Oncogene (2006) 25, 3834–3847

www.nature.com/onc

REVIEW

Viral hepatitis and liver cancer: the case of hepatitis C

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Chronic infection with the hepatitis C virus (HCV) is a major risk factor for the development of hepatocellular carcinoma (HCC) worldwide. The pathogenesis of HCC in HCV infection has extensively been analysed. Hepatitis C virus-induced chronic inflammation and the effects of cytokines in the development of fibrosis and liver cell proliferation are considered as one of the major pathogenic mechanisms. Increasing experimental evidence suggests that HCV contributes to HCC by directly modulating pathways that promote the malignant transformation of hepatocytes. Hepatitis C virus is an RNA virus that does not integrate into the host genome but HCV proteins interact with many host-cell factors well beyond their roles in the viral life cycle and are involved in a wide range of activities, including cell signaling, transcription, cell proliferation, apoptosis, membrane rearrangements, vesicular trafficking and translational regulation. At least four of the HCV gene products, namely HCV core, NS3, NS4B and NS5A, have been shown to exhibit transformation potential in tissue culture and several potentially oncogenic pathways have been shown to be altered by the expression of HCV proteins. Both HCV core and NS5A induce the accumulation of wild-type β-catenin and the Wnt–β-catenin pathway emerges as a common target for HCV (and HBV) in human HCCs, also independently from axin/β-catenin gene mutations. Induction of both endoplasmic reticulum stress and oxidative stress by HCV proteins might also contribute to HCV transformation. Most of the putative transforming functions of the HCV proteins have been defined in artificial cellular systems, which may not be applicable to HCV infection *in vivo*, and still need to be established in relevant infection and disease models.
Oncogene (2006) **25,** 3834–3847. doi:10.1038/sj.onc.1209562

Keywords: hepatocellular carcinoma; hepatitis C; HCV core; NS5A; Wnt/β-catenin pathway; p53-family; p73

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Introduction

Hepatocellular carcinoma (HCC) ranks among the most common cancers in many countries (Bosch *et al*., 1999). A recent estimate indicates that HCC represents the fifth most common cancer of males, and the eighth most common cancer in female candidates, with a total of 560 000 new cases each year, 83% of which occurring in developing countries, and more than one-half in China alone. Moreover, because of its very poor prognosis, HCC represents the third leading cause of cancer death worldwide. Chronic hepatitis B and C and associated liver cirrhosis represent major risk factors for HCC development, being implicated in more than 70% of HCC cases worldwide. The rise in the incidence of and mortality from HCC recently observed in most industrialized countries likely reflects the increased prevalence of HCV infection (Taylor-Robinson *et al*., 1997; Deuffic *et al*., 1998; El-Serag and Mason, 1999; El-Serag *et al*., 2003). A large analysis of HCC in Europe, based on both serology and molecular tests, has demonstrated the major impact of hepatitis B virus (HBV) and hepatitis C virus (HCV). Only 29% of HCC cases were found negative for these viruses. The hepatitis B surface antigen (HBsAg) and anti-HCV antibodies were detected in 19 and 40.1% of the patients, respectively, with HCV 1b being the most prevalent genotype (Brechot *et al*., 1998) Additional etiological factors that often represent co-factors of an underlying HBV- or HCV-related chronic liver disease include toxins and drugs (e.g., alcohol, aflatoxins, microcystin, anabolic steroids), metabolic liver diseases (e.g., hereditary haemochromatosis, α1-antitrypsin deficiency), steatosis (Ohata *et al*., 2003) and non-alcoholic fatty liver diseases (Brunt *et al*., 2004), diabetes (Davila *et al*., 2005). In general, HCCs are more frequent in men than in women and the incidence increases with age.

As for most types of cancer, hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to malignant transformation of the hepatocyte. The detailed analysis of HCC development in experimental animals and the comparison of the results with HCC in humans has identified a variety of genomic and molecular alterations in fully developed HCC (Thorgeirsson and Grisham, 2002) and to a lesser extent in morphologically defined pre-neoplastic precursor lesions (Llovet *et al*., 2003; Kojiro, 2005).

However, in most studies, single chromosome loci, oncogenes or tumor suppressor genes have been analysed in relatively small series of human HCCs and lesions from experimental animals. Indeed, although significant progress has been achieved in recognizing the sequence of events involved in other forms of cancer, notably colorectal cancer and certain hematopoietic malignancies, we still lack a coherent understanding of the mechanisms of HCC development. In particular, the contribution of the different etiological factors and their interactions in hepatocarcinogenesis are still poorly understood and we have not yet identified critical genomic and/or molecular aberrations that might improve diagnosis or be targeted for therapeutic interventions.

Malignant transformation of hepatocytes is believed to occur, regardless of the etiological agent, through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration, in a context of inflammation and oxidative DNA damage (Figure 1). This microenvironment facilitates the occurrence of genetic and epigenetic alterations. Chronic viral hepatitis, alcohol, metabolic liver diseases such as hemochromatosis and α1-antitrypsin deficiency, as well as non-alcoholic fatty liver disease may act predominantly through this pathway of chronic liver injury, regeneration and cirrhosis. Accordingly, the major clinical risk factor for HCC development is liver cirrhosis and 70–90% of all HCCs develop in a cirrhotic liver. The risk of HCC in patients with liver cirrhosis depends on the activity, duration and the etiology of the underlying liver disease. The co-existence of multiple etiologies, for example, HCV infection with overt or occult HBV, aflatoxin B1 (AFB1) and HBV infection, HCV infection and alcohol or HCV infection and liver steatosis, increases the relative risk of HCC development. Dysplastic nodules and macroregenerative nodules are considered pre-neoplastic lesions (Furuya *et al*., 1988; Terada *et al*., 1993). Several observations support this hypothesis. Firstly, direct outgrowth of HCC from cirrhotic nodules has been described as the nodule-in-nodule appearance. Secondly, 50–60% of the cirrhotic macronodules have a monoclonal origin when examined for the X-chromosome methylation pattern. Thirdly, chromosome aberrations and allelic losses are found in half of cirrhotic nodules and in small cell dysplasia, thus indicating that they represent true pre-malignant lesions of HCC. Only in less than 10% of the cases, HCCs, are observed in non-cirrhotic livers and even without inflammatory lesions. These HCCs developing in an otherwise normal livers are usually found in patients without well-established risk factors and some of these cases may correspond to the malignant transformation of liver adenoma rare benign hepatocellular tumors sometimes found in young women taking oral contraceptives (Bluteau *et al*., 2002).

