HCV, infecting only humans and chimpanzees, so far precludes the study of HCV infection in conventional small animal models. Different mouse models, including HCV transgenic mice, immunodeficient human liver chimeric mice and immunocompetent humanized mice have been developed to study defined aspects of HCV pathogenesis. While these mouse models provided first insights into HCV-induced fibrosis and carcinogenesis, a mouse model that closely mimics human liver disease including HCC is still lacking [11,12].

Oncogenic effects of HCV proteins

HCV is a single-strand RNA virus in the *Flaviviridae* family that encodes structural (core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [13]. The viral particle is formed by a nucleocapsid, comprising the core protein and viral genome and an envelope consisting of envelope glycoproteins E1 and E2. Following viral infection, the cellular expression of the nucleocapsid core protein localizes in the cytosol, lipid droplets, endoplasmic reticulum/Golgi apparatus, mitochondria and nuclei, and has been suggested to affect a variety of cellular functions. The envelope glycoproteins (E1 and E2) are involved in interactions with host cells and viral entry, and potential targets for vaccine development [14,15]. NS3 has serine protease and helicase activities, and cleaves downstream NS proteins together with NS4A. NS4B is a component of a membrane-associated cytoplasmic HCV replication complex. NS5A is an indispensable factor in the HCV replication complex and virion assembly. NS5B, an RNA-dependent RNA polymerase, synthesizes viral RNA. Due to its inability to stably integrate into the host genome, in contrast to HBV, HCV requires continuous replication for its viability. There are several clinical data, suggesting the role of HCV viral factors in disease progression, such as more frequent steatosis in genotype 3 and more frequent HCC development in genotype 1b, although some of the evidences are conflicting [16–19]. Nevertheless, several experimental models have suggested direct oncogenic effects of HCV proteins (Fig. 2).

Cellular proliferation and survival pathways

Artificial over-expression of HCV proteins, e.g., core, NS3, and NS5A promotes cellular proliferation, transformation, anchorage-independent growth, and/or tumour formation in mice, suggesting their direct contribution in activating oncogenic molecular pathways [20–23]. The core protein inhibits tumour suppressor genes *TP53*, *TP73*, and *RB1* as well as negative

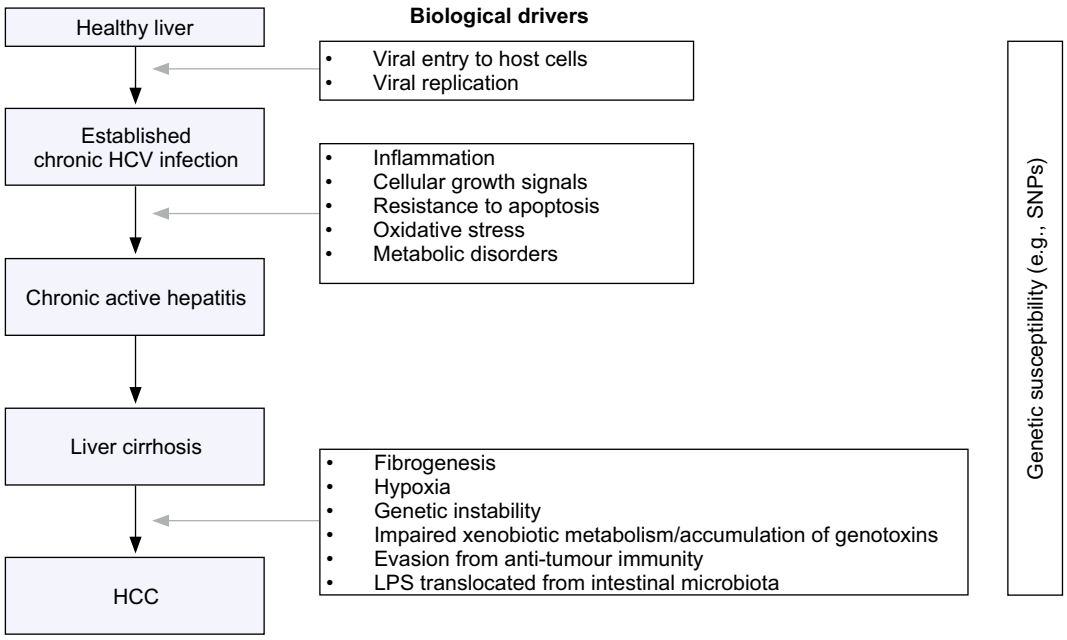

Fig. 1. Natural history and biological processes in HCV-induced HCC development. HCV, hepatitis C virus; HCC, hepatocellular carcinoma; LPS, lipopolysaccharide; SNP, single nucleotide polymorphism.

