REVIEW

Hepatitis C Virus Infection and Cholangiocarcinoma

An Insight into Epidemiologic Evidences and Hypothetical Mechanisms of Oncogenesis

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Hepatitis C virus (HCV) infection is a global public health problem because it is a main cause of liver cirrhosis and hepatocellular carcinoma. This human oncogenic virus is also associated with the development of non-Hodgkin lymphoma and cholangiocarcinoma (CCA). The association between HCV infection and CCA has been examined in a number of epidemiologic studies. However, *in vivo* and *in vitro* results demonstrating the oncogenic mechanisms of HCV in CCA development and progression are insufficient. Here, we review the epidemiologic association of HCV and CCA and recent publications of studies of HCV infection of cholangiocytes and CCA cell lines as well as studies of viral infection performed with liver samples obtained from patients. In addition, we also discuss the preliminary results of *in vitro* assays of HCV protein expression in CCA cell lines. Finally, we discuss the hypothetical role of HCV infection in CCA development by induction of epithelial–mesenchymal transition and up-regulation of hedgehog signaling, and consequently biliary tree inflammation and liver fibrosis. Further studies are required to demonstrate these hypotheses and therefore to elucidate the mechanisms of HCV as a risk factor for CCA. (*Am J Pathol* 2019, 189: 1122–1132; <https://doi.org/10.1016/j.ajpath.2019.01.018>)

Hepatitis C Virus (HCV) is remarkably efficient in the establishment of persistent infection. More than 60% of infected individuals experience chronic hepatitis C that is a main cause of liver cirrhosis and primary liver cancer.¹ Globally, 30% of hepatocellular carcinoma (HCC) cases, the most important primary liver cancer, are associated with persistent HCV infection, representing a significant public health problem.²

The HCV oncoproteins and the molecular mechanisms of carcinogenesis are well identified in the development of HCC associated with HCV infection as well as the role of chronic liver inflammation.³ One of the HCV oncoproteins is Core (21 kDa), the subunit of the viral capsid. This

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structural protein also has regulatory functions in processes such as cell growth, apoptosis, oxidative stress, senescence, mitochondrial functions, innate immune response, steatosis, signal transduction, and, in particular, cell transformation.⁴

This human oncogenic virus is also associated with the development of B-cell non-Hodgkin lymphoma, and recent epidemiologic studies have reported a link between HCV infection and cholangiocarcinoma (CCA).^{5,6}

CCA is a biliary epithelial tumor that represents approximately 15% of primary liver cancer cases.⁷ This neoplasia was classified in two categories: intrahepatic and extrahepatic (ECC), accounting for 20% and 80% of CCA, respectively.^{8–10} However, considering the heterogeneity of biliary tumors a new classification of CCA has been proposed: intrahepatic (iCCA), perihilar, and distal biliary tree. In addition, these tumors differ in their epidemiologic pattern, prognosis, pathogenesis, clinical management, and treatment.^{11–13}

Epidemiology

The incidence of iCCA has increased in the past decades in different regions such as Western and Central Europe, North America, Asia, and Oceania, whereas the incidence of ECC has not changed during the past decade.^{14,15} Indeed, the estimated incidence of iCCA increased to 128% between 1973 and 2012 (0.44 to 1.18 cases/100,000) in the United States, whereas the rank of ECC incidence was between 0.96 and 1.02 cases/100,000 during the same period.¹⁶ Moreover, the trends in mortality from iCCA showed a significant increase in mortality in some countries such as Australia, Canada, France, Germany, Japan, Ireland, Sweden, Portugal, Slovenia, and Spain.¹⁵ The increased incidence of iCCA could be associated with the epidemiologic pattern of chronic liver diseases such as viral hepatitis.¹⁷ However, the quality of cancer registry in some countries as well as differences in resources available for diagnosis, classification, and treatment of CCA have to be considered to establish the trends on incidence of this biliary epithelial tumor.^{7,13}

Classification and Histologic Subtypes

iCCA is a tumor developed by the transformation of the epithelial cells lining small and large intrahepatic bile ducts, the cholangiocytes. Two histologic subtypes of iCCA have been characterized, mucinous (bile duct type) and mixed (bile ductular type). The mucinous iCCA appears in large bile ducts that are characterized by mucin-producing cylindrical cells, similar to the perihilar extrahepatic bile duct. Mucinous iCCA and perihilar CCA have similar clinical, pathologic, and molecular profiles,¹⁸ whereas mixed iCCA arises from small intrahepatic bile ducts, such as interlobular bile ducts, and ductules that are characterized by mucin-negative cuboidal cholangiocytes.^{11,19,20}

Cell of Origin of CCA

One of the characteristics of the liver is the self-renewal of both cholangiocytes and hepatocytes.²¹ During tissue homeostasis, the self-renewal is limited, but in the context of a chronic liver disease, hepatocytes and cholangiocytes can divide largely to replace the damaged cells as a consequence of liver injury. Moreover, hepatic stem cells located at the portal region could be activated, generating oval cells that have qualities of both liver epithelial cells and then could be differentiated into mature hepatocytes. Interestingly, the ductular plasticity of mature hepatocytes has been found in chronic liver injury. Indeed, ductal epithelium could be derived from hepatocytes that exhibit the characteristics of oval cells.²²