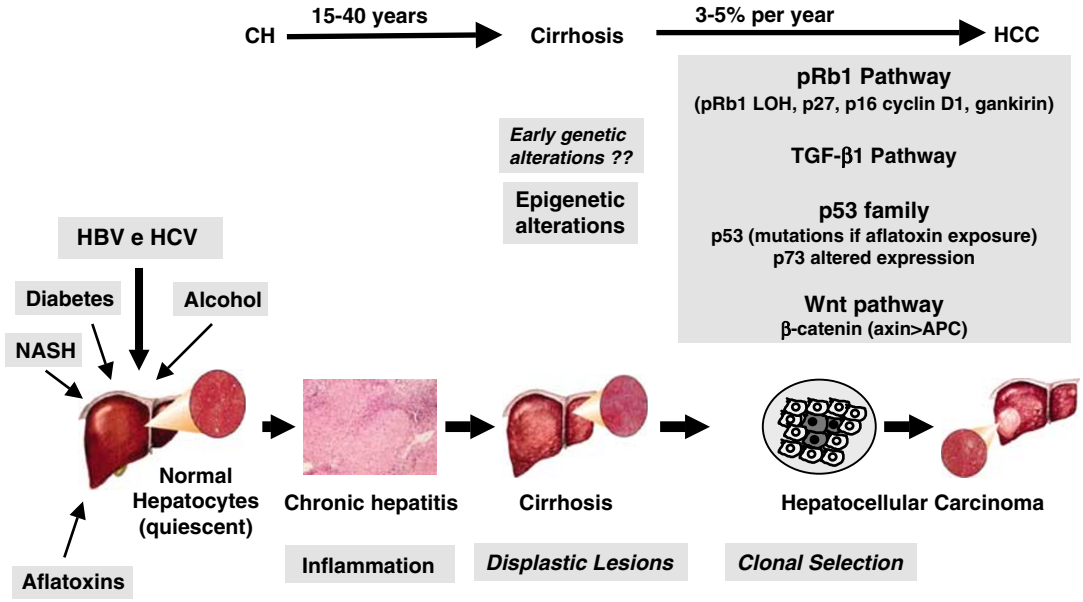

Figure 1 Pathogenesis of human hepatocellular carcinoma (HCC). Chronic hepatitis B and C and associated liver cirrhosis represent major risk factors for HCC development, being implicated in more than 70% of HCC cases worldwide. Additional etiological factors, which often represent co-factors of an underlying HBV- or HCV-related chronic liver disease, include toxins and drugs (e.g., alcohol, aflatoxins, microcystin, anabolic steroids), metabolic liver diseases (e.g., hereditary hemochromatosis, α1-antitrypsin deficiency), steatosis, non-alcoholic fatty liver diseases and diabetes. Hepatocarcinogenesis is a multistep process that may last for decades and involves the progressive accumulation of different genetic alterations ultimately leading to malignant transformation. Regardless of the etiological agent, malignant transformation of hepatocytes is believed to occur through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration, in a context of inflammation and oxidative DNA damage. Dysplastic nodules and macroregenerative nodules are considered as pre-neoplastic lesions. The detailed analysis of HCC development in experimental animals and the comparison of the results with HCC in humans has identified a variety of genomic and molecular alterations in fully developed HCC and to a lesser extent in morphologically defined pre-neoplastic precursor lesions. At least four pathways that regulate either cell proliferation or cell death (i.e., the phospho-retinoblastoma (pRb), p53, transforming growth factor-β (TGF-β) and β-catenin pathways) are affected in HCCs.

Molecular pathways in human hepatocarcinogenesis

At least four pathways that regulate either cell proliferation or cell death (i.e., the phospho-retinoblastoma (pRb), p53, transforming growth factor-β (TGF-β) and β-catenin pathways) are affected in HCCs (Ozturk, 1999; Moradpour and Wands, 2002; Moradpour and Blum, 2005). Although activated ras family oncogenes have been found in spontaneous and chemically induced rodent hepatocarcinogenesis models and the cyclin D has been found to be amplified in 10–20% of human cases (Nishida *et al*., 1994), no consistent pattern of proto-oncogene activation has emerged so far in human HCCs. Deregulated expression of β-catenin, resulting from Adenomatous Polyposis of the Colon (APC) defects, β-catenin gene mutations and/or Wnt signaling pathway alterations, appears to play a role in more than 50% of HCCs (Ozturk, 1999). The Wnt/Frizzled/β-catenin signaling is mediated by a complex interaction between a Wnt ligand (Wnt) and a Frizzled receptor (Fzd), mostly in cooperation with the low-density lipoprotein receptor (LDLR)-related protein (LRP-5 or -6) co-receptors. The Wnt/β-catenin pathway is involved in developmental control, cell adhesion and cell proliferation. Cellular levels of β-catenin are tightly regulated by proteasome-dependent degradation, which is in turn controlled by the activity of the APC and Axin1 proteins, and the glycogen synthase kinase-3β (GSK-3β). Accumulation of nuclear β-catenin-containing complexes leads to the unrestricted transcription of several cell-cycle control genes. Differently from colon carcinoma, somatic APC gene mutations appear to be rare in HCC, whereas activating mutations of β-catenin were reported in 18–41% of HCCs (De La Coste *et al*., 1998; Miyoshi *et al*., 1998) and Axin1 gene mutations have been found in a substantial proportion of HCCs with β-catenin accumulation in the absence of mutation of the β-catenin gene (Satoh *et al*., 2000a). A recent report indicates that the Frizzled type 7 receptor (Fzd-7) is overexpressed in above 90% of HCCs and in around 75% of the corresponding peritumorous/precancerous liver parenchyma as compared to normal livers and that Fzd-7 is also involved in wild-type β-catenin stabilization/activation (Merle *et al*., 2004). Interestingly, β-catenin mutations and/or deregulation in HCC correlate(s) with a low rate of loss of heterozygosity (LOH), suggesting that β-catenin pathway activation over-rides the need for multiple genetic/epigenetic events and loss of tumor suppressor genes in the multistep process of hepatocarcinogenesis and may lead more directly to the liver malignant phenotype (Legoix *et al*., 1999).

The upregulation of growth factors expression and the activation of components of their signaling pathways also play an important role in hepatocarcinogenesis. Insulin-like growth factor (IGF) II, insulin receptor substrate 1, hepatocyte growth factor (HGF) and TGF-α and β have been involved in the development of HCC (Moradpour and Wands, 2002). Unexpectedly, HGF, a mitogen for normal hepatocytes, inhibits the growth of hepatoma cell lines *in vitro* and the development of HCC in transgenic mouse models of hepatocarcinogenesis (Santoni-Rugiu *et al*., 1996). Overexpression of the angiogenic factors vascular endothelial growth factor (Mise *et al*., 1996; Yamaguchi *et al*., 1998) and angiopoietin-2 (Tanaka *et al*., 1999; Mitsuhashi *et al*., 2003) has been documented in HCC. These findings are particularly relevant as the evolution from pre-neoplatic to HCC nodules is accompanied by neovascolarization and HCC is often a highly vascular tumor.

Several studies of paired HCC and non-tumorous liver samples have revealed relatively frequent allelic losses (LOH) on chromosomes 1, 2q, 4, 5q, 6q, 8, 9, 10q, 11p, 13q, 14q, 16, 17 and 22q (Ozturk, 1999; Moradpour and Wands, 2002; Thorgeirsson and Grisham, 2002), suggesting that these sites may harbor tumor suppressor genes relevant for HCC pathogenesis. Recent large genomewide scans using microsatellite markers and comparative genomic hybridization studies have confirmed and extended these observations (Boige *et al*., 1997; Marchio *et al*., 1997; Nagai *et al*., 1997). However, only few tumor suppressor genes located in these deleted regions have been clearly involved in a significant subset of HCCs and the search for a liver-specific tumor suppressor has essentially failed. On the other hand, the pRb tumor suppressor pathway is altered in more than 50% of human HCCs owing to genetic and epigenetic changes (Ozturk, 1999). The Rb protein and its regulators p16INK4A and cyclin D are involved in the G1/S progression of the cell cycle. Loss of heterozygosity at the RB1 locus is quite frequent and RB1 gene mutations are found in about 15% of human HCCs. Both mutations of the p16INK4A gene and its inactivation by methylation have been described (Liew *et al*., 1999). Recently, overexpression of gankyrin, a six ankyrin repeat protein, that mediates pRb degradation by the 26S proteasome, has also been described (Higashitsuji *et al*., 2000), thus providing an additional, liver-specific mechanism of pRb inactivation.