regulators of cell cycle such as *CDKN1A* (also known as p21/CIP) through physical interaction, modulation of regulatory networks, or post-translational modifications [24–27]. NS3 and NS5A also inhibit p53 (*TP53*) [28,29], and NS5B inhibits the retinoblastoma-associated protein (*RB1*) [30]. HCV core, E2, NS5A, and NS5B activate cellular proliferative RAF/MAPK/ERK kinase pathways and the E2F1 pathway, which are associated with a more aggressive biological phenotype of HCC tumours [26,30–33]. HCV proteins, such as core, are known to induce the generation of reactive oxygen species (ROS) and to transactivate MAPK and AP1 pathways [34]. Insulin-like growth factor signalling is activated via the insulin-like growth factor 1 receptor (*IGF1R*) in early stage HCV-related HCC [35]. NS5A was found to be involved in activation of PI3K/AKT and beta-catenin/WNT pathways, and evasion from apoptosis by caspase-3 inhibition [36]. Transforming growth factor-beta (TGF-beta) is elevated in the serum of chronic hepatitis C patients [37]. HCV core directly interacts with Smad3 and inhibits the tumour suppressor activity of the TGF-beta pathway [38]. *YAP1* and *IGF2BP3* expressed in *TLR4*/*NANOG*-dependent tumour-initiating stem-like cells (TICs) also inhibit the tumour suppressing role of the TGF-beta pathway in HCV-related HCC [39]. NS5A inhibits TGF-beta signalling by preventing nuclear translocation of Smad proteins, resulting in downregulation of the tumour suppressor cyclin-dependent kinase inhibitor 1 (*CDKN1A*) [40]. NS5A downregulates abnormal spindle-like, microcephaly-associated (*ASPM*), a regulator of mitotic spindle, and induces mitotic dysregulation and chromosomal instability [41]. NS5A inhibits tumour necrosis factor-alpha (TNF-alpha) mediated apoptosis [42]. HCV induces cancer stem cell-like gene signatures in cell culture and murine tumour xenografts through *DCLK1* [43].

Retinoid X receptor-alpha (RXR-alpha), activated by RAF/MAPK signalling, is a nuclear receptor for retinoids, vitamin A analogues, involved in cell growth, differentiation, and apoptosis [44]. Acyclic retinoid counteracts this process and induces apoptosis. Silymarin, a herbal flavonoid, induces cell cycle arrest and apoptosis in HCC cells, suppresses N-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in rats, and shows anti-HCV activity [45,46]. An observational study showed that silymarin use was associated with reduced fibrosis progression, but an association with HCC incidence was not obvious during the follow-up of 5.5 years [47].

Genetic instability

Structural alterations of host genomic DNA, including somatic oncogenic mutations and deletions of tumour suppressor genes, are major drivers of carcinogenesis. HCV core inhibits mitotic spindle checkpoint function by reducing the retinoblastoma-associated protein, and increases chromosomal polyploidy [27]. Chronic oxidative stress induced by the core also leads to mitochondrial and chromosomal DNA damage, leading to HCC development [34]. NS3/4A interacts with serine-protein kinase (*ATM*), a cell cycle checkpoint kinase, and impairs DNA damage repair [48]. Perturbations of the endoplasmic reticulum (ER) lead to an evolutionarily conserved cell stress response called the unfolded protein response (UPR) to compensate for damage or eventually trigger cell death when ER dysfunction is severe or prolonged. HCV has been shown to induce ER stress [49]. It has been hypothesized that persistent ER stress induction could predispose a cell to mutagenesis, secondary to the intracellular and extracellular accumulation of DNA-damaging factors.

Immune response, inflammation pathways

Interferon pathway activation is a well-known innate immune response to HCV infection, and recent studies have elucidated its role in anti-tumour immunity [50]. The nuclear factor kappa-B (NF-κB) pathway was implicated in HCC development especially during progression of initiated tumour clones [51], although there is somewhat conflicting evidence regarding its role in hepatocarcinogenesis [52]. HCV core protein inhibits NF-κB-mediated immune responses [53]. The c-Jun N-terminal kinase (JNK) pathway, activated in non-parenchymal liver cells

