

Molecular viral oncology of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the fifth most common cancer, but the third leading cause of cancer death, in the world, with more than 500 000 fatalities annually. The major etiology of HCC/liver cancer in people is hepatitis B virus (HBV), followed by hepatitis C virus infection (HCV), although nonviral causes also play a role in a minority of cases. Recent molecular studies confirm what was suspected: that HCC tissue from different individuals have many phenotypic differences. However, there are clearly features that unify HCC occurring in a background of viral hepatitis B and C. HCC due to HBV and HCV may be an indirect result of enhanced hepatocyte turnover that occurs in an effort to replace infected cells that have been immunologically attacked. Viral functions may also play a more direct role in mediating oncogenesis. This review considers the molecular and cellular mechanisms involved in primary hepatocellular carcinoma, using a viral perspective.

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Introduction

The majority of cases of hepatocellular carcinoma (HCC) in the world are due to hepatitis B virus (HBV), with the number of hepatitis C virus (HCV)-associated cases growing in the Western world (Figure 1) and the incidence of nonviral HCC is also rising in the US (El-Serag and Mason, 1999; Parkin *et al.*, 2001). As shown in Figure 2, HCC is the fifth most frequent cancer in the world, with an estimate of more than 500 000 incidences in 2000. Primary hepatocellular carcinoma (PHCC) refers to HCC originating within the liver. Although some of the information discussed in this review is based upon studies that do not definitively identify the HCC as primary, since most of the HCC discussed here is associated with HBV and HCV and is PHCC, HCC and PHCC designations will be used interchangeably.

Despite being fifth in cancer incidence, worldwide, HCC is the third leading cause of cancer death (Figure 3). The high mortality associated with HCC is because it is often unresponsive to treatment. The high mortality may be in part because the noncapsular part of the liver is lacking in sensory fibers and symptoms of HCC often occur late PHCC and with a 5-year survival rate of less than 5% with or without therapeutic intervention (El-Serag *et al.*, 1999). Moreover, the number of deaths due to PHCC is expected to rise over the next 20 years.

Those chronically infected with HBV have a life risk of death to PHCC of between 10 and 25% (Evans *et al.*, 1998; El-Serag *et al.*, 1999; Montalto *et al.*, 2002). The epidemiology and natural history of HCV is somewhat less understood, but it appears that the lifetime risk of HCC in those chronically infected with HCV will be between 2 and 7% (Di Bisceglie, 1997; Liang *et al.*, 2000). Clearly, viral hepatitis B and C represent major public health problems and it is also worth noting that the number of deaths due to viral hepatitis is approximately 1 million per year, when death due to non-HCC viral causes are taken into account. Although there are certainly similarities between the pathogenesis, biology and even molecular biology of chronic HBV and HCV, these are two distinct viruses making the degrees of clinical similarity all the more striking.

Natural history of HBV HCV infection

Both HBV and HCV infection in people is characterized by the ability to cause either acute infection that is frequently clinically inapparent or an unresolved, long-term persistence (Lok *et al.*, 2001). In either outcome, the liver is the primary site of replication. 'Acute' infections of adults are usually inapparent, although in some cases (perhaps fewer than 1%) a severe life-threatening hepatitis occurs (Lok *et al.*, 2001). As implied, for both HBV and HCV, symptoms associated with long-term (chronic) infection may not be apparent for years but eventually present as fatigue, malaise and other conditions typical of hepatitis (Lok *et al.*, 2001). The long continuum from infection, through chronicity and disease, is shown in Figure 4. Cirrhosis and primary liver cancer, as mentioned, often follow, and may be the

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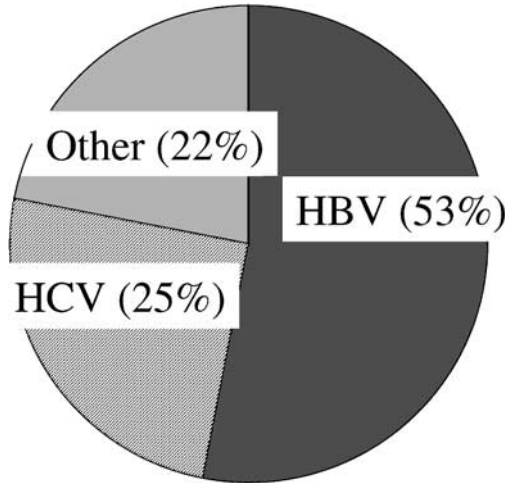


Figure 1 Etiology of HCC. The percentage of worldwide HCC associated with either HBV, HCV or other causes is shown. Data are from GLOBOCAN 2001 and Parkin *et al.* (2001)

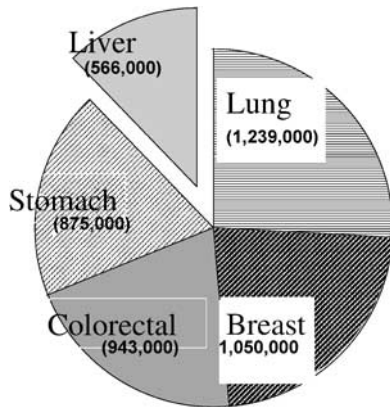


Figure 2 Relative incidence of the five leading cancer diagnoses, worldwide. The relative disease burdens of the five leading causes of cancer in the world in 2000 are compared. Data are from GLOBOCAN and Parkin *et al.* (2001)

result of, many years of unresolved, persistent infection. The rate at which HCC occurs in the individual chronically infected with HBV- or HCV-associated cirrhosis is between 1 and 6% per year (Di Bisceglie, 1997; El Serag and Mason, 1999; El Serag *et al.*, 2001; Lok *et al.*, 2001). Certainly, as experience with HCV becomes greater, more information about the differences between HBV and HCV clinical and pathobiological disease is likely to become appreciated. Nevertheless, it is the frequency with which acute and chronic outcomes result from the two viruses that are the best recognized characteristics of these infections. For example, although HBV infection of neonates usually results in chronic infection, in adults, only 10% of infections with HBV result in chronicity (Lok *et al.*, 2001). On the other hand, the majority of acute adult infections with HCV result in chronicity (Di

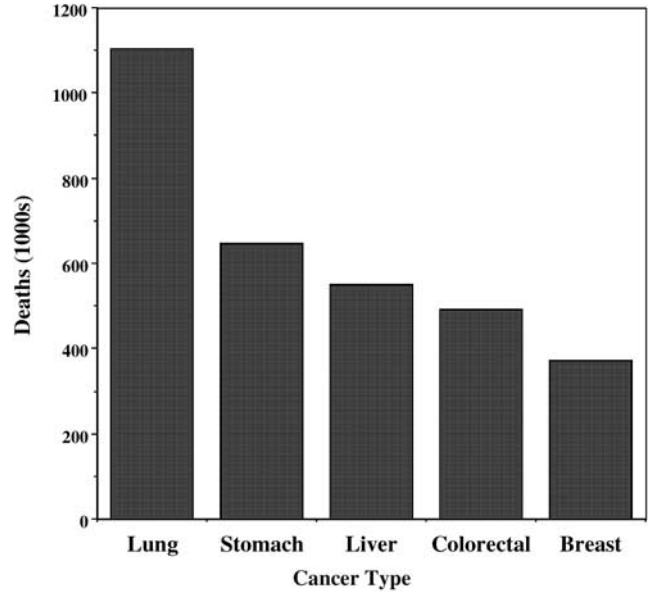


Figure 3 Deaths due to the five most common cancer diagnoses in the year 2000. Data is from GLOBOCAN (2001) and Parkin *et al.* (2001)

Bisceglie, 1997). In either case, it is the population of chronic carriers that is at risk for HCC.