A G-to-T mutation at the third base position of codon 249 of the p53 tumor suppressor gene is found in up to 50–70% of HCCs in patients from southern Africa and the Qidong area in China (Bressac *et al*., 1991; Hsu *et al*., 1991). This 'hot-spot' mutation, leading to an arginine to serine substitution (R249S) and the expression of a DNA-binding defective p53 mutant protein, is associated with high food contamination with the AFB1 mycotoxin (Aguilar *et al*., 1993). In other regions where aflatoxin levels in food are low or undetectable low p53 mutation rates are observed ($<4\%$ of HCCs) and no specific gene mutation pattern can been detected (Ozturk, 1999). Importantly, dietary AFB1 exposure and co-existing HBV infection appear to act synergistically and are associated with even higher rates of HCC development (Henry *et al*., 1999; Sun *et al*., 1999; Ming *et al*., 2002). Polymorphisms of enzymes involved in the biotransformation of environmental toxins such as AFB1, benz[*a*]pyrene and other polycyclic aromatic hydrocarbons may contribute a genetic susceptibility to the development of HCC (Chen and Chen, 2002). The p53-related tumor suppressor p73 is also deregulated in HCC. Mutations in the p73 gene have not been

described (Levrero *et al*., 2000), but the amino-terminal-deleted isoform DNp73, which acts as a dominant *trans*-repressor of both p53 and proapoptotic TAp73 proteins (Vossio *et al*., 2002), accumulates progressively in chronic hepatitis, cirrhosis and HCC, and confers to HCC cells a chemoresistant phenotype (Muller *et al*., 2005; Palescandolo *et al*., 2006). DNp73 is expressed from an alternative P2p73 promoter, which is controlled in hepatocytes by NF-κB and β-catenin (Palescandolo *et al*., 2006). This latter observation links β-catenin activation with p53 and TAp73 functional inactivation that is mediated by DNp73 overexpression and does not require the selection of p53 gene-inactivating mutations. Additional, transactivation-deficient NH-terminally truncated DTA-p73 oncogenic proteins are overexpressed from the P1p73 promoter in a subset of HCCs (Putzer *et al*., 2003).

Loss of pRb and p53 pathways leads to genomic instability, which plays an important permissive role in malignant transformation. Defective DNA mismatch repair and microsatellite instability occur in a subset of HCCs (MacDonald *et al*., 1998), although the frequency of such defects is debated (Piao *et al*., 2000). Moreover, recent evidence suggests that telomere dysfunction, leading to telomere-based chromosomal instability, occurs during the early stages of hepatocarcinogenesis, whereas activation of telomerase, the ribonucleoprotein enzyme that prevents the shortening of telomeres and extends cell life span, occurs later during HCC progression (Farazi *et al*., 2003).

The overall emerging picture is that of HCCs as genetically very heterogeneous tumors. This is not completely unexpected, given the heterogeneity of etiological factors implicated in the development of HCC, the complexity of hepatocyte functions and the late stage at which HCCs are usually detected and analysed. Genomewide analysis of genetic alterations in HCC showed that genetic alterations are not randomly distributed in tumors but are closely associated in clusters, and has enabled the definition of two main mechanisms of hepatocarcinogenesis. In the first, genetic alterations are accumulated through chromosome instability, and in the second, activation of the Wnt pathway by β-catenin mutation is predominant (Laurent-Puig *et al*., 2001). Associations between etiological factors of HCC and genetic alterations in tumors have been found, and globally, chromosome instability together with p53 and Axin1 mutations was closely related to HBV infection. In contrast, the second hepatocarcinogenesis pathway, defined by β-catenin mutation associated with chromosome 8p deletion in a context of chromosome stability, is significantly associated with the absence of HBV infection.

Hepatitis C virus and hepatocellular carcinoma

The notion of HCV, a completely cytoplasmic-replicating virus, that induces oncogenic transformation has challenged the current conventional biological models for viral oncogenesis. As HCC associated with HCV infection evolves after many years of chronic infection and is generally preceded by the development of cirrhosis (NIH Conference, 2002), the role of chronic liver injury followed by regeneration, cirrhosis and the development of HCC has been the leading hypothesis. Increasing experimental evidence, however, raises the possibility that HCV might also contribute through more direct pathways in promoting malignant transformation of hepatocytes. Different viral proteins, notably core (Moriya *et al*., 1998; Yoshida *et al*., 2002), NS3 (Sakamuro *et al*., 1995) and NS5A (Gale *et al*., 1999), have been implicated in transformation and HCC development.

The hepatitis C virus

Hepatitis C virus is a member of the Flaviviridae family of enveloped, positive-strand RNA viruses and is the only member of the genus *Hepacivirus* (Tellinghuisen and Rice, 2002). The HCV genome consists of an RNA molecule, of approximately 9.6 kb, that contains a large open-reading frame flanked by structured 5′ and 3′ non-translated regions (NTRs). Viral proteins are translated as a polyprotein precursor from an internal ribosome entry site (IRES) located in the 5′ NTR (Figure 2). The polyprotein undergoes a complex series of co- and post-translational cleavage events catalysed by both host and viral proteinases to yield the individual HCV proteins (Figure 3). The structural proteins include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins include the P7 polypeptide, the NS2-3 autoprotease and the NS3 serine protease, an RNA helicase located in the C-terminal region of NS3, the NS4A polypeptide, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (Tellinghuisen and Rice, 2002). An additional HCV protein, F (for frameshift protein) or ARFP (for alternate reading frame protein), generated by an overlapping reading frame in the core (C) protein coding sequence, has been proposed (Waleswski *et al*., 2001; Xu *et al*., 2001; Varaklioti *et al*., 2002)

The study of HCV life cycle and replication has been limited by the lack of efficient cell culture systems that support high-level HCV replication and HCV molecular biology has mainly been addressed by expressing individual viral proteins. The development of efficient cell culture HCV RNA replication systems based on the replicon technology (Lohmann *et al*., 1999, 2001; Blight *et al*., 2000; Guo *et al*., 2001) has allowed to perform molecular genetic studies to determine the function of individual HCV proteins during replication. Replicons are self-replicating HCV genomic RNA molecules. The prototype replicon usually consists of a self-replicating RNA that contains the 5′ and 3′ ends of the HCV genome (5′ NTR, 3′ NTR), a selectable marker gene (e.g., *neo*), and the non-structural genes of HCV that encode the viral replicase (NS3-5b). Translation of the *neo* marker gene is mediated by the natural HCV IRES, whereas translation initiation of HCV NS2-5b is mediated by an encephalomyocarditis virus (ECMV)