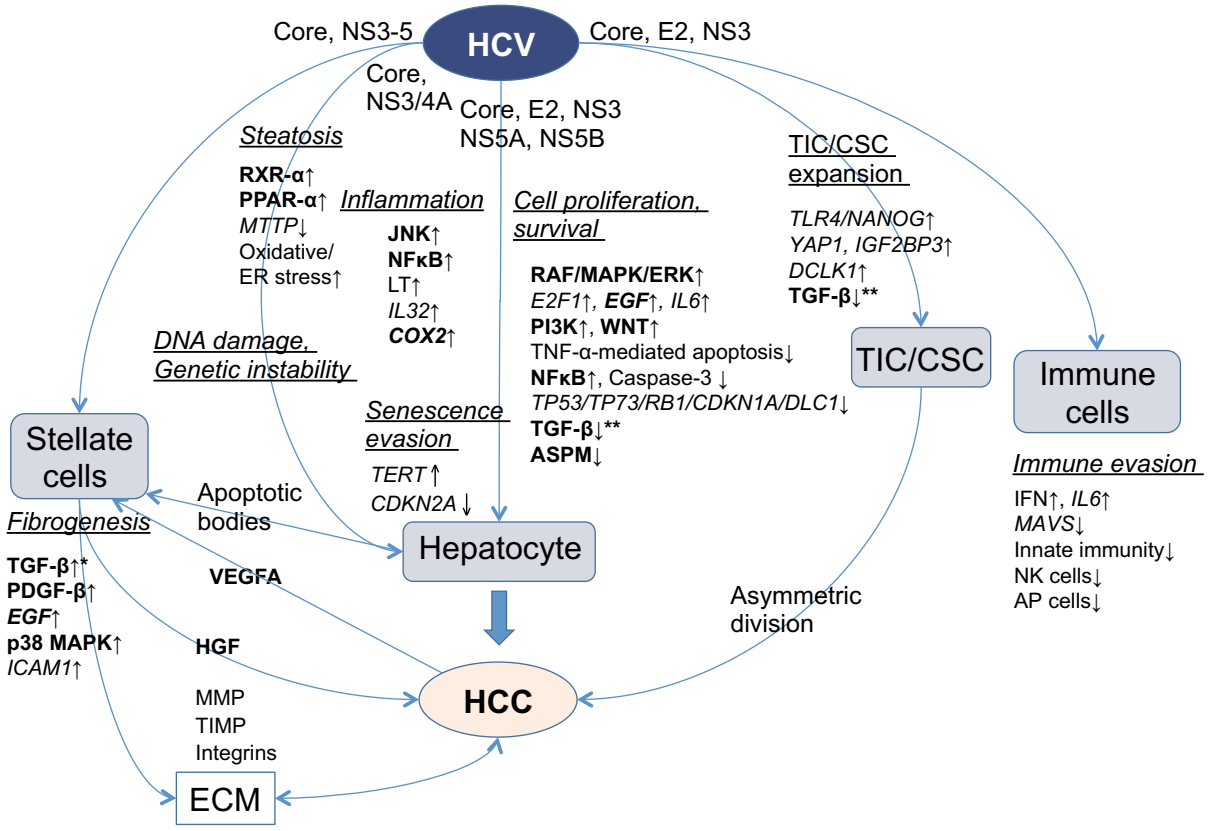

Fig. 2. Interactions of HCV with cellular components in cirrhotic tissue microenvironment that promote hepatocarcinogenesis. Potential HCC chemoprevention targets are extracted from the broader pathogenic involvement of HCV in the development of hepatitis, fibrosis, and cirrhosis. HCV proteins directly or indirectly promote cellular proliferation and survival, induce inflammation, metabolic pathway deregulation, leading to steatosis, oxidative stress, endoplasmic reticulum (ER) stress, DNA damage and genetic instability, expansion of tumour-initiating cell (TIC)/cancer stem cell (CSC) fibrogenesis by activating hepatic stellate cells, and attenuate immune cell response, leading to immune evasion. Genes/proteins and pathways for which pharmacological interventions have been clinically evaluated (not limited to liver diseases) are highlighted in bold. HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ECM, extracellular matrix.

by pro-inflammatory signals such as reactive oxygen species (ROS), generates an inflammatory hepatic microenvironment that supports HCC development [54]. NS5A activates the JNK pathway through interaction with the TNF receptor-associated factor 2 (*TRAF2*) [55]. A JNK inhibitor, SP600125, suppressed HCC development in diethylnitrosamine-treated rats [56]. Selective inhibition of cyclooxygenase-2 (COX2) prevents HCC in an experimental animal model [57]. Liver-specific expression of lymphotoxin (LT)-alpha and beta in mice caused hepatic inflammation and HCC, which was suppressed by inhibition of the LT-beta receptor [58].

Viral proteins also appear to subvert innate immune pathways. NS3 suppresses innate immunity by cleavage of the mitochondrial antiviral signalling protein (*MAVS*) responsible for induction of type-I interferon [59]. The inhibition of natural killer cells by E2 may contribute to immune evasion and establishment of chronic infection [60]. Interleukin-6 (*IL6*) is a multifunctional cytokine involved in oestrogen-regulated liver carcinogenesis [61]. Extracellular HCV core protein was suggested to impair antigen-presenting cells via the IL-6 pathway [62].

Metabolic pathways

Clinically, HCV-related HCC is often accompanied by steatosis within the tumours and non-tumourous liver, suggesting modulation of metabolic pathways [63]. HCV core protein co-localizes with apolipoprotein A2 on the surface of triglyceride containing lipid droplets *in vitro* and *in vivo*, suggesting its association with lipid metabolism [64]. Transgenic mice that express core protein develop progressive steatosis in the liver and then HCC [23]. Insulin resistance is another feature of the HCV core transgenic mice, which results in lipid accumulation in the liver [65]. HCV core protein suppresses microsomal triglyceride transfer protein (*MTTP*) activity and interferes with hepatic assembly and secretion of triglyceride-rich very low density lipoproteins (VLDL), further contributing to steatosis [66]. HCV core protein interacts with RXR-alpha and peroxisome proliferator-activated receptor-alpha (PPAR-alpha), and modulates cell differentiation, proliferation and fatty acid transport and catabolism in mice [67]. PPAR-alpha generally ameliorates steatosis, but in the presence of HCV core-induced mitochondrial dysfunction, it exacerbates steatosis, induces oxidative stress, and increases cell growth signals [68].