Virology of HBV and HCV

Although, as stated, there are similarities between the natural histories of chronic hepatitis due to HBV and HCV, they are two very distinct viruses. HBV is the prototype member of a virus family called ‘hepadnaviridae’ and is small, partially double-stranded DNA (~3.5 kb) (Seeger and Mason, 2000). As shown in Figure 5a, it specifies a small number of known gene products, including a reverse transcriptase/DNA polymerase (pol), capsid protein (core), envelope (env) proteins (L, M and S) as well as proteins of uncertain function such as ‘X’ and ‘e’. Since its replication depends upon reverse transcription of genome-length RNA, it is called a ‘para-retrovirus’. Since it gains its envelop by budding through the endoplasmic reticulum membrane and replicates near this organelle, we have called HBV ‘ER tropic’ (Jordan *et al.*, 2002b and Romana, P., personal communication).

HCV replication also occurs in the cytoplasm and, similar to HBV, utilizes the endoplasmic reticular (ER) as the primary site of genomic replication and virion assembly (Westaway *et al.*, 1997; Weiland *et al.*, 1999). Upon entry and uncoating, the 9 kb positive stranded RNA viral genome is translated by ER bound ribosomes into a polyprotein that is processed co- and post-translationally by viral and host proteases (Bartenschlager and Lohmann, 2000; Parkin *et al.*, 2001). As illustrated in Figure 5b, the nonstructural proteins of the virus (NS2, NS3, NS4a, NS4b, NS5a, and NS5b) associate with the ER membrane to form the viral replicase (Wolk *et al.*, 2000; Hugle *et al.*, 2001; Mottola

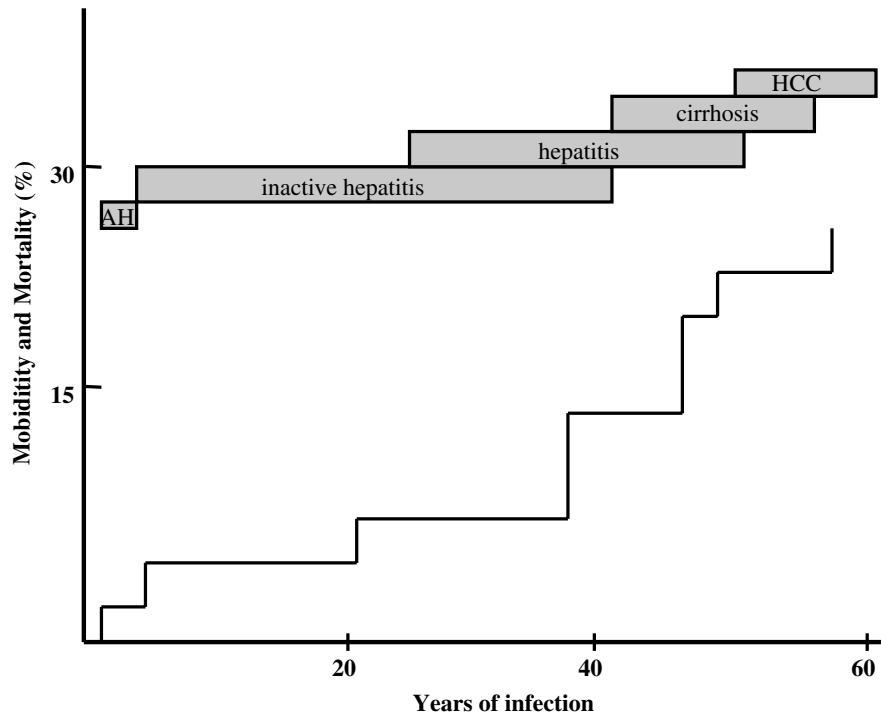


Figure 4 Time line of cumulative morbidity associated with viral hepatitis B or C infection. Years of infection are plotted on the x-axis with cumulative incidence of significant clinical events (cirrhosis or HCC) plotted on the y-axis. The time and incidence data are idealized and not based upon exact statistics, and are intended to show that the progression of disease ranging from acute hepatitis (AH) occurs within the first 6 months of infection and either resolves or leads to chronic infection characterized by possibly decades of inactive hepatitis

et al., 2001; Pietschmann *et al.*, 2001; Egger *et al.*, 2002). Ultrastructural studies of cells containing HCV replicons or the related flavivirus, Kunjin virus, show that the assembly of the viral replicase causes a proliferation of ER membranes (Westaway *et al.*, 1997; Mottola *et al.*, 2001; Egger *et al.*, 2002). Newly synthesized HCV viral RNA interacts with core protein to form the nucleocapsid and formation of the viral envelope occurs by budding into the ER (Weiland *et al.*, 1999; Mackenzie and Westaway, 2001). The viral envelope proteins (E1 and E2) accumulate in the ER as heterodimers and are retained by nonstandard ER retention signals located in the C-terminus of each molecule (Cocquerel *et al.*, 1998, 1999; Duvet *et al.*, 1999; Flint and McKeating, 1999). Virions egress through the normal secretory pathway and viral glycoproteins are thought to be further processed by Golgi enzymes (Jordan *et al.*, 2002a).

Study of the molecular biology of HCV replication has been hindered by the lack of efficient cell culture systems that support high-level HCV replication. Thus, studies of HCV molecular biology have usually been limited to the expression of individual viral proteins. The recent description of HCV replicons has been considered an important advance in HCV research (Lohmann *et al.*, 1999; Blight *et al.*, 2000). 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 nonstructural genes of HCV that

encode the viral replicase (NS3-5b) (Lohmann *et al.*, 1999; Blight *et al.*, 2000). Translation of the *neo* marker gene is mediated by the natural HCV internal ribosome entry site (IRES) while translation initiation of HCV NS2-5b is mediated by an EMCV IRES. Recently, replicons containing full-length HCV genomes have been constructed (Pietschmann *et al.*, 2002). Although these constructs express the entire complement of HCV genes, they replicate with lower efficiency and do not assemble into infectious virions. The advantage of the replicon system over other cell culture models of HCV replication is that the levels of replication and synthesis of HCV gene products are readily detectable by Northern and Western blotting, respectively. In addition, the replicon provides a means to perform molecular genetic studies to determine the function of individual HCV proteins during replication. A disadvantage is that infection from the point of attachment cannot be studied.