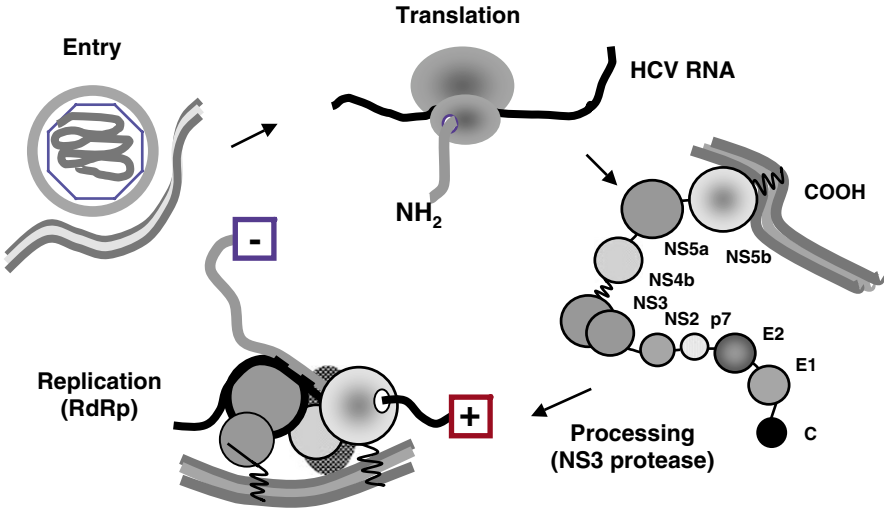

Figure 2 Hepatitis C virus (HCV) life cycle. The HCV genome consists of an RNA molecule, of approximately 9.6 kb. Viral proteins are translated as a polyprotein precursor from an internal ribosome entry site (IRES) located in the 5′ non-translated region (NTR). The polyprotein undergoes a complex series of co- and post-translational cleavage events catalysed by both host and viral proteinases to yield the individual HCV proteins. The non-structural proteins form the viral replicase complex. Once released from NS2, the amino-terminal domains of the NS3 proteins serve as serine proteinases for the release of the remaining non-structural proteins from the polyprotein. Viral RNA replication is thought to occur in the perinuclear membrane. The replication complex contains the viral polymerase, helicase as well as a number of host-cell factors.

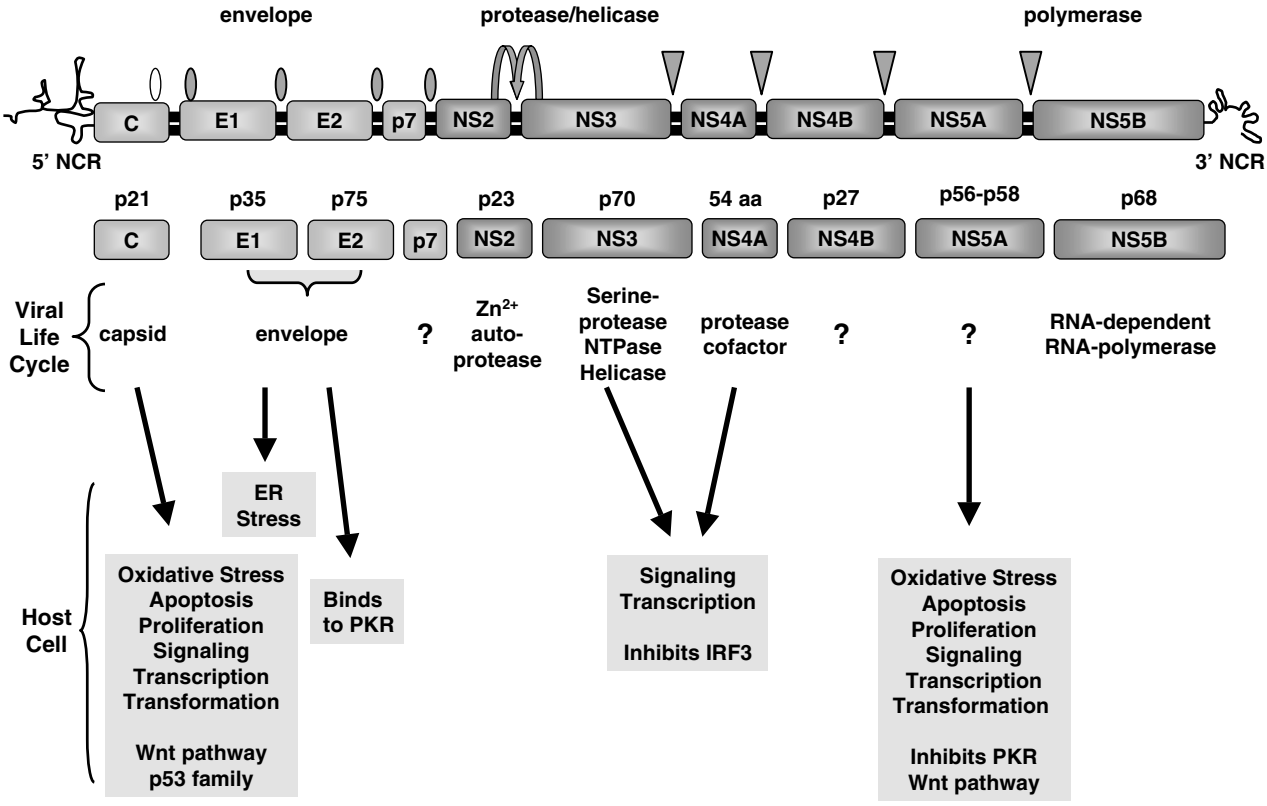

Figure 3 Hepatitis C virus (HCV) proteins. Genetic organization and polyprotein processing of HCV. The 9.6 kb positive-strand RNA genome is composed of a 5′ non-coding region (NCR), a long open-reading frame encoding a polyprotein precursor of about 3000 amino acids and a 3′ NCR. The polyprotein precursor is processed into structural and non-structural proteins by cellular and viral proteases. Solid rods denote cleavage sites of the endoplasmic reticulum signal peptidase. The open rod indicates the additional C-terminal processing of the core protein by signal peptide peptidase. Arrow heads indicate cleavages by HCV NS2-3 and NS3 proteases. Besides their roles in the viral life cycle, HCV proteins interact with many host-cell factors and impact on a wide range of cellular activities, including cell signaling, transcriptional modulation, transformation, apoptosis, membrane rearrangements, vesicular trafficking and translational regulation.

IRES. More recently, systems have also been developed for the expression of the full-length HCV polyprotein in the context of a replicating viral RNA (Ikeda *et al*., 2002; Pietschmann *et al*., 2002). Although these constructs may provide a system to evaluate some of the early aspects of assembly, they replicate with lower efficiency and do not assemble into infectious virions.

Replicons have been instrumental in defining the cellular localization and topology of the HCV non-structural proteins. The structural proteins, namely C, E1 and E2, are cleaved from the polyprotein by the endoplasmic reticulum (ER) signal peptidases and, after maturation in the ER, are assembled into the progeny virions in internal membrane compartments. E2 is also responsible for binding the putative host-cell receptor(s), including the CD81 tetraspannin. The function of the small hydrophobic p7 protein, located in the polyprotein at the junction of the structural and non-structural proteins, is unknown. The non-structural proteins form the viral replicase complex. The NS2 protein, together with the amino-terminal region of the NS3 protein, constitutes the NS2–NS3 proteinase, which catalyses a single autocatalytic cleavage between NS2 and NS3. Once released from NS2, the amino-terminal domains of the NS3 proteins serve as serine proteinases for the release of the remaining non-structural proteins from the polyprotein. The carboxy-terminal region of the NS3 protein has RNA helicase activity. The NS4A protein acts as a co-factor/enhancer for the activities of NS3. The function of the hydrophobic integral membrane protein NS4B is unknown, but it interacts with the viral replicase. NS5A is a hydrophilic membrane-associated protein and exists in multiple phosphorylation states. The NS5B protein is the RNA-dependent RNA polymerase. Viral RNA replication is thought to occur in the perinuclear membrane. The replication complex contains the viral polymerase, helicase as well as a number of host-cell factors.