Cellular senescence

Hepatocyte proliferation is generally decreased at the stage of cirrhosis after many rounds of regeneration accompanied by telomere shortening that triggers cellular senescence though *ATM*, *TP53*, and *CDKN1A* as a safeguard to prevent carcinogenesis [69]. Activating somatic mutations in the telomerase reverse-transcriptase (*TERT*) promoter is a frequent early neoplastic event

in HCC with mixed aetiologies including HCV [70]. HCV core protein overcomes stress-induced hepatocyte senescence by downregulating *CDKN2A* expression via DNA methylation [71]. Senescence of hepatic stellate cells has also been shown to limit liver fibrosis [72]. HCV does not infect stellate cells but could have an indirect role in this process.

Fibrogenic pathways

Irrespective of the aetiology, established cirrhosis serves as a milieu/microenvironment that fosters initiation and promotion of neoplastic clones by facilitating genetic aberrations and cellular transformation, which is often referred to as "field cancerization" or "field effect" [73]. Liver fibrosis is an excessive wound healing response to chronic liver injury that results in increased production and deposition of extracellular matrix (ECM). Dynamic balancing between fibrogenesis and fibrolysis determines liver fibrosis as a result of a complex interplay between various cell types in the liver, including hepatic stellate cells, Kupffer cells, hepatocytes, cholangiocytes, sinusoidal endothelial cells, and infiltrating immune cells. Severity of liver fibrosis is tightly correlated with an increasing risk of HCC in patients with chronic HCV infection, suggesting that cirrhosis-driven carcinogenesis is the major mechanism in the development of HCV-related HCC [6,74]. Although sustained virological response (SVR) from HCV improves histological fibrosis, a subset of patients is still at risk of fibrosis progression and HCC development [75], indicating the necessity of anti-fibrotic therapies to prevent HCC [76].

Activation of hepatic stellate cells, or myofibroblasts, is the major driver of liver fibrogenesis [77]. HCV broadly infects hepatocytes, monocytes, lymphocytes and other secretory cells, and contributes to stellate cell activation. HCV core and non-structural proteins stimulate profibrogenic mediators such as TGF-beta [78]. HCV infection induces *TGFB1* through ROS production, p38 MAPK, JNK, ERK, and NF-κB pathways [79], although concerns regarding toxicities have been raised about targeting the TGF-beta pathway exclusively [80]. Platelet-derived growth factor (PDGF) is the most potent mitogenic signal, inducing expression of beta PDGF receptor expression in stellate cells together with other cell surface receptors of growth signalling such as integrins [81]. Transgenic mice, expressing PDGF-C, develop liver fibrosis and HCC [82], and the acyclic retinoid, peretinoin, represses fibrosis and HCC development in the model [83].

In cell culture models, HCV non-structural proteins and, to a lesser extent, core protein stimulate production of pro-inflammatory chemokines such as IL-8, MCP-1, and RANTES and induce expression of ICAM-1, a cell adhesion molecule known to activate T cells [78]. JNK-pathway activation by the pro-inflammatory cytokine IL1-beta can shift TGF-beta signalling from tumour suppression to oncogenesis through accelerated fibrogenesis [84,85]. *IL32* expression in hepatocytes is associated with hepatic inflammation and fibrosis in HCV infection [86]. Hepatocyte death serves as a stimuli activating stellate cells [87], and is a potential therapeutic target in chronic hepatitis C [88]. Apoptotic bodies with HCV infection could amplify fibrogenic signals [89]. Bacterial lipopolysaccharide (LPS), permeabilized from intestinal microbiota, elicits fibrogenic response and carcinogenesis through the Toll-like receptor 4 (TLR4), expressed on stellate cells, by inducing TGF-beta, which can be prevented by gut sterilization [90]. Multiple variants in *TLR4* modulate the risk of fibrosis in HCV-infected Caucasian patients [91]. Matrix metalloproteinase-2 (*MMP2*), a major ECM-degrading enzyme, is induced by interaction of E2 with CD81, a member of the receptor complex for HCV cellular internalization, which may exacerbate inflammatory infiltration and parenchymal damage [92].

Adipokines, including leptin, adiponectin, and resistin are implicated in liver fibrogenesis in hepatitis C and NAFLD [93]. Suppression of the heat shock protein 47 (*HSP47*), a collagen-specific chaperon, by siRNA in stellate cells reduced fibrosis in rodent models of fibrosis, and is now under early clinical evaluation [94]. Renin-angiotensin system (RAS) is suggested to be involved in hepatocarcinogenesis [95]. Inhibition of angiotensin-II (AT-II) by angiotensin-converting enzyme inhibitor (ACE-I) downregulates angiogenic factors such as VEGF, and ACE-I administration, combined with branched-chain amino acids (BCAA), has been shown to attenuate insulin resistance-related hepatocarcinogenesis in a diabetic rat model [96].