Viral oncology of HBV and HCV

Two central questions regarding HCC are (1) which, if any, viral gene products are necessary for the establishment of HCC and (2) at what point, if any, in the development of HCC does the cancer become independent of the virus? These questions remain mysteries for both HBV and HCV. HBV studies have benefited greatly from the study of an animal hepatitis B-like virus as described below.

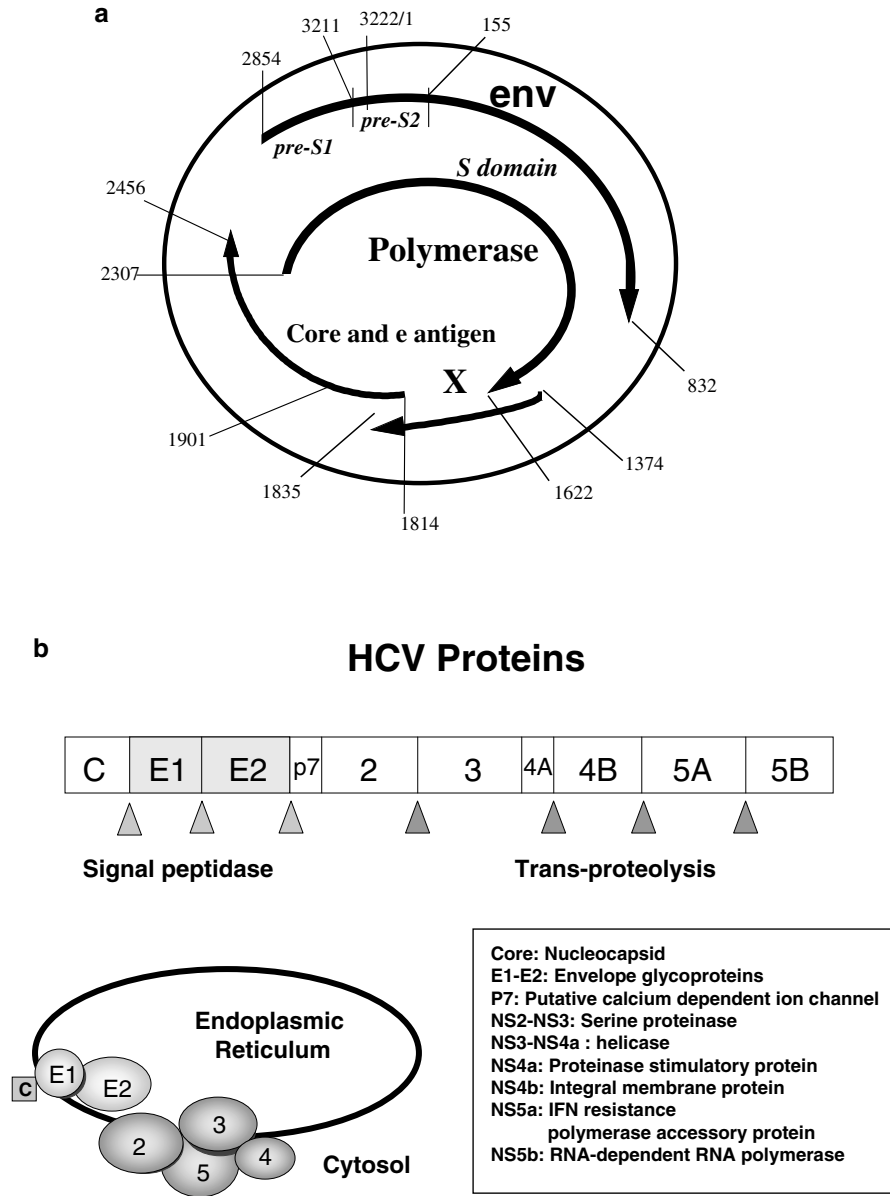


Figure 5 Hepatitis B and C genes and genes products. The HBV genome is shown in a circular form, with bold lines representing transcripts corresponding to the env (envelope or surface), polymerase, 'X', and core products. The numbers indicate the nucleotide positions using the EcoRI site as an arbitrary beginning. The nucleotide positions of the AUG (first codons) of envelope polypeptides PreS1, preS2 and S are shown. The linear, 9 kb, HCV genome is shown, separated into distinct coding regions. A possible scheme for interaction and organization of HCV polypeptides at the ER is also shown and described in the text. Abbreviations are explained in the box

HBV

The hepadnavirus woodchuck hepatitis virus (WHV) is an HBV-like virus that infects and causes chronic hepatitis infection in woodchucks (Tennant and Gerin, 1994; Menne and Tennant, 1999). Although WHV is not HBV and does not infect people, and human HBV does not infect woodchucks, there are biological and genetic similarities between WHV and HBV (reviewed in Menne and Tennant, 1999). Both specify homologous gene products, and both are associated with HCC in their natural hosts. Indeed, chronic infection of neonatal

woodchucks with WHV results in HCC in almost all animals, after a period of years (Tennant and Gerin, 1994). Thus, this has been a very attractive model to study hepadnavirus disease.

Most cancer cells isolated from WHV-induced HCC contain WHV genomes integrated adjacent to cellular N-myc genes (Fourel *et al.*, 1990; Brechot *et al.*, 2000). It is assumed that this juxtaposition of WHV promoters and cellular proto-oncogenes causes an insertional activation responsible for the transformed cell phenotype, analogous to that seen with avian leukosis virus-mediated transformation (Menne *et al.*, 2002). It was

thus tempting to assume that human HBV-mediated HCC would follow a similar pattern. This, however, does not appear to be the case. Indeed, although human HBV can be found to be integrated into the genomes of most cells derived from human HCC in HBV carriers, unlike the situation with WHV HCC, the integration is usually not specific and not associated with activation of any cellular proto-oncogenes (Matsubara and Tokino, 1990; Brechot *et al.*, 2000). The molecular etiology of HBV-induced HCC remains for the most part unclear. It is worth pointing out that there have been reports of HBV integrations near cellular proto-oncogene genes in tumor-derived tissue; thus, promoter integration may indeed occur, but is unusual in people and may be responsible for only a minority of HBV-induced HCC in people (Gozuacik *et al.*, 2001).

Perhaps the reason for the differences between natural human HCC and woodchuck HCC, with respect to insertional activation, may be explained by differences in viremia. WHV infection is usually associated with a very high viremia where viral genomes are in excess of 10^{11} per ml. The high copy number of viral genomes may provide an environment conducive for viral genomic integrations. Although associated with significant viremia, viremia levels in chronic HBV infection in people are usually less than 10^9 genomes per ml. This is substantially less than that seen in woodchuck.