Hepatitis C virus proteins and host-cell factors

The proteins and RNA of HCV also interact with many host-cell factors besides their roles in the viral life -cycle. Interactions of HCV proteins with the translation machinery and post-translational modification systems have been described. In addition, HCV proteins have been proposed to be involved in a wide range of activities, including cell signaling, transcriptional modulation, transformation, apoptosis, membrane rearrangements, vesicular trafficking and translational regulation (Figure 3). Indeed, at least four of the HCV gene products (core, NS3, NS4B and NS5A) have been shown to exhibit transformation potential in tissue culture (Sakamuro *et al*., 1995; Ray *et al*., 1996; Gale *et al*., 1999; Park *et al*., 2000). Owing to the enormous amount of information that has been generated regarding the properties of HCV structural and non-structural proteins and their interactions with cellular proteins and functions, only the findings that are potentially relevant for malignant transformation are summarized below.

Hepatitis C virus core

Hepatitis C virus core is involved in binding viral RNA, regulating HCV RNA translation, making homotypic interactions for particle assembly and interacting with the glycoproteins to generate a complete virion. In addition to these more predictable functions, the core gene product has been proposed to be also involved in cell signaling, transcriptional activation, apoptosis, lipid metabolism and transformation. An extensive list of cellular proteins has been shown to interact with HCV core, but, in most cases, it is still unclear whether these interactions occur in the course of a normal infection or reflect protein overexpression.

Hepatitis C virus core binds to the p53 (Ray *et al*., 1997; Lu *et al*., 1999), p73 (Alisi *et al*., 2003) and pRb (Cho *et al*., 2001) tumor suppressor proteins, but the functional consequences of these interactions have not fully been elucidated. p73/core interaction results in the nuclear translocation of HCV core protein in the presence of the either p73α or p73β tumour suppressor proteins. The interaction with HCV core protein prevents p73α-, but not p73β-dependent cell growth arrest in a p53-dependent manner (Alisi *et al*., 2003). Hepatitis C virus core protein also modulates the expression of the cyclin-dependent kinase (CDK) inhibitor p21/Waf (Wang *et al*., 2000). p21/Waf is a transcriptional target of p53 and regulates the activities of cyclin/CDK complexes involved in cell-cycle control and tumor formation. Downregulation of $p21^{WAF1}$ expression is due to a decrease in the $p21^{WAF1}$ gene transcription and of the $p21^{WAF1}$ protein half-life. Hepatitis C virus core protein is produced as an innate form (amino acids 1–191) that is then processed to produce a mature form (amino acids 1–173). The innate core protein in the cytoplasm increases the amount of $p21^{WAF1}$ by activating p53, and the mature core protein in the nucleus decreases the amount of $p21^{WAF1}$ by a p53-independent pathway (Varaklioti *et al*., 2002; Yamanaka *et al*., 2002).

Additional proteins that interact with C include the LZIP protein (Jin *et al*., 2000), the hnRNP K (Hsieh *et al*., 1998), the RNA helicase DEAD box DDX3 protein (Mamiya and Worman, 1999; Owsianka and Patel, 1999; You *et al*., 1999) and the 14-3-3 protein (Aoki *et al*., 2000). The tumor necrosis factor receptor (Zhu *et al*., 1998) and the lymphotoxin β receptor (Matsumoto *et al*., 1997) have been shown to interact with C and a role in inhibiting apoptosis has been proposed for HCV core through these interactions (Ruggieri *et al*., 1997; Balachandran *et al*., 1998; Ray *et al*., 1998; Zhu *et al*., 1998, 2001; Marusawa *et al*., 1999; Machida *et al*., 2001). Hepatitis C virus core also has been proposed to have immunosuppressive activities through its interaction with the complement receptor C1qR on T cells, thus contributing to chronic infection (Kittlesen *et al*., 2000).

The mechanism by which HCV core regulates transcription has been proposed to be indirect, with the core protein interacting with cytoplasmic signal-transduction molecules and leading to modulation of transcription for genes dependent on these cascades. The

Raf1/mitogen-activated protein kinase (MAPK) pathway has consistently been reported to be activated by HCV core (Aoki *et al*., 2000; Hayashi *et al*., 2000; Tsuchihara *et al*., 2000; Fukuda *et al*., 2001; Giambartolomei *et al*., 2001; Erhardt *et al*., 2002), resulting in relieve from serum starvation growth arrest and cell proliferation. Conflicting reports have shown both activation (Tai *et al*., 2000a, b; Ray *et al*., 2002, Soo *et al*., 2002) and repression (Joo *et al*., 2005) of the NF-κB pathways by HCV core. Although full-length HCV core protein does not translocate to the nucleus (Chang *et al*., 1994; Ravaggi *et al*., 1994; Suzuki *et al*., 1995), a number of reports suggest a more direct, nuclear role of HCV core on transcriptional elements. Recent reports have also implicated HCV core in the activation of the Wnt/β-catenin pathway. Indeed, expression of HCV core protein has been shown to induce cell proliferation DNA synthesis, and cell-cycle progression either alone or in the context of HCV replication, which is mediated by transcriptional upregulation of growth-related genes, in particular wnt-1 and its downstream target gene wisp-2 (Fukutomi *et al*., 2005). Abrogation of wnt-1 expression by specific small interfering RNA blunts core-mediated cell growth and, consistent with secretion of the wnt-1 protein, conditioned medium from wnt-1-transfected cells accelerated cell growth. Microarray analysis revealed significant transcriptional changes in 372 of 12 500 human genes in core protein-expressing cells, with upregulation, besides wnt-1 and wisp-2 of many genes involved in cell growth, oncogenic signaling and cell lipid metabolism and downregulation of genes associated with immunity, cellular defense systems and inflammatory responses (Fukutomi *et al*., 2005).

Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF-β pathway (Pavio *et al*., 2005), suggesting that during chronic infection, viral variants that promote cell transformation by providing clonally expanding cells with resistance to TGF-β antiproliferative effects are actively selected. Indeed, HCV exists as quasi-species in patient sera or tissues and the switch from acute to chronic infection has been associated with a wider variety of viral quasi-species (Thimme *et al*., 2002). Hepatitis C virus genetic variability is well characterized for the hypervariable region 1 of E2 and NS5A (Polyak *et al*., 1998), and it has been involved in viral escape from the immune system and resistance to interferon therapy. Hepatitis C virus quasi-species seem to be compartmentalized into different cell types such as the liver or peripheral blood mononuclear cells (Lerat *et al*., 1998), and, in the liver, different viral variants have been isolated in tumor (T) and non-tumor (NT) regions, suggesting that these may contribute to HCV-induced carcinogenesis (Ruster *et al*., 2001; Alam *et al*., 2002). TGF-β signaling not only controls cell proliferation, differentiation and apoptosis but also plays an important role in liver repair processes and fibrogenesis through its action on the extracellular matrix. Hepatitis C virus-infected patients have high levels of TGF-β, which correlate with the degree of fibrosis (Nelson *et al*., 1997; Marcellin *et al*., 2002; Neuman *et al*., 2002). On the other hand, wild-type core has been shown to upregulate TGF-β expression at the transcriptional level (Taniguchi *et al*., 2004) and to induce TGF-β synthesis in hepatic stellate cells, thus promoting fibrogenesis (Bataller *et al*., 2004). Induction of TGF-β would at the same time favor viral persistence by limiting the antiviral immune response. To reconcile these apparently conflicting findings, it has been proposed that HCV core might have a dual action on the TGF-β system depending on the phase of the disease. Early in HCV infection, HCV core would contribute to fibrogenesis by increasing TGF-β synthesis, and then, after long-term of fibrosis and inflammation and in the context of cirrhotic livers, HCV core variants that contribute to clonal cell expansion and cellular transformation by inhibiting TGF-β-dependent antiproliferative pathways may arise and be selected (Pavio *et al*., 2005).