The hepatic progenitor cell could be the cell of origin of iCCA by transformation of cholangiocytes and/or hepatocytes during their differentiation.^{14,23} The tumors derived from hepatic progenitor cells (mixed iCCA and cholangiocellular carcinoma) can present different histologic characteristics with focal hepatocyte differentiation and/or ductular areas and diverse clinicopathologic features while they display similar gene expression profiles.¹⁸

As described before, the ductular plasticity of mature hepatocytes is a characteristic that could be important in oncogenesis. Indeed, normal human hepatocytes have been recently proposed as another cell type of origin for iCCA.²² The transdifferentiation and conversion of normal hepatocytes into cholangiocyte precursors was demonstrated in a mouse model by overexpression of Notch1 and thymoma viral proto-oncogene (AKT). Moreover, the Notch-mediated conversion of normal hepatocytes into cholangiocytes preceded the development of iCCA and macronodular cirrhosis in the mouse model.^{24,25}

Risk Factors

Although not identified in all cases, many risk factors for CCA development, whether infectious or otherwise, indicate a common pathogenesis pathway, the development of chronic biliary inflammation.^{11,26,27}

Liver infection by flukes *Clonorchis sinensis* and *Opisthorchis viverrini* is an important risk factor in Southeast Asia, particularly in Thailand, where CCA is the main primary liver cancer. According to the International Agency for Research on Cancer, infection with *C. sinensis* or *O. viverrini* with subsequent development of chronic bile duct inflammation is recognized as carcinogen type I and is probably carcinogenic to humans, respectively.²⁸ Primary sclerosing cholangitis has been established as a risk factor for CCA in developed countries, especially in Europe. The lifetime incidence of CCA in patients, predominantly men, with primary sclerosing cholangitis is 5% to 10%. This disease is characterized by chronic inflammation of bile ducts and consequently biliary strictures that could evolve to cirrhosis and the development of CCA, mainly mucinous iCCA.^{26,29,30}

Table 1 The Pathway to Demonstrate the Association of HCV Infection and Cholangiocarcinoma

Study	Characteristics of the study	Results	Reference
Epidemiologic link between markers of viral infection and the risk of cancer			
Retrospective	United States, population-based cancer registries, 535 patients ≥ 65 years with diagnosis of iCCA	HCV infection was associated with iCCA (OR = 4.4; 95% CI, 1.4–14).	27
Cohort study	Japan, 1980–1997, 600 patients with HCV infection, median follow-up 7.2 years	1.8% patients experienced cholangiocellular carcinoma, 0.5% type of hepatocellular and cholangiocellular carcinoma, and 34.3% HCC	32
Case–control	Japan, 50 patients with diagnosis of iCCA and 205 matched controls	HCV infection was associated with risk of iCCA (OR = 6.02; 95% CI, 1.51–24.1)	33
Retrospective	Cambodia, 2003–2015, 553 cases of primary liver cancer, including 42 cases of CCA	HCV infection in 11.5% of CCA cases	34
Retrospective	United States, 1995–2007, 244 liver explants, patients with cirrhosis, and controls	HCV infection is a risk factor for a precursor lesion, the biliary intraepithelial neoplasia	35
Meta-analysis	13 case–control studies and three cohort studies; Thailand, Republic of Korea, China, Taiwan, Italy, United States, and Japan	HCV infection was associated with risk of iCCA (OR = 3.42; 95% CI, 1.96–5.99)	36
Meta-analysis	16 case–control studies; Thailand, Republic of Korea, China, Taiwan, Italy, United States, and Japan	HCV infection was associated with risk of iCCA (OR = 3.42; 95% CI, 1.96–5.99)	37
Meta-analysis	Eight case–control studies. Japan, United States, Italy, Republic of Korea, and China	HCV infection was associated with risk of iCCA (OR = 4.84; 95% CI, 2.41–9.71)	38
Retrospective	United States, Medicare, 1993–2011, 1,623,538 cases of cancer and 200,000 controls	HCV infection was associated with risk of intrahepatic bile duct cancer (OR = 3.40; 95% CI, 2.52–4.58) and extrahepatic bile duct cancer (OR = 1.90; 95% CI, 1.41–2.57)	39
Case–control	China, 2004–2006, 312 cases of iCCA and 438 controls	HCV infection was not associated with risk of iCCA (OR = 0.933; 95% CI, 0.281–3.1)	40
Case–control	Republic of Korea, 2000–2004, 622 cases of iCCA and 2488 controls	HCV infection was not associated with risk of iCCA (OR = 1; 95% CI, 0.5–1.9)	41
Case–control	Republic of Korea, 1990–1993, 41 cases of CCA, 203 cases of HCC, and 406 controls	HCV infection was not associated with risk of CCA	42
Case–control	Republic of Korea, 2003–2004, 185 cases of CCA and 185 controls	HCV infection was not associated with risk of iCCA (OR = 1; 95% CI, 0.04–25.26)	43
Detection of viral sequence in tumor samples			
Descriptive	Bile duct epithelium samples obtained from seven cases of chronic hepatitis C (autopsy)	Detection of HCV genome in six bile duct epithelium samples obtained by laser capture microdissection	44
<i>In vitro</i>	HCV infection of primary cholangiocytes	Primary cholangiocytes are refractory to HCV infection	45
	HCV infection of CCA cell lines	Entry of HCVpp was demonstrated; however, scarce viral replication was detected in the Sk-ChA-1 cell line	
Descriptive	Liver biopsies obtained from 12 patients seropositive for anti-HCV and three controls	Detection of HCV genome in the bile and viral protein expression in epithelial cells of middle-sized bile ducts	46
Liver EMT modulation by HCV			
<i>In vitro</i>	Human primary hepatocytes infected with HCV	Induction of EMT in infected hepatocytes (Vimentin, S100A4, Snail, Slug, and Twist)	47