Thus, for most HBV-induced HCC, the molecular mechanisms are unknown. The 154-amino-acid viral gene product 'X' is probably the viral function most frequently implicated in oncogenesis. It is named 'X' because of uncertainty about its function, but it does appear to be important for either viral WHV and HBV replication (Chen *et al.*, 1993; Zoulim *et al.*, 1994). With regard to oncogenesis, X can inactivate or complex with the cellular antioncogene product, p53, which is frequently disabled in HCC (Feitelson, 1999). This activity alone could account for any cellular transformation properties. However, p53 inactivation may occur in only a minority of HBV-induced HCCs, and the relevance of the X : antioncogene observations, however logical, controversial. The 'X' gene product has been shown to transactivate HBV promoters (S, C and X gene promoters) as well as cellular functions associated with cellular growth such as c-fos, c-jun, c-myc and EGF (Feitelson, 1999; Yeh, 2000; Wu *et al.*, 2002). There are also reports that X can influence cytosolic proteasome function and MHC expression (Hu *et al.*, 1999) and perhaps these two phenomena are linked, given the role of proteasome degradation in MHC function (Sirma *et al.*, 1998). X function has also been implicated in influencing DNA repair, suggesting yet another 'hit and run' type means of predisposing the hepatocyte to oncogenesis (Feitelson, 1999). It is difficult to determine which, if any, of the possible ways in which X contributes to HCC are relevant.

Taken together, one model is that X acts indirectly on NF- κ B and possibly AP-1 by activating protein kinase C. NF- κ B activation has also been associated with HCV polypeptides (see below). There is now evidence and

growing interest in the observation that 'X' influences intracellular calcium mobilization, possibly resulting in activation of calcium-dependent kinases that in turn can have multiple cell regulatory effects, including activation of NF- κ B and other various cellular polypeptides and genes, although indirectly (Brechot *et al.*, 2000; Bouchard *et al.*, 2001).

Other circumstantial evidence for the role of 'X' in oncogenesis is that although both the human and woodchuck hepadnaviruses specify an 'X' and are oncogenic in their natural hosts, Duck hepadnavirus is both nononcogenic and lacking 'X' (reviewed in Feitelson, 1999). However, since HCC usually occurs after many years of chronic infection and even then only in a minority of those infected, there has been resistance to the idea that 'X' expression alone is sufficient to explain HBV-induced cancer. Moreover, 'X' expression is variable (even undetectable) in HCC-derived tissue.

The number of different functions that have been attributed to 'X' makes it hard to build a specific hypothesis about its possible mechanism of action. Nevertheless, since the way in which HBV increases the risk of HCC is certainly complex, one should not be too dismissive of the possibility that a single viral gene product is multifunctional.

There may be multiple ways in which HBV enhances the risk of liver cancer. Transgenic mice producing only HBV L envelope protein uniformly develop HCC, suggesting that even the viral envelope polypeptide produced may be hepatotoxic and sufficient to induce malignancy (Chisari *et al.*, 1987; Chisari and Ferrari, 1995). Cytologically, overproduction of viral envelope proteins, particularly L and possibly M, results in their intracellular accumulation and may predispose the cell to stress, which in turn may lead to the development of cancer (Xu *et al.*, 1997). In addition, HBV envelope protein mutants that overaccumulate envelope polypeptides within the cell have been observed to be associated with advancing liver disease and may be, in part, responsible for ground glass hepatocytes and perhaps even HCC lesions (Tai *et al.*, 2002). In general, viral variants containing mutations within core, precore and envelope genes have been, as is indicated, associated with advancing liver disease, although it is not always clear if their occurrence is 'cause' or 'effect' (Feitelson, 1999; Preikschat *et al.*, 2002).

HBV replication appears to involve heat shock proteins (Hu *et al.*, 1997) and viral envelope gene transcription may be actually upregulated by ER stress (Xu *et al.*, 1997). It is possible, as discussed below, that an ER or other cellular stress, induced by the accumulation of viral glycoproteins, could, over a period of time (if not resolved), lead to a cell response that involves mutagenic reactants (see Figures 6 and 7).

Alternatively, perhaps envelope polypeptide-mediated malignancies could be a result of the relentless occurrence of cellular mutations occurring as hepatocytes are induced to divide in an effort to replace dying cells. The role of viral mutants in the pathogenesis of HBV disease is gaining greater appreciation.

HCV

The situation with HCV-induced HCC is even less clear and the notion of a completely cytoplasmic replicating virus inducing oncogenic transformation presents a challenge to conventional biological models. Of course, the theory that the relentless virus-induced immunological killing of infected cells results in chronic hepatocytes growth and opportunities for mutations in the hepatocytes as they replace the killed cells, remains a leading hypothesis, as it is for HBV. However, immunological invasion of livers in chronic HCV

carriers appears to be less prominent than with HBV, and other virological factors that may be cofactors in oncogenesis are receiving attention.

Quasi-species, persistent infection, and HCC Persistent infection is likely to be of central importance to the development of HCC, regardless of whether transformation is induced by immune-mediated turnover of infected cells or caused directly by viral gene products (as discussed below). HCV has evolved a variety of mechanisms to establish persistent infections and evade the host immune response. One mechanism is the generation of viral quasi-species which arise as the result of error-prone replication and are selected for by their ability to replicate in the presence of a robust immune response (Forns *et al.*, 1999). Quasi-species generation and persistence in the face of an active host immune response could be a leading cause of diseases associated with HCV (Cerny and Chisari, 1999; Forns *et al.*, 1999). Indeed, viral clearance during acute infection correlates with reduced populations of quasi-species (Gerotto *et al.*, 1999).

However, the generation of a quasi-species, in this case, is only an explanation for how the virus persists, not how it causes cancer. Several HCV proteins could play a role and have been shown to cause alterations in cellular metabolism or cellular signal transduction pathways that could contribute directly to hepatocyte transformation and HCC.