However, it is important to note that most of these findings have been derived from experimental cell culture or animal models, overexpressing individual proteins. Since a robust infectious small animal model, recapitulating the virus-induced carcinogenesis, is not yet available [11], the functional relevance of these observations for hepatocarcinogenesis in humans is still unclear and needs to be confirmed. The development of immunocompetent animal models, fully recapitulating the viral life cycle and virus-induced liver disease, in combination with studies in liver tissue from HCV-infected patients will ultimately be required to validate these findings and concepts. Also, ancillary assessment of HCC development as an additional end point in clinical trials of the anti-fibrotic agents may provide insight into their potential role as HCC chemoprevention therapies [97].

Host factors affecting susceptibility to HCV-related HCC

Growth signalling pathways

Kinase signalling pathways represent druggable/targetable molecular pathways that have been extensively studied. In HCV-related HCC, genome-wide profiling of genomic DNA variants as well as RNA transcripts has identified several candidate genes and pathways. Epidermal growth factor (*EGF*) is a mitogen involved in cellular growth, proliferation, differentiation, and carcinogenesis. In rodent models of cirrhosis-driven HCC, the EGF pathway was activated in hepatic stellate cells, and pharmacological inhibition with a small molecule EGF receptor (*EGFR*) inhibitor, erlotinib, regressed fibrosis and inhibited HCC development [98]. Interestingly, there was no inhibition of the EGF pathway in the tumours, suggesting that the HCC preventive effect was through regression of the cirrhotic tissue microenvironment that supports initiation of neoplastic clones. In contrast, another small molecule EGFR inhibitor, gefitinib, suppressed growth of initiated HCC clones in rats [99]. EGFR was recently identified as a co-factor for HCV cellular entry, and erlotinib inhibited HCV infection, suggesting its role as anti-HCV drug [100,101]. The role of the EGF pathway in HCV-related liver diseases might be complicated though because HCV infection induces the expression of other EGFR ligands such as amphiregulin (*AREG*) and heparin-binding EGF-like growth factor (*HBEGF*), and while AREG enhances liver fibrosis, HB-EGF suppresses liver fibrosis [102–105]. A multi-kinase inhibitor, sorafenib, improved portal hypertension in cirrhosis patients, supposedly due to its anti-angiogenic activity [106]. Sorafenib showed its anti-HCC effect by blocking paracrine hepatocyte growth factor (*HGF*) from stromal cells in response to vascular endothelial growth factor-A

(*VEGFA*) secreted from HCC cells [107]. Selective inhibitors of these growth signalling pathways have been clinically evaluated mostly as cancer therapeutics. There may be opportunities to repurpose this class of drugs for HCC chemoprevention if the toxicity concern is satisfactorily addressed.

Immune pathways

An *IL28B* variant (rs12979860), initially identified as an interferon response predictor [108], may be associated with increased risk of HCV-related HCC [109]. Interferon effector genes (IEGs) such as *BCHE* were identified through high-throughput RNAi screening [110]. A genome-wide association study (GWAS) comparing HCV-related HCC patients with chronic hepatitis C patients in Japan identified a SNP in the MHC class I polypeptide-related sequence A (*MICA*) (rs2596542), which is involved in response of dendritic cells to type-I interferon in chronic hepatitis C [111,112]. Another SNP in the *MICA* promoter (rs2596538) was associated with increased serum soluble MICA protein [113]. Because the controls are patients without cirrhosis, it is possible that the variants indirectly contribute to carcinogenesis through increased inflammation and/or fibrogenesis [114]. A subsequent study in Caucasian hepatitis C patients in Switzerland did not replicate the association with HCC for this locus, but for a nearby locus in *HCP5* (rs2244546), suggesting that the *MICA*/*HCP5* region contains a potential susceptibility locus [115]. An additional GWAS-identified locus in another Japanese patient series is in *DEPDC5* (rs1012068) [116], which was not replicated in the Caucasian patients [115].

Metabolic pathways

A SNP in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene (rs738409) associated with alcoholic and non-alcoholic steatohepatitis may have weak association with HCV-related HCC [117]. In patients with chronic hepatitis C with advanced fibrosis, positive association between liver iron deposition and higher incidence of HCC and poor prognosis was observed [118]. Hepatic iron overload was associated with elevated levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), which signifies hepatic oxidative DNA damage in patients with chronic hepatitis C [119]. With an excess iron diet, transgenic mice expressing HCV polyprotein developed hepatic steatosis, ultrastructural alterations of mitochondria, and HCC, accompanied with elevated levels of hepatic 8-OHdG [120]. *HFE* gene mutations, in particular H63D, were associated with increased SVR [121].

microRNAs

microRNAs (miRNAs) are small non-coding RNA that negatively regulate gene expression by binding to complementary sites within the 3′UTR of multiple target protein-coding mRNAs. miRNA expression profiling of HCV-related HCC tissues revealed deregulated miRNAs including *MIR122*, *MIR100*, *MIR10A*, *MIR198*, *MIR145*, and *MIR517A* as well as distinct expression patterns compared to HBV-related HCC [122–124]. *MIR122* is a liver-specific miRNA that promotes replication of HCV [125]. In contrast, *MIR122* is under-expressed in HCC and associated with a more aggressive biological phenotype, including overexpression of alpha-fetoprotein [126]. Therapeutic delivery of *MIR122* inhibits MYC-driven mouse HCC [127]. Infection of HCV genotypes 1a, 1b, and 2a in primary human hepatocytes revealed that *MIR141* targets a tumour suppressor gene *DLC1* [128].