(table continues)

Table 1 (continued)

Study	Characteristics of the study	Results	Reference
Liver samples	From patients with HCV infection	Up-regulation of Snail and Twist in patients compared with controls	47
<i>In vitro</i>	Human CCA cell line transfected with HCV Core protein	Induction of EMT in infected QBC939 cells (Vimentin, Fibronectin) and enhanced ability of migration	48
<i>In vitro</i>	Nonmalignant human cholangiocytes cell line and human CCA cell line transfected with HCV Core protein (HCV genotype 2a)	Preliminary results: induction of EMT in infected H69 cells (α -SMA, S100A4) and in KMCH-1 cells (TGF- β 1)	49
<i>In vitro</i>	Overexpression of HCV Core protein in hepatocytes	Up-regulation of TGF- β in hepatocytes	50
Liver Hedgehog pathway activation by HCV			
<i>In vitro</i>	Nonmalignant human cholangiocyte cell line and human CCA cell line transfected with HCV Core protein (HCV genotype 2a)	Preliminary results: enhanced expression of <i>Gli2</i> , <i>Shh</i> , and <i>SMAD4</i> genes in H69 cells and KMCH-1 cells	49
Liver samples	From patients with HCV infection	Increased expression of Hh ligands (<i>Shh</i> , <i>Ihh</i>) and <i>Gli2</i> ; Hh signaling in epithelial cells of ductular reaction	51

α -SMA, α -smooth muscle actin; CCA, cholangiocarcinoma; EMT, epithelial–mesenchymal transition; *Gli2*, glioblastoma transcription factor 2; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVpp, hepatitis C virus pseudovirus particles; Hh, hedgehog; iCCA, intrahepatic cholangiocarcinoma; *Ihh*, Indian hedgehog; OR, odds ratio; *Shh*, sonic hedgehog; TGF, transforming growth factor.

Moreover, other biliary tract diseases such as congenital biliary tract malformations, hepatolithiasis, and cystic fibrosis have been associated with CCA risk.^{11,26,27} Other risk factors such as alcohol liver disease, tobacco smoking, and exposure to the radiopaque medium Thorotrast, 1,2-dichloropropane, and dichloromethane have also been associated.^{11,26,27} Furthermore, viral hepatitis has been proposed as a CCA risk factor, in particular mixed iCCA. Indeed, HCV infection is related to the incidence of both primary liver cancer, HCC, and CCA.^{11,14,30}

Epidemiologic Association of HCV Infection and CCA

To demonstrate the oncogenic potential of a human virus is necessary to establish the epidemiologic link between markers of viral infection and the risk of cancer.³¹

The association of HCV and CCA has been investigated in a number of studies^{27–51} (Table 1). One of the first studies was conducted in Japan between 1980 and 1997 in a cohort of 600 HCV patients. During the follow-up period (median, 7.2 years), 1.8% of patients experienced cholangiocellular carcinoma, 0.5% a type of hepatocellular and cholangiocellular carcinoma, and 34.3% of patients experienced HCC. This is one of the first studies to show the epidemiologic association between HCV-related liver cirrhosis and CCA development.³²

The results of this study were confirmed by a case–control study in Japan with 50 patients with a diagnosis of iCCA and 205 matched control subjects. Serologic evidence of HCV infection was demonstrated in 36% of iCCA patients and 3% of control subjects. The analysis of

risk factors demonstrated an odds ratio (OR) of 6.02 (95% CI, 1.51–24.1) for iCCA development with positivity of anti-HCV antibodies.³³ Moreover, a retrospective study performed in Cambodia identified the serologic marker of HCV infection in 11.5% of CCA cases.³⁴

Interestingly, HCV infection had also been identified as a risk factor for biliary intraepithelial neoplasia, a precursor lesion of CCA, including mucinous iCCA and mixed iCCA. The analysis of 244 liver explants obtained between 1995 and 2007 at the Mayo Clinic from cases of liver cirrhosis and controls demonstrated the relation between HCV infection and/or alcohol cirrhosis and biliary intraepithelial neoplasia. The retrospective study provides histologic evidence of HCV infection as a risk factor for precursor lesion of CCA.³⁵