HCV core protein HCV core protein has been shown to modify intracellular signaling pathways which inhibit immune-mediated cell killing (Chen *et al.*, 1997; Matsumoto *et al.*, 1997; Kittlesen *et al.*, 2000; Zhu *et al.*, 2001). HCV core inhibits TNF- α -mediated apoptosis through a mechanism that involves interactions with the TNF- α receptor (Kittlesen *et al.*, 2000; Tai *et al.*, 2000). TNF- α is a major inflammatory cytokine

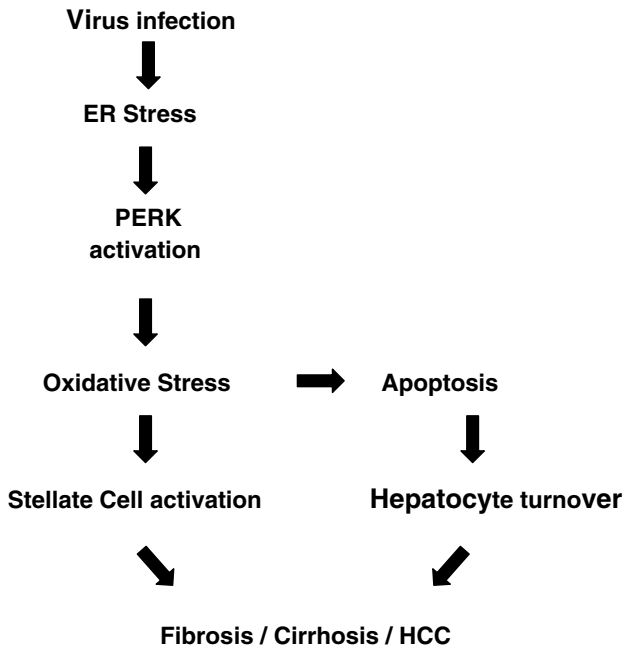


Figure 6 Chronic virus infection and cell stress. Sequential steps from virus infection through cell stress and leading to liver injury and HCC are shown. See text for details

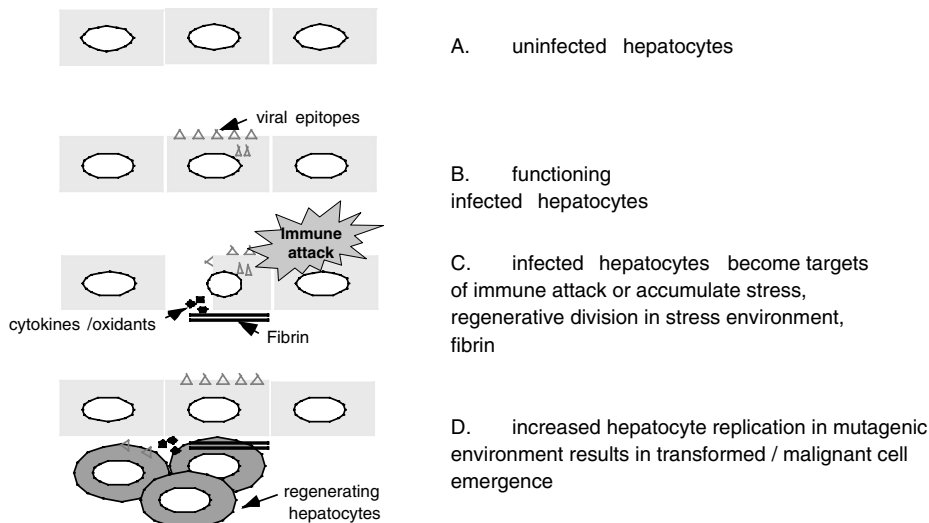


Figure 7 Scheme for how cell infection, injury and turnover can result in emergence of malignant hepatocytes

secreted by activated macrophages and T-cells, which plays a central role in resolving acute infections. TNF- α stimulates FAS-mediated apoptosis and facilitates clearance of infected cells. HCV core binds to the cytoplasmic domains of tumor necrosis factor receptor 1 (TNFR1), lymphotoxin b receptor, and gC1q receptor and blocks FAS/TNF α receptor signaling (Chen *et al.*, 1997; Matsumoto *et al.*, 1997; Kittlesen *et al.*, 2000; Zhu *et al.*, 2001). Blocking TNF- α -mediated signaling would result in the survival of infected hepatocytes and thereby promote persistent HCV infection.

Interfering with cellular signaling pathways may also contribute to cellular transformation by activating cellular transcription factors or interacting with cellular proteins involved in cell growth regulation (Shrivastava *et al.*, 1998). Expression of HCV core has also been shown to activate NF- κ B, a transcription factor that is involved in regulating the immune response (Baldwin, 1996; Zhu *et al.*, 2001). Moreover, hepatocytes from patients chronically infected with HCV show elevated levels of NF- κ B protein and increased NF- κ B DNA binding activity (Tai *et al.*, 2000). In addition, these cells are less responsive to TNF- α (Tai *et al.*, 2000). Based upon these observations, it was hypothesized that HCV core protein promotes persistent infection by down-regulating the host immune system. However, in transgenic mouse studies, comparison of the immune response to intrahepatic challenge with adenovirus showed that expression of core protein did not alter the immune response to viral infection (Sun *et al.*, 2001).

While modulation of transcription factor activity may not alter the immune response to infections, these activities may promote transformation. Transgenic mice expressing HCV core have higher rates of HCC (Moriya *et al.*, 1998). The mechanism of core-induced HCC is not well understood, but may involve interaction with one or more cellular proteins required for the control of cell growth. Indeed, yeast two-hybrid analysis has demonstrated that core protein interacts with a putative cellular helicase (You *et al.*, 1999). While the major function of HCV core is to encapsidate newly synthesized viral RNA, the core has been shown to modulate the activity of transcription factors and cytokines that could promote cellular transformation and HCC.

NS5A NS5a is a nonstructural protein with no known enzymatic activities, but has been implicated in conferring resistance to interferon and altering cellular signaling pathways. Blocking interferon action not only promotes persistent infection but may also circumvent normal cellular signaling pathways that could ultimately lead to cellular transformation. Sequence analysis of NS5a from interferon nonresponder patients showed a high degree of sequence diversity that localized to a region of the NS5a gene (Gale *et al.*, 1998). This region was termed the interferon sensitivity determining region (ISDR). Mutational analysis of the ISDR showed that this region was required for NS5a interaction with PKR, a protein kinase induced by interferon that down-regulates viral translation and mediates interferon's antiviral effects (Gale *et al.*, 1997, 1998). Several lines

of evidence suggest that expression of NS5a in NIH 3T3 cells can partially block the antiviral effects of interferon (Gale *et al.*, 1997, 1998; Song *et al.*, 1999). Consistent with the ability of NS5a to block the inhibitory effects of interferon and activated PKR, selection of cell culture adaptive mutations localize to NS5a in the ISDR element (Blight *et al.*, 2000). However, these cell culture adapted replicons are still sensitive to high-dose interferon treatment. In addition, mutations in other regions of the HCV genome also contribute to increased replication in cell culture (Lohmann *et al.*, 2001). Thus, the ISDR element of NS5a mediates important virus host interactions required for replication and resistance to interferon.

Activated PKR has been shown to be a mediator of apoptosis in response to certain types of cellular stress. Introduction of dsRNA into certain cell types activates PKR and leads to apoptosis (Kaufman, 1999a). Expression of NS5a in NIH 3T3 cells can suppress dsRNA-mediated apoptosis and is dependent upon intact ISDR elements for this effect (Tan and Katze, 2001). In a similar study, NS5a prevented TNF- α -mediated apoptosis. Thus, like core protein, NS5a may suppress the immune-mediated turnover of infected cells (Ghosh *et al.*, 2000). While NS5a can suppress dsRNA-dependent apoptosis in NIH 3T3 cells, NS5a failed to block dsRNA-mediated apoptosis of HeLa cells, suggesting that cell-type specific factors may also contribute to the dsRNA-dependent apoptotic phenotype.