Prevention of HCV-induced HCC

It has been noted that early detection and prevention is the most effective and rational approach to substantially impact the prognosis of cancer patients rather than starting the treatment at advanced/terminal stage [129]. However, development of cancer prevention therapies is more challenging compared to cancer therapeutics, due to the requirement for larger and longer clinical trials because of the lower incidence of clinical events. In addition, a safer toxicity profile is required as preventive medicine, administered to asymptomatic, cancer-free patients potentially for long durations. HCV-related HCC is one of the most rational targets for cancer preventive intervention because of the well-established risk factor, HCV infection and cirrhosis, which in fact enabled conduction of cancer chemoprevention trials with significantly smaller sample size compared to other cancer types [130–133]. Although the trials failed to demonstrate a satisfactory effect and toxicity profile as a standard of care, the HCC preventive effect in patients with established or more advanced cirrhosis provides the proof of concept of HCC chemoprevention as a valid option for further exploration.

Molecular biomarkers of HCC risk in HCV-related cirrhosis

Molecular biomarkers of HCC risk and/or poor prognosis will enable further enrichment of the high-risk population and boost statistical power in HCC chemoprevention trials [134]. HCC risk biomarkers will also significantly contribute to the improvement of early HCC detection. The current practice guidelines recommend regular tumour surveillance with biannual ultrasound to increase the opportunity to identify lesions at a stage where potentially curative radical therapies can be applied [135]. However, the sizable cirrhosis population poses a challenge in implementing the surveillance program: only 12% of new HCV-related HCC patients are diagnosed through the surveillance in the U.S. [136] Growing numbers of early-stage, asymptomatic cirrhotics identified by non-invasive fibrosis detection methods such as elastography will also add to the HCC screening burden [137]. Clinical variable-based prediction models for HCC development have been explored, although their performance is limited and none of them has been established in practice [138,139].

Numerous germline SNPs have been reported as HCC risk variants, although very few of them are replicated in independent patient series/cohorts [140]. The *EGF* 61*G allele (rs4444903) was associated with HCC risk in a prospective cohort of patients with HCV-related advanced fibrosis or cirrhosis with a hazard ratio (HR) of 2.10 for the G/G genotype in comparison to A/A (Table 1) [141,142]. Despite diverse allele frequencies across patient populations, association between the *EGF* genotype and HCC risk remains significant and independent of patient race [143]. A SNP in an antioxidant enzyme, myeloperoxidase, (*MPO* −463*G, rs2333227) was associated with HCC risk in a prospective study (HR = 2.80) [144]. A panel of 7 SNPs (cirrhosis risk score) was shown to be associated with the risk of fibrosis progression in male Caucasian patients with chronic hepatitis C, although association with long-term outcomes, including HCC, is yet to be determined [145]. A 186-gene-expression signature was associated with HCC risk in prospectively followed patients with early-stage HCV-related cirrhosis (HR = 2.65) [146]. Annual HCC incidence in patients with poor-prognosis signature (5.8%) was nearly 4 times higher than the incidence in patients with

Table 1. Molecular biomarkers of HCV-related HCC risk.

Molecular biomarker	Type	No. of patients	HR	Race/ethnicity	[Ref.]
EGF 61*G (rs4444903)	SNP	816	2.10	White, Hispanic, Black, Asian	[142]
MPO -463*G (rs2333227)	SNP	205	2.80	White	[144]
CAT -262*C (rs1001179)	SNP	205	1.74	White	[144]
186-gene poor prognosis signature	Gene expression	216	2.65	White, Hispanic, Black, Asian	[146]

Molecular biomarkers demonstrating HR >1.50 in independent prospective or prospective-retrospective cohort (n >100) are shown.
rs numbers indicate accession numbers in the NCBI dbSNP database (www.ncbi.nlm.nih.gov/snp).
HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HR, hazard ratio.

good-prognosis signature (1.5%). The signature reflects activation of NF-κB, IL6, EGF, and interferon pathways, suppression of DNA damage repair genes such as *GSNOR*, and hepatic stellate cell activation. In rodent models of cirrhosis-driven HCC, the signature was induced from the inception of liver fibrosis, reversed in response to an EGFR inhibitor, erlotinib, and accompanied its HCC chemopreventive effect, suggesting its role as a pharmacogenomic companion biomarker [98]. With the recent emergence of highly selective molecular targeted agents, the tissue-based assessment of predictive biomarker for response is now recommended in practice guidelines [135]. Circulating cells or biomolecules such as miRNAs may be alternative sources to obtain similar molecular information less invasively [147]. In addition, molecular imaging of collagen could potentially be used to monitor fibrosis regression, which may correlate with decreased HCC risk [148].