Exogenously expressed HCV core protein associates with cellular membranes (Barba *et al*., 1997; Hope *et al*., 2002) and lipid vesicles (Moriya *et al*., 1997), binds to apolipoprotein II (Sabile *et al*., 1999; Perlemuter *et al*., 2002; Shi *et al*., 2002) and reduces microsomal triglyceride transfer protein (MTP) activity, leading to defects in the assembly and secretion of very-low-density lipoproteins (Perlemuter *et al*., 2002) and steatosis. The relevance *in vivo* of this interaction is supported by the development of steatosis (Moriya *et al*., 1997; Perlemuter *et al*., 2002) and liver cancer (Moriya *et al*., 2001; Lerat *et al*., 2002) in transgenic mice expressing HCV core.

E2 protein

The impact of other structural proteins on malignant transformation is more indirect. The E2 glycoprotein has been shown to interfere with interferon actions *in vitro* by inhibition of protein kinase R (PKR), an important intermediate of interferon effects (Taylor *et al*., 1999; Crotta *et al*., 2002; Tseng and Klimpel, 2002). In addition, a soluble form of E2 (with the transmembrane domain truncated) interacts specifically with the cell surface marker CD81 and exerts inhibitory effect on the activation of T and natural killer cells *in vitro* (Crotta *et al*., 2002; Tseng and Klimpel, 2002). E2 interaction with CD81 as well as with the LDLR on the cell surface activates the MAPK/extracellular signal-regualted protein kinase (ERK) pathway, including the downstream transcription factor ATF-2 and promotes cell proliferation and cell survival (Zhao *et al*., 2005).

NS3

The NS3 serine protease domain alone can transform mammalian cells, although the link between this interaction and HCC is not clear (Sakamuro *et al*., 1995). The oncogenic properties of NS3 may involve an interaction with the tumor suppressor p53 (Ishido *et al*., 1997; Ishido and Hotta, 1998). A portion near the C-terminus of wt–p53 (amino acids 301–360), which has been reported to contain the oligomerization domain, is important for the complex formation with NS3 (Ishido

and Hotta, 1998). The functional outcome of this interaction is a dose-dependent NS3 repression of the $p21^{WAF1}$ gene transcription. NS3 also contains a histone-binding site and binds histones H2B and H4 (Borowski *et al*., 1999b). The NS3 protein modulates various signal-transduction pathways that have transformation potential. NS3 interacts with protein kinase A (PKA) and inhibits its ability to translocate to the nucleus and catalyse phosphorylation in response to stimulation (Borowski *et al*., 1999b, c). Sequences in NS3 have been show to serve as substrates for protein kinase C (PKC) phosphorylation and can inhibit PKC signaling via competition with normal substrates (Borowski *et al*., 1999b, c). NS3 has also been shown to inhibit interferon response factor (IRF)-3-mediated induction of type I interferon in response to viral infection, which may be important in the ability of HCV to escape immune surveillance (Foy *et al*., 2003). Clearly, more work is needed to characterize and critically evaluate these interactions, particularly those requiring nuclear-localized NS3.

NS5A

NS5A has been implicated in diverse cellular functions, including apoptosis, signal transduction, transcriptional activation and cellular transformation. NS5A has widely been studied for its potential role in blocking IRFs. *In vitro* NS5A is a potent inhibitor of protein kinase R activity (Gale *et al*., 1997). Although the initial studies on PKR–NS5A interaction involved the expression of NS5A in yeast or mammalian cells, a recent publication has also observed some of these phenomena in the context of an HCV replicon (Pflugheber *et al*., 2002). Whether the interaction between NS5A and PKR mediates interferon resistance *in vivo* remains unknown. NS5A also induces interleukin-8, leading to the inhibition of the antiviral effects of IFN (Polyak *et al*., 2001). The binding to the SNAP receptor (SNARE)-like protein hVAPA (Tu *et al*., 1999) mediates NS5A association to cellular membranes and its interaction with lipid droplets (Shi *et al*., 2002), whereas the interaction with the karyopherin β3 protein suggests a role of NS5A nuclear import/transport phenomena (Chung *et al*., 2000).

NS5A has been implicated both in the modulation of cytoplasmic signaling pathways and in the regulation of the cellular transcriptional machinery. Amino-terminal NS5A–Gal4 fusion proteins have *trans*-activating properties (Tanimoto *et al*., 1997). Although intact NS5A is cytoplasmic and membrane-bound (Brass *et al*., 2002), caspase-mediated cleavage exposes a cryptic nuclear localization signal (Ide *et al*., 1996) and leads to NS5A nuclear localization, where it functions as a PKA-regulated transcription factor (Satoh *et al*., 2000b; Goh *et al*., 2001). Truncated versions of NS5A modulate transcriptional. NS5A forms a heteromeric complex with TATA box-binding protein (TBP) and tumor suppressor protein p53 (Qadri *et al*., 2002). NS5A inhibits the binding of both p53 and TBP to their DNA consensus binding sequences *in vitro* and also inhibits the p53–TBP and p53–excision repair cross complementing factor 3 protein–protein complex formation (Qadri *et al*., 2002). In addition, NS5A protein interacts with and sequestrates hTAF(II)32 and hTAF(II)28, components of TFIID and essential co-activators of p53, *in vivo* (Otsuka *et al*., 2000; Lan *et al*., 2002). NS5A and p53 co-localizes in the perinuclear region and the functional consequence of these complex interactions is the inhibition of both transcriptional transactivation by p53 and p53-induced apoptosis (Ghosh *et al*., 2000; Arima *et al*., 2001; Majumder *et al*., 2001; Lan *et al*., 2002). Modulation of cell growth and differentiation by the NS5A protein also occurs through its interaction with the growth-factor-receptor-bound protein 2 adaptor protein (G2b2) (Tan *et al*., 1999) and with the CDK2 (Arima *et al*., 2001). In addition, NS5A protein binds and activates the phosphoinositide 3-kinase (PI3K), resulting in the activation of both the downstream effector serine/threonine kinase Akt/protein kinase B and Akt-dependent survival pathways (Street *et al*., 2004). More recently, it has been reported that NS5A expression in the context of HCV polyprotein results in the inhibition of the transcription factor Forkhead as well as in the phosphorylation and inactivation of the GSK-3, leading to accumulation of β-catenin and stimulation of β-catenin-dependent transcription. It is noteworthy that the HBV-encoded protein HBx also activates the β-catenin pathway through an ERK-mediated inactivation of GSK-3β (Ding *et al*., 2005). This ERK-GSK-3β pathway is activated by IGF1, TGF-β and deregulated tyrosine kinase receptor HER2 activity, and it is responsible for β-catenin upregulation *in vivo* not only in HCCs but also in kidney, stomach and breast cancers (Ding *et al*., 2005). Altogether, these observations strongly suggest that the Wnt/β-catenin pathway is a common target for HCV and HBV proteins (independently from axin/β-catenin gene mutations) in human HCCs.