Suppression of dsRNA-mediated or TNF- α -induced apoptosis could also contribute to induction of HCC. Consistent with this hypothesis, constitutive expression of NS5a in NIH 3T3 cells induced transformation and tumor formation after injection of the NS5a-expressing cells into nude mice. While ISDR mutations also showed a growth stimulatory phenotype, they did not cause tumors in mice (Gale Jr *et al.*, 1999; Ghosh *et al.*, 1999). Expression of NS5a in U2OS cells, however, leads to cytopathic effects (Polyak *et al.*, 1999). Moreover, NS5a expression in Chang human liver cells causes a decrease in proliferation through interaction with CDK1/2-cyclin complex (Arima *et al.*, 2001). Thus, it is unclear what role NS5a-dependent inhibition of PKR plays in causing cellular transformation and HCC.

HCV replicase Formation of the viral replicase on the cytosolic face of the ER is likely to play a role in the molecular pathogenesis of HCV. The nonstructural proteins of HCV and Kunjin virus localize to the ER and to virus-specific, ER-like membrane structures that are readily visible by electron microscopy, consistent with viral influences upon the ER (Westaway *et al.*, 1997; Mottola *et al.*, 2001; Egger *et al.*, 2002). Structural alteration in ER morphology may be a generalized property of RNA virus replication (den Boon *et al.*, 2001). Genetic analysis of positively stranded RNA virus replication has shown that cellular genes involved in lipid biosynthesis are essential for the formation of an active replicase (Lee *et al.*, 2001). Moreover, inhibitors of lipid biosynthesis downregulate RNA virus replication (Schlesinger and Malfer, 1982). Although the effects of increased lipid biosynthesis and ER membrane

expansion on cellular physiology are unknown, recent evidence suggests that both processes may contribute to virus-induced ER stress.

ER stress and viral pathogenesis Viruses such as HCV and HBV, which use the ER as an integral part of their replication strategy, must contend with the ER stress response and the downstream consequences of ER stress signaling (see Figure 6). ER 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). The ER stress response works at the level of protein translation and appears to be graded to the level of stress; mild ER stress modulates protein synthesis initiation and can slow down cell growth, while extreme or prolonged ER stress can lead to apoptosis mediated by the ER-associated caspase 12 (Kaufman, 1999b). In addition, transcription factors induced by ER stress regulate ER expansion (Gass *et al.*, 2002).

HCV and other flaviviruses have been shown to induce ER stress (Jordan *et al.*, 2002b). In fact, apoptosis induced by cytopathic strains of BVDV or JEV has been linked to induction of ER stress signaling (Jordan *et al.*, 2002b; Waris *et al.*, 2002). While apoptosis is one extreme outcome of virus-induced ER stress, noncytopathic viruses like HCV, which induce ER stress signaling at sublethal levels, likely cause alterations in cell physiology that can lead to cellular transformation. The long-term consequences of low-level ER stress signaling on the pathogenesis of HCV infection are not well understood, but it can be hypothesized that persistent stress induction that results in intra- and extracellular accumulation of DNA damaging factors could predispose a cell to mutagenesis.

ER stress and oxidative stress

Even low-level ER stress signaling may be a significant cofactor in the pathogenesis of HCV and HBV infection. ER stress signaling is intimately linked to cellular metabolism through connections involving changes in the intracellular redox state. These connections can lead to downregulation of glutathione (GSH) synthesis, which in turn leads to oxidative stress (McCullough *et al.*, 2000) (Figures 6 and 7). Oxidative stress has profound effects on cellular metabolism that can lead to increased mutation rates, changes in cellular proliferation and, at extreme levels, apoptosis (Collins, 1999; Finkel and Holbrook, 2000). Because oxidative stress is linked to changes in cellular proliferation rates and accumulation of DNA damage, a number of studies have implicated oxidative stress in the development of HCC caused by chronic viral hepatitis. Patients with chronic HCV have elevated levels of serum thioredoxin, a marker of acute intracellular oxidative stress (Sumida *et al.*, 2000). In addition, DNA from patients suffering from chronic viral hepatitis have elevated levels of 8-hydroxydeoxyguanosine, a DNA modification that is caused by oxidative stress (Shimoda *et al.*, 1994).

Transgenic mice expressing HCV core protein or intact full-length HBV (another ER-tropic virus) show increased accumulation of ROS that correlates with an increased rate of HCC (Moriya *et al.*, 1998, 2001). Moreover, transgenic mice expressing HBV show increased oxidative damage in hepatocytes destined to develop HCC (Hagen *et al.*, 1994; Moriya *et al.*, 2001). Transient expression of HCV NS5a alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF- κ B (Gong *et al.*, 2001). Thus, oxidative stress plays an important role in hepatitis virus-induced liver diseases and HCC.

Oxidative stress activates intracellular signaling pathways including the mitogen-activated protein kinases (MAPKs) that can have profound effects on cell growth regulation and may also promote transformation. MAPKs regulate cellular proliferation and apoptosis in response to external and internal stimuli and may contribute to diseases associated with oxidative stress (Finkel and Holbrook, 2000). MAPKs are a group of signaling enzymes that include extracellular signal-regulated protein kinase (ERK), c-JUN N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 subfamilies. These signaling cascades are responsible for phosphorylating a variety of other kinases and transcription factors that regulate cell growth, differentiation, and apoptosis. Each signaling cascade responds to a unique set of stimuli, with ERK pathways stimulated to a higher degree by mitogens while JNK/SAPK and p38 are preferentially stimulated by a variety of stresses (Robinson and Cobb, 1997). High-level activation of JNK/SAPK and p38 is associated with apoptosis whereas stimulation of the ERK pathway promotes cell survival (Guyton *et al.*, 1996; Wang *et al.*, 1999; Potapova *et al.*, 2000; Benhar *et al.*, 2001). The relative levels of activation of these various pathways may determine the outcome of the stimuli (Xia *et al.*, 1995). The degree with which ER-tropic viruses interact with these pathways will have profound consequences on the pathogenesis of infection.

Oxidative stress may also activate stellate cells that regulate hepatocyte growth and differentiation. Stellate cells are a major fibrogenic cell in the liver that respond to cytokines, growth factors, and chemokines in response to liver injury. Their normal function is to produce an extracellular matrix to provide a scaffold for normal growth and differentiation of hepatocytes in response to liver damage. Chronic stellate cell activation in response to oxidative stress induced by virus replication could contribute to fibrogenesis and increased proliferation of hepatocytes characteristic of persistent HCV and HBV infection. This increased production of extracellular matrix and hepatocyte turnover coupled with activation of MAPKs may ultimately lead to HCC.