In addition, a study performed with the data of population-based cancer registries in the United States found that HCV infection was not associated with ECC (OR = 1.5; 95% CI, 0.2–11.0), whereas this viral infection was significantly associated with iCCA (OR = 4.4; 95% CI, 1.4–14). All of the cases of this study corresponded to patients ≥ 65 years with diagnosis of iCCA ($n = 535$) or ECC ($n = 535$) in the period 1993 to 1999.²⁷

Considering conflicting results of some studies, three meta-analyses were performed to test the association between HCV and/or hepatitis B virus infection and CCA (Table 1). A first meta-analysis identified three cohort studies and 13 case–control studies that evaluated HCV and hepatitis B virus infection as a risk factor for iCCA in different countries.³⁶ The case–control studies were conducted in Thailand, Republic of Korea, China, Taiwan, Italy, United States, and Japan. The cohort studies were developed in three populations, veterans in the United States, blood donors in Japan,

and pregnant women in Taiwan. The analysis of the 16 studies found that HCV infection was associated with diagnosis of iCCA (OR = 3.42; 95% CI, 1.96–5.99); the association persisted when the case–control (OR = 3.63; 95% CI, 1.86–7.05) and cohort (OR = 2.60; 95% CI, 1.36–4.97) studies were analyzed separately.³⁶

Furthermore, a second meta-analysis confirmed the link between HCV and CCA using the data of 16 studies from 1991 to 2012 with data of HCV infection serology marker (anti-HCV) and diagnosis of CCA confirmed by histopathology (iCCA or ECC). In addition to 10 case–control studies previously analyzed,³⁶ four case–control studies conducted in China, one in Thailand, and one in the United States were included in the analysis.³⁷ This meta-analysis found that the risk of CCA increased 5.44-fold in patients with diagnosis of HCV infection compared with control subjects. In addition, the analysis by type of tumor established the association of HCV infection with iCCA with an OR of 3.38 (95% CI, 2.72–4.21) compared with ECC with an OR of 1.75 (95% CI, 1.00–3.05). Interestingly, the risk of HCV and iCCA was higher in studies from North America (OR = 6.48) than Asia (OR = 1.06). A similar geographic difference was observed for the risk of HCV and ECC in North America (OR = 4.88) compared with Asia (OR = 1.06).³⁷

Another systematic review and meta-analysis (eight case–control studies previously analyzed³⁷) showed the increased risk of iCCA in HCV-infected patients (OR = 4.84; 95% CI, 2.41–9.71). HCV infection was established in five of these studies by the presence of anti-HCV antibodies as well as in most of the surveys included in these meta-analyses; however, in two of these studies the marker of HCV infection was the detection of the viral genome.³⁸

Indeed, one of the limitations of the meta-analyses is the definition of status of HCV infection; unfortunately, the only marker available for most of the studies was the presence of anti-HCV antibodies. This is an important limitation to discriminate between individuals exposed but not infected (infection with resolution) and individuals with chronic hepatitis C and the risk of developing end-stage liver disease such as cirrhosis and liver cancer.¹ In addition, tumor heterogeneity and misclassification, selection bias, pattern of incidence and prevalence, and population demographic characteristics of each country could be considered as limitations of these meta-analysis.³⁸

Analyses performed recently confirmed the increased risk of CCA in HCV-infected patients. HCV prevalence in cases of cancer (1,623,538) was compared with cancer-free controls (200,000), using the data of Medicare registries of patients aged ≥66 years from 1993 to 2011 in the United States. The HCV infection was found as a risk factor for intrahepatic bile duct cancer (OR = 3.40; 95% CI, 2.52–4.58) and extrahepatic bile duct cancer (OR = 1.90; 95% CI, 1.41–2.57) in an elderly population.³⁹ However, not all of the studies on liver cancer could demonstrate this association, as described in four case–control studies performed in China

and in Republic of Korea in which HCV infection was not significantly associated with iCCA.^{40–43} In addition, the role of HCV promoting the bile duct neoplasia has so far not been described and characterized (Table 1).

Susceptibility and Permissibility of Cholangiocytes for HCV Infection

The next evidence on the pathway to demonstrate the oncogenic properties of a human virus is the detection of viral sequence in tumor samples.³¹

HCV infection of cholangiocytes and therefore induction of transformation by viral oncoprotein activity could be a direct mechanism of CCA development and progression (Figure 1). The plausibility of this hypothesis is that cholangiocytes and hepatocytes have the same liver progenitor cell; therefore, the cholangiocyte could express the receptors and cellular factors related to susceptibility to HCV infection and permissibility to HCV replication.^{23,44,52} Moreover, as described before, cholangiocytes could be also derived from hepatocytes in conditions of chronic liver injury.²²