Hepatitis C virus, endoplasmic reticulum stress and oxidative stress

Hepatitis C virus and other flaviviruses have been shown to induce ER stress (Jordan *et al*., 2002; Tardif *et al*., 2002; Waris *et al*., 2002). Endoplasmic reticulum stress is a homeostatic mechanism that regulates cellular metabolism and protein synthesis in response to perturbations in protein folding and biosynthesis (Ma and Hendershot, 2001). Mild ER stress modulates protein synthesis initiation and causes a reduction in cell growth, whereas extreme or prolonged ER stress leads to apoptosis mediated by the activation of the ER-associated caspase 12 (Kaufman, 1999). Although the long-term consequences of low-level ER stress signaling in the pathogenesis of HCV infection are not well understood, it has been hypothesized that persistent ER stress induction results in intra- and extracellular accumulation of DNA-damaging factors that could predispose a cell to mutagenesis. Indeed, ER stress signaling is intimately linked to changes in the intracellular redox state. Markers of acute intracellular

oxidative stress are elevated in patients with chronic HCV (Sumida *et al*., 2000) and they accumulate the DNA adduct 8-hydroxydeoxyguanosine (Shimoda *et al*., 1994). Transgenic mice expressing HCV core protein show an increased accumulation of ROS that correlates with HCC development (Moriya *et al*., 1998, 2001). Transient expression of HCV NS5A alters intracellular calcium levels, induces oxidative stress and activates STAT-3 and NF-κB (Gong *et al*., 2001; Waris *et al*., 2002). Oxidative stress activates intracellular signaling pathways, including the MAPKs that can have profound effects on cell growth regulation and may also promote transformation.

Hepatitis C virus transgenic mice

The transgenic mouse system has widely been used to study HCV proteins and carcinogenesis (Table 1). Hepatitis C virus gene products have been expressed either alone or in combination in the liver of transgenic mice by using different liver-specific promoters. As already mentioned, three different HCV core transgenic lines develop liver steatosis and HCCs (Moriya *et al*., 1997, 1998; Lerat *et al*., 2002); but other animals show only steatosis (Perlemuter *et al*., 2002) or different phenotypes (Okuda *et al*., 2002), depending on the promoter used, the context of expression and the mouse strain background. NS5A transgenic mice, in spite of the pleiotropic functions of the protein *in vitro*, do not have any significant phenotype (Majumder *et al*., 2002, 2003). The transgenic mice reported so far in HCV transgenes have always been expressed from constitutive promoters. Besides from not being amenable to any postnatal regulation, constitutive expression of HCV proteins *in utero* may easily induce adaptive or compensatory epigenetic that can profoundly affect the animal phenotype.

Occult HBV infection

Cryptic or occult HBV infection is defined as the persistence of HBV DNA into the liver ($\pm$serum) of individuals negative for the HBsAg. This peculiar form of infection occurs frequently in HCV-infected patients, with the highest prevalence reported in Asian populations (reviewed by Torbenson and Thomas, 2002). In the Mediterranean basin, about one-third of the patients with chronic HCV carry such cryptic infection (Cacciola *et al*., 1999; Pollicino *et al*., 2004). Most studies indicate an increased frequency of cirrhosis in occult HBV-infected patients, particularly if HCV co-infected (Torbenson and Thomas, 2002). Cryptic HBV infection is an important risk factor for the development of the HCC in patients with HCV-related cirrhosis (Pollicino *et al*., 2004; Squadrito *et al*., 2006). As occult HBV may persist in the infected livers both in integrated and episomal forms (Pollicino *et al*., 2004), it may contribute to hepatocellular transformation through the same mechanisms traditionally considered the basis of the tumorigenic properties of the HBV, including the capacity of the integrated virus to rearrange the host genome and the potential pro-oncogenic activity of the X protein that shares many molecular targets with HCV core and NS5A, including the ERK kinases, the β-catenin pathway, NF-κB and p53.

Gene expression profile in hepatitis C virus-related hepatocellular carcinomas

Expression profile analysis of HCC samples by comparative gene expression clustering has identified a number of transcripts 'upregulated' (mainly cell growth genes) or 'downregulated' (mainly growth inhibition genes) in HCC tissues (Okabe *et al*., 2001; Shirota *et al*., 2001; Chen *et al*., 2002; Lee and Thorgeirsson, 2002; Smith *et al*., 2003; Ye *et al*., 2003; Breuhahn *et al*., 2004; Lee *et al*., 2004; Neo *et al*., 2004; Nam *et al*., 2005; Patil *et al*., 2005; Thorgeirsson *et al*., 2006). Examples, derived from several studies, of genes whose transcripts are prominently altered, in HCCs relative to non-cancerous liver tissue, include cyclin gene family members, CDC20, CDK4, myb homologs, members of the Wnt/β-catenin pathway, as well as many matrix metalloproteinases. Downregulation was observed for many genes involved with biotransformation, such as

Table 1 Transgenic mice expressing HCV gene products

Viral proteins	*Promoter*	*Pathology*	*References*
E1, E2	HBV	Sjogren-like exocrinopathy	Koike *et al*. (1997)
Core, E2 truncated	MUP	None	Pasquinelli *et al*. (1997)
Core, E1, E2	MUP	None	Kawamura *et al*. (1997)
Core, E1, E2	CAG Cre/loxP	Hepatitis	Wakita *et al*. (1998, 2000)
Core, E1, E2	MHC	Hepatitis	Honda *et al*. (1999)
Core	HBV	Steatosis, adenomas, HCC (two lines) (insulin resistance)	Moriya *et al*. (1997, 1998, 2001), Shintani *et al*. (2004)
Core	ALB	None (oxidative injury)	Okuda *et al*. (2002)
Core	HBV	Steatosis	Perlemuter *et al*. (2002)
NS5A	ApoE	None (resistance toTNF)	Majumder *et al*. (2002)
NS5A	ApoE	None	Majumder *et al*. (2003)
HCV polyprotein or structural proteins	ALB	Steatosis, HCC (resistance to apoptosis)	Lerat *et al*. (2002), Disson *et al*. (2004)
HCV polyprotein	AIAT minigene	None (interference on IFN signaling)	Blindenbacher *et al*. (2003)

Abbreviations: ALB, mouse albumin promoter; ApoE, murine ApoE promoter; CAG, CMV-actin promoter; HBV, hepatitis B virus regulatory elements; HCV, hepatitis C virus regulatory elements; MHC, major histocompatibilty complex; MUP, mouse major urinary protein promoter.

glutathione transferases (Zhou *et al*., 1997), monoamine oxidases and cytochrome genes (Xu *et al*., 2001; Kinoshita and Miyajima, 2002). A number of over-expressed genes encoding for secreted (e.g., GPC3, LCN2 and DKK1) or membrane-bound proteins (e.g., GPC3, IGSF1 and PSK-1), which may be attractive candidates for the diagnosis of HCC, have also been identified (Patil *et al*., 2005). In the analysis of 102 tumors from 82 HBV and HCV patients, Chen *et al*. did not find any consistent distinction between the two groups, whereas other studies could identify at least some distinctive trends between HBV- and HCV-related HCCs (Iizuka *et al*., 2002; Delpuech *et al*., 2002). However, as for the study of viral genes, no specific gene signature has been found that might clearly explain as to how HBV or HCV mediates oncogenesis.