Cellular stress induced by intracellular accumulation of viral polypeptides

As with HBV, described above, it is possible that persistent, unresolved, stimulation of cellular stress responses by accumulation of viral proteins within

hepatocytes can predispose the cell to mutation. BVDV, a pestivirus closely related to HCV, induces ER stress in the infected cell (Jordan *et al.*, 2002b), and HCV polypeptides themselves have been observed to induce ER stress (Tardif *et al.*, 2002; Waris *et al.*, 2002). Induction of these stress pathways, in the extreme, would be expected to cause cell death by apoptosis (Figure 6). However, if the infected cell can compensate, as we suspect, and avoid apoptosis, mediators of cellular DNA damage (e.g. oxidative agents) may be induced (Figure 6), providing a cofactor for transformation. These could be a mechanism of HCV (and perhaps HBV)-induced cellular transformation that does not involve the circulating immune system or specific viral transformation genes. It is noted, however, that despite compelling laboratory evidence for induction of cellular stress by HBV and HCV, there has been very little information from *in vivo* studies. Certainly, *in vivo* work will be more difficult, but is essential to validate the tissue culture work. Perhaps the recently discovered Golgi polypeptide gp73, whose expression in livers has been correlated with HBV or HCV infection, is actually a Golgi 'stress' function (Kladney *et al.*, 2002).

Pathobiology of viral HCC

Immunobiology of chronic HBV and HCV

Both circulating and resident T lymphocytes (T cells) are believed to play major roles in the natural history of chronic HBV (and presumably HCV) as well as in the development of cancer. An active cytotoxic killer T cell (CTL) response, selective for viral polypeptide epitopes such as pol and envelope, is thought to be important in the successful resolution of acute hepatitis (Rehermann *et al.*, 1996), and the level of the CTL response is perhaps the primary factor in mediating the clearance of virus in people (Chisari and Ferrari, 1995; Liang *et al.*, 2000). Chronic carriers of HBV appear to have an inadequate (in quality and quantity) T cell level to viral antigens (Rehermann *et al.*, 1996; Guidotti *et al.*, 1999, 2002; Webster *et al.*, 2000; Webster and Bertolotti, 2001). Indeed, recent work with the woodchuck model, as well as studies in people, are consistent with the notion that reduction of circulating viral envelope antigens, coupled with induction of T cell recognition of viral antigens, is associated with a form of recovery from the chronic carrier state (Chisari and Ferrari, 1995).

It has been recently postulated that immunological management of at least HBV also involves noncytolytic T cells (Guidotti *et al.*, 1999, 2002). There is evidence in animal studies, particularly in transgenic mice expressing the HBV genome, that a subset of CD8 positive T cells can mediate reduction of HBV replication (reduction of viral RNA, nucleocapsids) in the absence of significant hepatocyte killing (Guidotti *et al.*, 1999). This appeared to involve TNF- α and interferon γ .

Thus, it is the orchestration of the T cell response as much as the precise components of the response that is critical in determining outcome. On the other hand, killing of infected hepatocytes may also be responsible for

liver damage, causing liver failure in one extreme, and responsible for persistent liver cell regeneration in which opportunities for oncogenic mutation occur. It is worth noting that, compared to uninfected individuals, the half-life of hepatocytes is much less and the turnover rate is much greater (Seeger and Mason, 2000). The environment in which the hepatocytes replicate, in response to injury, is likely to be enriched for mutagens such as oxidants, which are present during, and as a consequence of, cell injury (Friedman, 2000) and immunological attack. These complications are all aggravated during fibrosis, which is also a predisposition for liver cancer.

Taken together, there are thus at least three arms of the immune system that are engaged in the management and perhaps pathogenesis of HBV-associated liver disease in people, including the circulating T and B cell systems as well as the NK/NKT cell system (Kakimi *et al.*, 2001). Each of these systems requires involvement of myeloid, nonhepatocyte cells, in the recognition and response to infection in the hepatocyte. We have recently shown that small glycolipid mimetics, such as alkyl α -aza galactose and even α -galactosyl-ceramide, can directly activate cellular defense genes (such as the small 2',5'-oligoadenylate synthase gene) and reduce the amount of HBV replication, without recruitment of any cells other than those infected (Mehta, Lu, Dwek and Block, in preparation). Since the alkyl sugars that stimulate this response could be mimetics for pathogen glycolipids, these data suggest that hepatocytes have the ability themselves to autogenously recognize and react defensively to foreign pathogen molecules without assistance from any other immunological cells. It is tempting to speculate that these glycolipid-like molecules are activating a very primitive arm of the host defense system. It is noted that this system is, in many ways, analogous to activation of the innate host defense pathway as seen with dsRNA (Guidotti *et al.*, 1999). In contrast to the situation with dsRNA, however, activation with alkylated aza sugars and α -galactosyl-ceramide appears to induce only a small subset of interferon specific genes and hence is associated with much less toxicity (Mehta, Dwek, and Block, in preparation).

Gene expression profile of HCC

Microarray analysis of 102 tumors from 82 HBV and HCV patients showed no consistent distinction between the two groups, although there were clear distinctions between tumor and nontumor tissue in this (Chen *et al.*, 2002) and other microarray studies (Xu *et al.*, 2001).

Briefly, HCCs, as analysed by gene array, have been shown to have patterns that reveal individualities of the particular clonal tumor as well as smaller subsets of genes whose expression seemed to be associated with HCC as a family (Xu *et al.*, 2001; Chen *et al.*, 2002). Again, as with the viral genes, no specific cellular 'smoking guns' have been found that clearly explain how HBV or HCV mediate oncogenesis. Comparative gene expression clustering analysis has, for example, identified transcripts associated with cell growth as

'upregulated' in HCC tissue, and those associated with growth inhibition as 'downregulated'. Examples, derived from several studies, of the genes whose transcripts are prominently altered, relative to noncancerous liver tissue, are summarized in Table 1. Curiously, although transcripts homologous to α feto protein (AFP) were found to be elevated in some reports (Xu *et al.*, 2001), it is noted that other studies suggested that elevation of AFP did not consistently occur in all samples (Chen *et al.*, 2002). AFP polypeptide detection is a standard laboratory assay for detection of HCC risk, but consistent with the report from Chen *et al.* (2002), only detects cancer in a minority of cases. Some caution must be used in interpreting differential gene expression data, since the pattern of expression in HCC may reflect the degree of de-differentiation of the cells, including a return to fetal-like programs, as well as a 'transformation' expression pattern. Moreover, it may be difficult to distinguish contributions from cancer and surrounding (often stellate) cells, although Chen *et al.* (2002) has employed impressive cluster analysis that permits the identification of these transcript families to serve as 'footprints' of immunological and extrahepatocyte cell presence at the tumor site.