The main target cell of HCV is the hepatocyte; however, HCV infection has also been found in other cells such as peripheral blood mononuclear cells, dendritic cells, B lymphocytes, and enteroendocrine cells of gastrointestinal mucosa.^{53–58}

The adhesion and penetration of HCV virions into the hepatocyte depends on a sequence of events in which viral particles bind in a specific order to five receptors: LDL receptor, scavenger receptor class B type 1, CD81, tight-junction proteins claudin-1, and occludin.⁵⁹

To evaluate the susceptibility of bile duct epithelial cells to HCV infection (Table 1), the expression of scavenger receptor class B type 1, CD81, and occludin was found in CCA samples and in two human cell lines derived from CCA (Sk-ChA-1) and iCCA (CC-LP-1).⁵³

Furthermore, the susceptibility of these cell lines, Sk-ChA-1 and CC-LP-1, was also explored and found with the use of pseudovirus particles with the HCV glycoproteins E1E2 (HCVpp); nevertheless, viral replication was scarcely detected in Sk-ChA-1 cells infected with cell-culture–derived HCV particles (HCVcc), indicating that this cell line has a restricted permissiveness for HCV replication.⁵³ The results of entry of HCV particles but with a low level of viral replication obtained in CCA cell lines could explain the detection of the viral genome in bile samples and of viral proteins (Core and NS5) in biliary epithelial cells in biopsies obtained from patients.^{44,54}

However, the infection assays on primary cholangiocyte cultures that used HCVpp were unsuccessful; the absence of viral replication in cholangiocytes could indicate that this cell population is refractory to HCV infection.⁵³

The expression of the receptors for HCV infection in CCA and the detection of viral proteins in bile duct epithelial cells suggest the susceptibility of CCA cell lines to HCV infection (Table 1). However, the results of *in vitro*

infection assays indicated that cholangiocytes and some of the CCA cell lines are not permissive to HCV replication.⁵³

It is important to consider that *in vitro* replication of HCV in hepatoma cell lines and primary hepatocytes was a challenge until some years ago.⁶⁰ This virus is not available to replicate efficiently *in vitro* with the use of clinical isolates for this is necessary to infect with HCVpp or HCVcc as described in the infection assay of CCA cell lines. Even more, efficient replication of HCV in the hepatoma cell line Huh-7 was achieved after several attempts and the identification of some host permissive conditions such as alterations of the innate antiviral response, depending on the retinoic acid inducible gene I pathway.⁶⁰

Several cellular factors could facilitate or restrict HCV replication in cholangiocytes, including the liver-specific miRNA-122 that is the most abundant liver miRNA, and its function is the maintenance of lipid and glucose homeostasis.⁶¹ Of interest, miR-122 is involved in enhancement of HCV replication by recruitment of argonaute-2 and then binding to two sequences in the 5' untranslated region (UTR) of the viral genome. The 5'-UTR of the HCV genome contains an internal ribosome entry site that mediates viral genome translation in a cap-independent manner. Contrary to the normal function of miRNA, the interaction of miR-122 and the viral genome stabilizes HCV RNA in infected cells by protecting the viral genome from 5' exonucleases and evading the cellular RNA sensors. In addition, this interaction

increases viral genome translation and therefore viral particle production.^{62,63} According to the low level of viral replication described in SK-ChA-1 cells infected with HCVcc, miR-122 was not expressed in this human cell line derived from CCA not in the CC-LP-1 cell line.⁵³

Epithelial–Mesenchymal Transition in CCA

The interaction of cholangiocytes with HCV proteins and the induction of chronic inflammation that results in liver fibrosis could be an indirect mechanism of CCA development (Figure 1).

The development and progression of fibrosis is regulated by several factors and the mechanism, including the epithelial–mesenchymal transition (EMT). Under EMT, polarized epithelial cells gradually change to exhibit morphologic, structural, and functional characteristics of mesenchymal cells such as invasiveness, migratory capacity, and enhanced production of extracellular matrix components.^{64–66}

EMT had been described in *in vivo* models as well as in cholangiocytes of bile ductules within fibrotic portal tracts in samples from patients with cholangiopathies such as primary sclerosing cholangitis, biliary atresia, and hepatolithiasis.^{67–71}

The switch from E-cadherin to N-cadherin expression is described in iCCA and ECC. Indeed, low-level expression

Figure 1 Model depicting the role of hepatitis C virus (HCV) infection on induction of biliary epithelial tumor. HCV infection of bi-potential hepatic progenitor cells and/or cholangiocytes could be the direct role of this hepatocellular carcinoma (CCA) development. Moreover, the modulation of epithelial–mesenchymal transition (EMT) and hedgehog (Hh) pathway by HCV and/or viral proteins and the resulting chronic biliary inflammation could be the indirect role of this viral infection on the process associated with fibrosis and CCA. Finally, down-regulation of miR-125b by HCV could be an additional viral strategy for induction of EMT and up-regulation of Hh pathway, therefore contributing to iCCA development in cases of chronic HCV infection. Gli2, glioblastoma transcription factor 2; HSC, hepatic stellate cell; iCCA, intrahepatic CCA; Shh, sonic hedgehog; SMAD, small mothers against decapentaplegic; TGF, transforming growth factor.