Strategies to prevent HCV-related HCC

Primary prevention, i.e., HCV vaccination, is currently unavailable due to the high variability in the viral genomic structure and envelope proteins, the large number of quasispecies, and the lack of a neutralizing antibody. Secondary prevention aims at preventing HCC development in established HCV-related advanced fibrosis or cirrhosis. To date, several relatively large phase 3 trials have been conducted, which demonstrated limited efficacy and utility of the tested therapies [130–132]. Tertiary prevention targets recurrence of *de novo* second primary HCC after curative treatment of initial primary HCC, but available evidence is still scarce [149–151]. Theoretically, secondary and tertiary prevention could be achieved by anti-HCV therapies and/or non-aetiology-specific therapies, targeting inflammation, fibrogenesis, and/or carcinogenesis, which have been extensively studied in the past decades. However, there are still several undetermined study design issues, including appropriate sample size, study duration, and elusive primary and surrogate study end points according to the preventive strategies. These points need to be clarified to streamline and facilitate design and conduction of HCC chemoprevention trials in HCV cirrhosis. Targeted disease stage/severity, e.g., compensated or decompensated cirrhosis, should be specified in the inclusion criteria especially in secondary prevention trials because of the distinct difference in expected outcome. Enrichment of high-risk patients with the use of HCC risk biomarkers and/or prognostic indices is critical to boost HCC incidence and keep the required sample size and study duration within practically feasible range. Testing candidate chemoprevention therapies in the setting of tertiary instead of secondary prevention could be a way to further boost HCC incidence because post-surgical recurrence is approximately three times more frequent compared to the first HCC in cirrhosis, although diagnosis of *de novo* HCC recurrence should be unambiguously determined based on explicit criteria [152]. Also, a consensus needs to be developed on acceptable toxicities in the context of preventive intervention in patients with advanced fibrosis or cirrhosis.

Anti-HCV therapies

Recent clinical trials have reported SVR rates greater than 90% with the use of DAA-based interferon-free regimens even in patients with cirrhosis [153,154]. Interferon-based therapies have shown that SVR is consistently associated with gradual regression of fibrosis and lower risk of HCC in retrospective studies [9,155]. However, the clinical utility of achieving SVR with the use of anti-HCV therapies in the context of HCC prevention needs to be clarified especially in patients with comorbid conditions, e.g., decompensated cirrhosis and older age, in future studies. It also needs to be determined whether DAAs have any role in tertiary prevention. Nevertheless, the cost of DAAs could be prohibitive in their use as preventive drugs. Also, because the patients are still at risk of HCC even after SVR, additional measures of secondary/tertiary prevention are needed. In liver transplantation for HCV-related HCC, HCV reinfection in grafted liver could lead to progressive fibrosis and *de novo* HCC, which may be prevented by inhibition of HCV entry [100].

Non-aetiology-specific HCC chemoprevention

Anti-inflammatory, immune therapies

Suppression of hepatic inflammation could delay disease progression and reduce HCC risk; biochemical response, i.e., normalization of liver enzymes, such as alanine aminotransferase (ALT), achieved by either glycyrrhizin or ursodeoxycholic acid (UDCA), have been suggested to reduce HCC risk [4]. Interferon has been extensively evaluated as a chemopreventive agent in HCV-related HCC. In two relatively large randomized trials of maintenance low-dose interferon, HCC risk was modestly reduced in patients with more advanced fibrosis/cirrhosis (HALT-C trial), and the composite of first liver-related clinical events was reduced in patients with portal hypertension (EPIC3 trial) in *post hoc* subgroup analyses [131,156]. However, the modest effects and poor tolerability (nearly 40% drop out and excess mortality in HALT-C trial) of Peg-interferon preclude its wide application as standard of care. The HCC suppressive effect in these studies was not evident during the first two to three years of treatment, which may reflect a latent period for newly initiated cancer clones to be clinically detected. Interferon has also been also assessed as tertiary prevention in retrospective and prospective studies, which consistently showed a trend of reducing post-treatment recurrence or death [4]. Immunosuppression after liver transplantation with sirolimus, an mTOR inhibitor, reduced HCC recurrence and

Table 2. Ongoing HCC chemoprevention trails relevant to HCV-related HCC.

Trial number	Agent	Type of agent	Phase	Type of prevention	Participants	Completion
NCT00513461	S-adenosylmethionine (SAMe)	Dietary supplement	2	Secondary	Advanced chronic hepatitis C	Dec 2013
NCT01956864	High-dose vitamin D	Dietary supplement	1	Secondary	Cirrhosis without HCC	Sep 2014
MAY2013-02-02	Erlotinib	Kinase (EGFR) inhibitor	1	Secondary/ tertiary	HCC after resection	2015-2016
NCT00355862	Sirolimus (SiLVER trial)	Immune modulator	3	Tertiary	HCC after transplantation	May 2014
NCT01924624*	Thalidomide	Immune modulator, anti-angiogenesis	n.a.	Tertiary	HCC after resection	Dec 2019
NCT01717066*	Ginsenoside Rg3	Chemo-sensitizer, anti-angiogenesis	n.a.	Tertiary	HCC after resection	May 2015
NCT01770431*	Huaier Granule	Traditional herbal medicine	4	Tertiary	HCC after resection	Dec 2014
NCT01964001*	Vitamin B6, Coenzyme Q10	Dietary supplement	2/3	Tertiary	HCC after resection	Dec 2015