This is not to trivialize these results, but rather to put those observations in perspective. For example, transcripts from cyclin gene family members, CDC20 CDK4 and myb homologues have consistently been shown to be elevated in HCC-derived tissue (Table 1), but it is not clear if this is the result of primary mutations in these genes or, more likely, a consequence of the growth relief generally associated with malignant transformation.

Downregulation was observed for many genes involved with biotransformation, such as glutathione

transferases (Zhou *et al.*, 1997), monoamine oxidases and even members of the cytochrome series (Xu *et al.*, 2001; Kinoshita and Miyajima, 2002). In previous work it has been shown that increased levels of iron correlate with risk of HCC in HBV carriers and this could be imagined to play a role in creating an environment of chemical radicals in or near the liver (Israel *et al.*, 1989; Stevens *et al.*, 1994).

Although these may be more 'effect' than 'cause', it is not difficult to imagine that the downregulation of expression of these genes could be consequential.

On the other hand, cellular genes whose expression appears to be influenced in an HCC-specific manner that have functions that could be directly associated with pathogenesis include members of the wnt catenin (β) pathways, as well as matrix metalloproteinases (MMT). β -catenin is a component of cell adherence junctions but can also mediate activation of the Wnt signaling pathway (Xu *et al.*, 2001). Dysfunction of this pathway has been implicated in a number of malignancies (Sekine *et al.*, 2002).

Other functions that could contribute to disease that are known to be elevated during cirrhosis are TGF- β , increased gelatinases, fibroblast activating proteins, and members of the interferon response pathway (particularly those associated with a Th1 immune response) (Xu *et al.*, 2001; Shackel *et al.*, 2002).

The situation regarding tumor suppressors in general, and p53 in particular, is also complex. p53 expression and detection is rare in nontumor tissues, but often mutant, presumably more stable forms have been detected in HCC tissue (Hsu *et al.*, 1993); the characteristic HCC p53 mutation, present in 10–20% of the tumors characterized, is at an Arg to Ser

Table 1 Examples of transcripts found to be aberrantly expressed in HCC tissue that might have biological consequences¹

<i>Category (italicized) with change (up or down) in HCC compared to non-HCC tissue</i>	<i>Transcript ID</i>	<i>References</i>
<i>Growth</i>		
Up	PCNA	Chen <i>et al.</i> (2002)
Up	Cyclin family members	Chen <i>et al.</i> (2002)
Up	CD34, CDC20, CDK4	Chen <i>et al.</i> (2002)
Up	Myb	Xu <i>et al.</i> (2001)
Up	AV683086	Xu <i>et al.</i> (2001)
<i>Signal transduction/transcriptional regulation¹</i>		
Up/down	Wnt/bcatenin pathway members	Xu <i>et al.</i> (2001), Sekine <i>et al.</i> (2002)
<i>Biotransformation/metabolism¹</i>		
Down	GST (glutathione S transferase)	Zhou <i>et al.</i> (1999), Xu <i>et al.</i> (2001)
Down	Monoamine oxidases	Xu <i>et al.</i> (2001)
Down	Cytochrome series	(Xu <i>et al.</i> , 2001)
<i>Other¹</i>		
Up	MMT	Xu <i>et al.</i> (2001), Chen <i>et al.</i> (2002)
	AV integrins	Xu <i>et al.</i> (2001) Nejari <i>et al.</i> (2002)
	Geletinases	Xu <i>et al.</i> (2001)
	Interferon response family members	Xu <i>et al.</i> (2001), Friedman (2000)
	AFP	Xu <i>et al.</i> (2001)
Down	x, pol	Xu <i>et al.</i> (2001)
Down	Albumin	Chen <i>et al.</i> (2002)

¹Summary of reports of RNA transcripts determined to be altered (up or down) by at least 3 fold in malignant versus non malignant tissue by various methods as described in references.

substitution at codon 249, and is often associated with aflatoxin exposure. This suggests that p53 mutation-associated HCC may have a significant environmental and even geographical influence. In that scenario, HCC in areas where aflatoxin exposure is great would be enriched for p53-related mutations. This does seem to be the case, and thus HCC in China and parts of Africa would be expected to have a molecular mechanism somewhat distinct from HCC in more developed areas of the world.

The tissue levels of several polypeptides, such as antitrypsin, aldehyde-dehydrogenase (ADH), chymotrypsin and c-reactive protein (Hurlimann and Gardiol, 1991), have been reported to correlate with HCC tissue, as determined by immunostaining. Recently, high-resolution two-dimensional gel electrophoresis (proteomic) analysis of HCC tissue has been used (Yu *et al.*, 2000; Steel *et al.*, 2001; Park *et al.*, 2002). In one report, the earlier observations correlating the rise in ADH with HCC were confirmed and extended to show that specific variants appear to be tumor associated, while other variants, such as ADH 2, decline in abundance in HCC tissue (Park *et al.*, 2002). Given the role of ADH in managing alcohol toxicity and the possibility of alcohol consumption as a risk factor for HCC, it is tempting to suggest that ADH 2 may have a special importance in reducing HCC risk.

We have been systematically comparing the proteomes of serum of individuals chronically infected with HBV or HCV as a function of their disease status (Steel *et al.*, 2001). The results are preliminary, but a pattern is unfolding suggesting that various liver-derived polypeptides decline in amount as disease progress. These include complements, apolipoprotein isoforms, and haptoglobins (Steel *et al.*, work in progress). Curiously, a polypeptide with homology to serum amyloid polypeptide is reduced in serum derived from those with an HCC diagnosis (Communale, Block, Mattu, Steel, and Mehta, in progress). An explanation consistent with all the data is that a protease activity that has become elevated with increasing disease severity is responsible

for the proteolysis of various liver-derived polypeptides. It remains to be determined if the polypeptides we have identified are the result of proteolysis and, if so, if common cleavage sites or domains suggest common proteases. It is not a stretch to think that protease levels would be increased in individuals with advancing cirrhosis and HCC, since MMTs, for example, have been consistently found to be elevated in malignant states. In that case, and perhaps not surprisingly, it would be the proteases that most directly reflect the disease progression, with the polypeptide fragments merely serving as reporters.

Conclusions

Although it is often difficult to distinguish between the clinical presentations resulting from chronic hepatitis B and C, and both cause PHCC, these are two very distinct viruses. There may be multiple molecular etiologies of the cancer they cause. There are, however, possible unifying themes, such as causing malignant hepatocyte transformation by indirect, extra virological factors such as inducing hepatocyte regeneration secondary to either virological damage or immunological attack, in a setting that may be enriched for mediators of mutation. All this highlights the interplay between virus and host that often involves a role of the dice of time and environment that determine the ultimate clinical outcome. The central question of which viral gene products are essential to these processes and when in the process do they become un-needed, remain the subject of study.

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