Figure 2 H69 cells expressing hepatitis C virus (HCV) Core protein induced epithelial–mesenchymal transition (EMT) markers and activated Hedgehog (Hh) pathway. H69 cells were transfected with pTracerHCV_Core (HCV genotype 2a) by electroporation with the use of Nucleofector (Nucleofector, Lonza, Switzerland). Transfection efficiency was determined by expression of the green fluorescent protein with the use of flow cytometry. Isolation of miRNAs was performed at 72 hours after transfection with the Ambion mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA). The mRNA level of fibrosis, EMT, and Hh pathway genes were analyzed by real-time quantitative PCR. The *t*-test was performed to compare the means between HCV Core–transfected H69 cells (pTracerCore) and the empty vector–transfected H69 cells (pTracer), using Prism version 5 (GraphPad Inc., San Diego, CA). Data are expressed as means $\pm$ SD. *n* = 2 independent experiments. ****P* < 0.01, *****P* < 0.001. α -SMA, α -smooth muscle actin; Gli2, glioblastoma transcription factor 2; S100A4, S100 family of small calcium-binding proteins; Shh, sonic hedgehog; SMAD, small mothers against decapentaplegic.

of E-cadherin, that is critical for epithelium up-keep, is associated with poor tumor differentiation and metastasis in CCA cases.^{66,72} This switch causes the release of β -catenin from the membranous complex; therefore, its translocation to the nucleus where β -catenin can regulate the transcription of EMT genes, among others by interacting with transcription factors. Overexpression of β -catenin in the cytoplasm and the nucleus has been observed in CCA, more frequently in iCCA cases.^{66,72}

Another well-known EMT marker is S100A4, a member of the S100 family of small calcium-binding proteins. Up-regulation of *S100A4* is described in 30% to 50% of iCCA and 11% of ECC cases and is not detected in normal cholangiocytes.⁶⁶ The expression of S100A4 is induced in intrahepatic biliary epithelial cell cultures under stimulation of the transforming growth factor (TGF)- β 1; these epithelial cells change to fibroblastic structure after treatment with TGF- β 1, demonstrating that biliary epithelial cells can undergo EMT.⁷³ This protein is also a relevant factor for motility and invasiveness, demonstrated by silencing of

S100A4 in the CCA cell line EGI-1. In addition, nuclear expression of S100A4 in bile ducts was related to decreased survival of patients after surgery and with metastasis.⁷⁴

Finally, a number of transcription factors that induce EMT have been studied in CCA development and progression. Nuclear expression of *Snail1*, *Slug*, *Twist1*, *ZEB1*, and *ZEB2* genes has been demonstrated in tumor cells in iCCA and ECC; whereas in normal biliary epithelium it is not detected. The findings of these studies are positively correlated with the different EMT markers described in cases of iCCA and ECC and demonstrate a key role of EMT in cancer progression.⁶⁶

Liver Modulation of EMT by HCV

EMT induction in hepatocytes by HCV infection was reported.⁷⁰ Human primary hepatocytes infected with HCVcc particles genotype 1a or 2a displayed senescence, detected by β -galactosidase activity, and down-regulation of the gene and

Figure 3 Transitory expression of hepatitis C virus (HCV) Core protein in KMCH-1 cells up-regulated epithelial–mesenchymal transition (EMT) and hedgehog (Hh) pathway. Transfection of KMCH-1 cells was performed with pTracerHCV_Core (HCV genotype 2a) and Lipofectamine (Thermo Fisher Scientific, Waltham, MA) or TransIT-LT1 (Mirus Bio LLC, Madison, WI). Transfection efficiency was determined by expression of the green fluorescent protein with the use of flow cytometry and Western blot analysis, using the human monoclonal antibodies anti-HCV Core B12-F8 antibody. Isolation of miRNAs was performed 72 hours after transfection with the use of the Ambion mirVana miRNA Isolation Kit (Thermo Fisher Scientific). The mRNA expression level of EMT and Hh pathway activation markers were analyzed by quantitative real-time PCR. The *t*-test was performed to compare the means between HCV Core–transfected KMCH-1 cells (pTracerCore) and the empty vector transfected KMCH-1 cells (pTracer), using Prism version 5 (GraphPad Inc., San Diego, CA). Data are expressed as means $\pm$ SD. *n* = 2 independent experiments. *****P* < 0.001. Gli2, glioblastoma transcription factor 2; Shh, sonic hedgehog; SMAD, small mothers against decapentaplegic; TGF, transforming growth factor.