Concluding remarks

Hepatocyte transformation most often occurs in the setting of chronic liver injury, regeneration and cirrhosis. Increased cell turnover in this context of inflammation and oxidative DNA damage facilitates the accumulation of genetic and epigenetic alterations that include the activation of cellular oncogenes and proliferative pathways, telomerase activation, the inactivation of tumor suppressor genes, the over-expression of growth and angiogenic factors.

A central question in HCC research over the last two decades has been whether HCV (and HBV), which account together for the majority of HCC cases world-wide, play an additional direct role in the molecular pathogenesis of HCC, besides their ability to trigger chronic inflammation in the liver. An overwhelming amount of information is now available regarding functions and properties of different HCV-encoded proteins, namely HCV core, E2, NS3 and NS5A, that are potentially relevant for HCC development and tumor progression. However, it is important to under-line that most, if not all, of the putative transforming functions of HCV proteins have been defined and characterized in systems that are artificial, with high-level expression of the viral proteins. Thus, these *in vitro* findings may not be applicable to infection *in vivo*, where HCV proteins are expressed in much lower concentrations, and the true biologic relevance of these

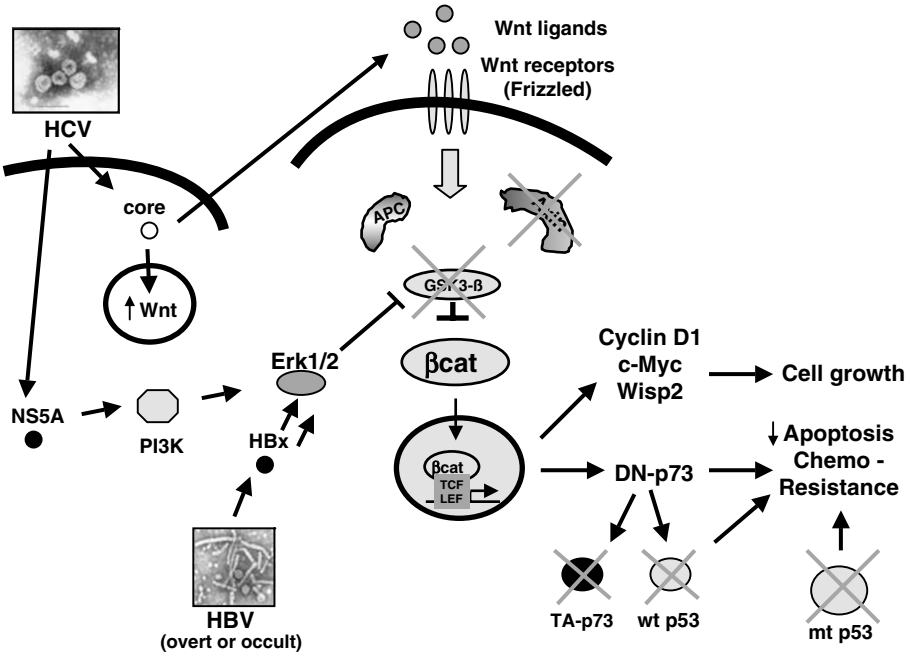

Figure 4 The Wnt pathway is targeted by genetic and epigenetic events in hepatitis C virus (HCV)-related hepatocellular carcinomas (HCCs). β-Catenin accumulation in human HCC may be triggered by multiple mechanisms. Activating mutations of β-catenin and Axin1 gene mutations occur in a substantial proportion of HCC patients but do not account for all HCC cases with β-catenin nuclear accumulation. Frizzled type 7 receptor (Fzd-7) is overexpressed in many human HCCs and induces wild-type β-catenin stabilization/activation. Hepatitis C virus core protein upregulates transcription of several growth-related genes, including wnt-1 that is secreted and activates signaling through the frizzled receptors. NS5A activates the PI3K that in turn phosphorylates and inactivates the glycogen synthase kinase-3 (GSK-3), leading to the accumulation of β-catenin and stimulation of β-catenin-dependent transcription. Interestingly, the hepatitis B virus (HBV)-encoded protein HBx also activates the β-catenin pathway through an extracellular signal-regulated protein kinase (ERK)-mediated inactivation of GSK-3β. Activated β-catenin increases transcription of several target genes that control cell proliferation, including cyclin D1 and c-myc. β-Catenin also potentiate the expression of DN-p73, a dominant-negative p73 isoform that act as a dominant *trans*-repressor of both p53 and proapoptotic TAp73 proteins and confers to HCC cells a chemoresistant phenotype. Thus, in human HCCs, the Wnt/β-catenin pathway is a common target for HCV (and HBV) proteins, independently from axin/β-catenin gene mutations. Conversely, β-catenin-dependent overexpression of DNp73 leads to p53 and TAp73 functional inactivation without requiring the selection of p53 gene-inactivating mutations.

observations remains still to be established in relevant infection and disease models. Indeed, despite all the evidence accumulated, we still do not know whether, and which, viral gene products are necessary *in vivo* for the establishment of HCCs. Another open question is whether and at what point in the development of HCC cancer become independent of the virus, as the relatively low and slow impact on HCC development of successful antiviral treatments in HCV patients with already established cirrhosis would suggest. Although many studies have now outlined a number of genetic and functional differences between HBV- and HCV-related HCCs, much more work is still needed to define whether the heterogeneity of etiological factors implicated in the development of HCC translates into specific genetic and epigenetic mechanisms that may be also be detected as specific molecular signatures. Systematic search for all genetic and epigenetic alterations in large series of tumors, including all grade, stages, etiologies and also pre-neoplastic lesions certainly need to be performed. However, to gain a deeper understanding of the basic mechanisms that control and determine HCC transcriptome and HCC phenotype, descriptive (phenotypic) oncogenomics has proven only partially useful and must be integrated with multiple and innovative functional approaches. From the dissection of the molecular and signaling pathways that may be, according to *in vitro* experiments and animal models, or are altered in human HCCs and/or in subsets of HCC patients, we must now progress to understand how these pathways build-up more complex networks that are relevant for transformation. An example of successful integration of old and new data generated with different approaches into a larger network is the emerging connection between viral proteins, components of the Wnt/β-catenin and members of the p53 family (Street *et al*., 2005; Muller *et al*., 2005; Fukutomi *et al*., 2005; Palescandolo *et al*., 2006) (Figure 4). Combining genomewide and functional approaches will lead not only to a better understanding of the cellular events involved in hepatocyte transformation but also in all likelihood to improve preventive measures and innovative therapies for one of the most devastating human malignancies in the world today.

Acknowledgements

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), from the 'Ministero dell'Istruzione dell'Università e della Ricerca Scientifica' (PRIN, FIRB) and from the European Community (LSHC-CT-2004-503576).

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