*Likely to enrol mainly hepatitis B virus-infected patients.
From www.ClinicalTrials.gov and cancerpreventionnetwork.org accessed May 2014. Verified trials after 2012 are shown.
HCC, hepatocellular carcinoma; HCV, hepatitis C virus; EGFR, epidermal growth factor; NCT, National Clinical Trial number; MAY, Cancer Prevention Network protocol number; n.a, not available.

improved survival [157]. The result of an ongoing multicentre trial of sirolimus (SiLVER study) is anticipated (Table 2). Aspirin may elicit cancer preventive effects through inhibition of cyclooxygenase-2 (*COX2*), although there are conflicting data about the HCC chemopreventive effect with COX-2 inhibition [158].

Treatment of metabolic disorders, dietary supplements

Statins, HMG-CoA reductase inhibitors, have been suggested to have an anti-proliferative effect through inhibition of RAS/MAPK and cell cycle pathways and a pro-apoptotic effect. Observational studies suggest an HCC preventive effect by statins, which is not yet verified in a clinical trial [159]. Diabetes is associated with prognosis in HCV-related cirrhosis, and an anti-diabetic drug, metformin, inhibits the mTOR pathway by activating AMPK, may reduce HCC risk and improve survival [160]. Coffee and green tea polyphenol, epigallocatechin gallate (EGCG), show a modest HCC preventive effect, supposedly by activating anti-oxidant and detoxification pathways in experimental and epidemiological studies [4,161]. EGCG is also reported to inhibit HCV entry [162]. S-adenosylmethionine (SAMe), a major methyl donor, inhibiting hepatocyte growth factor (*HGF*), is being tested in HCV-related HCC for AFP reduction in a phase 2 trial (Table 2). Other phytochemicals such as curcumin, resveratrol, silymarin, and genistein showed HCC preventive effects in animal models, but clinical evidence in HCV-infected patients is limited [4]. The HCC preventive effect of this type of drugs is generally expected to be modest. Therefore, enrichment of high-risk patients as well as utilization of epidemiological data/resources will be critical in determining their clinical utility.

Molecular targeted agents

Given the rapidly expanding inventory of selective molecular targeted agents, newly synthesized or identified through high-throughput screening, molecular targeted cancer chemoprevention is now an increasingly feasible option. An acyclic retinoid, peretinoin, was tested in a large-scale phase 3 trial, enrolling HCV-related cirrhosis patients, which showed modest HCC preventive effect [132]. Interestingly, an HCC reduction was observed after 2 years of enrolment as seen in the previous interferon trials. The multi-kinase inhibitor, sorafenib, was tested in the setting of tertiary prevention, although no clear HCC preventive effect was observed (Table 2). It is assumed that the "all-comer" approach without biomarker-based enrichment is the major basis for failure [163]. Nevertheless, a *post hoc* exploration of predictive biomarkers is currently underway. An *EGFR* inhibitor, erlotinib, is being tested in a phase 1 trial, in which the 186-gene signature is assessed as a companion biomarker [151]. A clinical trial of another EGFR inhibitor, gefitinib, is also registered.

New chemopreventive targets in HCV-related HCC

Genome-wide profiling of various biomolecules and high-throughput drug screens have facilitated unbiased, large-scale surveys of new molecular targets and therapeutics [164]. *In vivo* high-throughput RNAi screening will be another powerful tool to identify functional targets [165]. Recent advancement in the *in vitro* and *in vivo* modelling of HCV infection has allowed more physiological and functional assessment of the HCV-host interactions and viral life cycle and has allowed to identify and verify candidate target genes and pathways [13]. Transcriptome signatures have been successfully utilized to identify new drugs or indications, i.e., drug repurposing, in a variety of diseases [166]. Regulatory transcriptome network analysis could be a complementary approach in identifying key driver genes in hepatocarcinogenesis [167]. Genome-scale mathematical metabolic modelling of hepatocytes led to the identification of serine deficiency as a potential target in non-alcoholic steatohepatitis-related HCC [168]. This may suggest a potential utility for the construction of a model of the HCV-infected hepatocyte to explore HCC chemoprevention targets.

Conclusions

HCV-related HCC will remain a major health problem in the coming decades. Although prevention of HCV-induced HCC is not yet established, direct and indirect oncogenic roles of HCV and candidate targets genes and molecular pathways have been suggested

in experimental and clinical studies. Integration of genome-wide association studies, high-throughput and unbiased target/drug screens against libraries of RNAi/selective targeted agents, and more physiological HCV infection and liver disease models are expected to facilitate the development of molecularly targeted HCC chemoprevention, which may be widely applicable to cirrhosis-driven HCC, caused by other aetiologies, as well as inflammation-driven cancer in other organs, such as gastric, cervical, and colon cancers. Clinical assessment of antiviral, anti-inflammatory, and anti-fibrosis drugs in the context of HCC chemoprevention will be a challenge. Molecular biomarkers that could be used to select target patients and/or predict response will be the key in designing clinically feasible trials of HCC chemoprevention therapies.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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