protein expression of E-cadherin, occludin, and desmoplakin (Table 1). Up-regulation of EMT markers, Vimentin and S100A4, and EMT-inducing transcription factors, Snail, Slug, and Twist, was demonstrated.⁷⁰ Up-regulation of Snail and Twist was also described in liver biopsies from cases of HCV infection. Moreover, the researchers found that the EMT induction in HCV-infected hepatocytes was driven by phosphorylation at Thr 308 and Ser 409 of thymoma viral proto-oncogene (Akt) and subsequently activation of β -catenin by phosphorylation at Ser 552. The consequence of this phosphorylation is the release from the E-cadherin– α -catenin– β -catenin complex and therefore the dissociation of cell–cell contacts and finally β -catenin translocation to the nucleus. As described before, β -catenin is a transcription factor of a number of genes, including fibronectin and matrix metalloproteinase 7, that promotes EMT.⁷⁰ This activation could also be related to up-regulation of the Snail, Slug, and Twist factors observed in this *in vitro* model as well as in cells undergoing EMT.^{66,75}

Furthermore, HCV Core protein in cholangiocytes and CCA human cell lines has also been related to EMT (Table 1).⁷² The expression of the HCV Core protein in QBC939 cells, a human CCA cell line, modified the level expression of epithelial (E-Cadherin) and EMT markers (Vimentin and Fibronectin). In addition, Core protein-enhanced ability of migration was demonstrated in transfected QBC939 cells.⁷²

Recently, preliminary results of EMT induction by the HCV Core protein were obtained in a nonmalignant human cholangiocyte cell line (H69) and in a human CCA cell line (KMCH-1). Up-regulation of α -smooth muscle actin and *S100A4* gene expression at the mRNA level was detected 72 hours after transfection in HCV Core–transfected H69 cells compared with control cells (empty vector-transfected) ($P < 0.05$) (Figure 2). In addition, a differential gene expression of the glioblastoma transcription factor 2 (*Gli2*), sonic hedgehog ligand (*Shh*), and small mothers against decapentaplegic (SMAD) 2 and 4 genes was observed in HCV Core–transfected H69 cells ($P < 0.05$) (Figure 2 and Table 1).⁷³

As described in H69 human immortalized cholangiocytes (Table 1), preliminary results of enhanced expression of TGF- β 1, *Shh*, *Gli2*, *SMAD3*, and *SMAD4* at mRNA levels have been obtained in HCV Core–transfected KMCH-1 cells compared with control cells (empty vector transfected) ($P < 0.05$) (Figure 3). These findings could suggest that the HCV Core protein induces EMT and the activation of the Hedgehog (Hh) pathway in nonmalignant human cholangiocyte cells as well as in CCA cells (Figure 1).⁷³

These preliminary results are in agreement with the interaction of HCV Core protein with the *TGFB1* promoter and the further induction of the TGF- β response described in hepatocytes.⁷⁴ Furthermore, viral protein overexpression activates the TGF- β pathway demonstrated by Smad2/3 phosphorylation in hepatocytes and induces the activation of hepatic stellate cells (HSCs). These data indicate the role of HCV Core protein in up-regulation of TGF- β signaling

during the EMT process and therefore in liver fibrosis and HCC/CCA progression (Figure 1).^{74,76}

In addition, the activation of the Hh pathway observed in H69 and KMCH-1 cells induced overexpression of *Gli1* and *Gli2* as also described in CCA tumors (Figure 1).⁷⁷ Furthermore, activation of Hh signaling is related with metastasis and poor survival of patients with iCCA.⁶⁶

Hh Pathway in Response to Liver Injury and to HCV Infection

In response to liver injury, Hh ligands [sonic Hh (Shh), Indian Hh, and desert Hh] are released, inducing proliferation and differentiation of hepatic progenitor cells. Hh also induces proliferation and activation of quiescent HSCs into myofibroblastic HSCs, playing an important role in hepatic fibrogenesis.^{68,78}

The role of HCV infection to regulate the Hh pathway has been found in liver samples from cases of chronic hepatitis C by up-regulation of the Hh ligands, Shh and Indian Hh, and *Gli2*. In addition, the level of expression of Shh and *Gli2* correlates with the fibrosis stage of liver disease.⁵¹ Interestingly, in liver samples with advanced fibrosis and viral infection, epithelial cells positive for Shh were found in the ductular reaction (Table 1). The key role of HCV was also indicated by induction of *Shh* mRNA expression in cultured liver epithelial cells expressing the viral proteins. Thus, HCV infection is an important factor for Hh pathway activation that affects liver fibrosis, EMT, and liver epithelial progenitor density and subsequent liver disease progression and hepatocarcinogenesis.⁵¹

The role of the Hh pathway in fibrosis and liver regeneration has been also studied in animal models with liver injury as well as in liver samples from patients.^{68,79} Co-expression of EMT markers (*S100A4* and Vimentin) and of Hh pathway activation (nuclear localization of *Gli2*) markers have been found in ductular cells in liver samples obtained from patients with primary biliary cholangitis.⁶⁸ This result provides evidence of EMT regulation by the Hh pathway in bile ductular cells during chronic cholestasis. These findings were confirmed in rats after extrahepatic bile duct ligation by up-regulation of *Gli2* and down-regulation of *Hhip*, a Hh ligand inhibitor in the liver. In addition, the co-expression of *S100A4* and receptors of Hh ligands (Patched) was found in primary cholangiocytes isolated from rats with bile duct ligation.⁶³ The confirmation of Hh activation in cells undergoing EMT was obtained with the use of Hh-neutralizing antibodies in cultures of primary cholangiocytes treated with conditioned medium from Hh-producing myofibroblastic HSCs. This treatment abrogated not only the expression of the EMT marker but also the migratory phenotype in cholangiocytes.⁶⁸

However, liver regeneration and down-regulation of the Hh pathway were induced in rats treated with carbon tetrachloride after receiving human mesenchymal stem cells. The mRNA expression levels of *Shh*, *Gli2*, *Gli3*, and the signaling-competent coreceptor smoothened (*Smo*) genes

were reduced in these rodents, together with a decrease in liver fibrosis. Indeed, the 3'-UTR of the *Smo* gene is one of the targets of miR-125b.⁷⁹ The Hh pathway down-regulation was related with the presence of miR-125b in exosomes released by mesenchymal stem cells; furthermore, the function of miR-125b was demonstrated in co-culture of human mesenchymal stem cells and HSCs obtained from the treated rats by down-regulation of Hh signaling and also down-regulation of profibrotic genes. These data indicated that miR-125b suppresses Hh pathway activation and therefore plays a key role in this specific miRNA on regression of liver fibrosis and liver regeneration.⁷⁹

Of interest, we have demonstrated that miR-125b regulates biliary proliferation by targeting the 3'-UTR of vascular endothelial growth factor A (*VEGFA*) mRNA in rats with bile duct ligation.⁸⁰ This model of human cholangiopathies features large cholangiocyte proliferation in response to liver injury⁶²; this proliferation is stimulated by secretin, a neuro-endocrine hormone secreted mainly by S cells and also secreted by cholangiocytes as a mechanism of biliary growth regulation during cholestasis.⁸¹ The treatment with secretin of mice increased biliary expression of VEGFA and nerve growth factor and concomitantly down-regulated expression of miR-125b and miR-Let5a. miR-Let7a also regulates hepatobiliary proliferation by targeting the 3'-UTR of the nerve growth factor mRNA. The targets of these miRNAs were confirmed with the use of luciferase reporter constructs and by transfection of the specific inhibitors of miR-125b and miR-Let1a. Moreover, treatment with morpholinos against miR-125b induced liver expression of VEGFA and therefore increased intrahepatic bile duct mass.⁸⁰

Conclusions

Taking in account the pathway that establishes the link between a human virus and the development of a neoplasia,³¹ the epidemiologic association of the HCV infection marker (anti-HCV antibodies) and iCCA described in different studies is consistent with the hypothesis of induction of biliary epithelial tumors by this hepatocivirus (Table 1).

After the establishment of the epidemiologic link, the oncogenic properties of a human virus have to be demonstrated by detection of viral sequences of tumor cells.³¹ Unfortunately, the data available for this important evidence are not sufficient for HCV and iCCA.^{44,46} Thus, additional studies with the use of liver biopsies or even better explants from patients with diagnosis of iCCA have to be evaluated to demonstrate HCV sequences in biliary epithelial tumors (Table 1).

HCV infection of bipotential hepatic progenitor cells as well as the infection of hepatocytes with subsequent trans-differentiation into cholangiocyte precursors could be one of the mechanisms of CCA induction by HCV (Figure 1). Further studies are necessary to evaluate this hypothesis, considering that the study performed by Fletcher et al⁴⁵ indicated that primary cholangiocytes are refractory to

HCV infection. However, the expression of receptors for HCV entry observed in CCA samples obtained from patients and in CCA cell lines (Sk-ChA-1 and CC-LP1) and the low level of viral replication in Sk-ChA-1 cells are important findings that have to be taken into account for this hypothesis (Figure 1).

The modulation of EMT and the Hh pathway by HCV and the resulting chronic biliary inflammation and iCCA development is an interesting hypothesis corroborated somewhat by one study⁴⁸ and our preliminary results. EMT induction by the HCV Core protein in a nonmalignant human cholangiocyte cell line as well as in the human CCA cell lines suggests the role of the viral protein in this process associated with fibrosis and cancer (Table 1 and Figure 1).

Furthermore, the preliminary results of Hh pathway activation in a nonmalignant human cholangiocyte cell line (H69) and a human CCA cell line (KMCH-1) show by transitory expression of the HCV Core protein that is according to the induction of TGF- β response (Figure 1). Moreover, the activation of this pathway has been also described in liver samples from patients with chronic hepatitis C as well as in CCA tumors^{74,76} (Table 1).

Considering the targets of miR-125b, one of the mechanisms of HCV-induced CCA could be down-regulation of this miRNA by HCV; the subsequent induction of the TGF- β response; the activation of Snail, Slug, and Twist factors and Smo and its target (Gli1 and Gli2); and, finally, the induction of EMT in cholangiocytes (Figure 1).

Future studies are mandatory to elucidate whether the hypothesis explored in this review correspond to the direct and/or indirect mechanisms of HCV as the oncogenic virus that induces the transformation of cholangiocytes.

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