

REVIEW

Genetic mechanisms of hepatocarcinogenesis

Mark A Feitelson^{*,1,2}, Bill Sun¹, N Lale Satiroglu Tufan¹, Jie Liu¹, Jingbo Pan¹ and Zhaorui Lian¹¹Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, PA 19107, USA;²Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, PA 19107, USA

The development of hepatocellular carcinoma (HCC) is a multistep process associated with changes in host gene expression, some of which correlate with the appearance and progression of tumor. Preneoplastic changes in gene expression result from altered DNA methylation, the actions of hepatitis B and C viruses, and point mutations or loss of heterozygosity (LOH) in selected cellular genes. Tumor progression is characterized by LOH involving tumor suppressor genes on many chromosomes and by gene amplification of selected oncogenes. The changes observed in different HCC nodules are often distinct, suggesting heterogeneity on the molecular level. These observations suggest that there are multiple, perhaps redundant negative growth regulatory pathways that protect cells against transformation. An understanding of the molecular pathogenesis of HCC may provide new markers for tumor staging, for assessment of the relative risk of tumor formation, and open new opportunities for therapeutic intervention.

Oncogene (2002) 21, 2593–2604. DOI: 10.1038/sj/onc/1205434

Keywords: hepatocellular carcinoma; hepatitis viruses; loss of heterozygosity; mutations

Introduction

One of the most frequent tumor types worldwide is hepatocellular carcinoma (HCC). It is most commonly associated with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, with chronic exposure to the mycotoxin, aflatoxin B₁ (AFB₁), and is a complication of alcoholic cirrhosis. Combined with other risk factors (Table 1), there are 0.25–1 million newly diagnosed cases of HCC each year (Feitelson, 1986). The incidence of HCC is geographically variable, with the highest frequencies observed in sub-Saharan Africa and in the Far East among countries

where HBV and/or HCV are endemic, and in regions where aflatoxin contaminated peanuts are consumed. HCC is also predominantly male associated, with M:F ratios of up to 2:1 (Beasley and Hwang, 1984). HCC is rapidly fatal, with a life expectancy of about 6 months from time of diagnosis, and a <3% survival rate for untreated cancer over 5 years. Death is often due to liver failure associated with cirrhosis and/or rapid outgrowth of multilobular HCC. Although early HCC is cured by surgical resection, the fact that many tumors are asymptomatic, means that most patients at risk will not be diagnosed in time. These patients often present with inoperable HCC, which usually reflects the presence of large and/or multiple HCC nodules. Despite the high incidence and mortality, the development of an effective vaccine for HBV, combined with its universal administration to newborns in endemic countries, will significantly lower the incidence of HCC within the next generation.

There is accumulating evidence for the multistep nature of HCC. For example, mutations in p53, Rb, and/or the insulin-like growth factor receptor 1 (IGF_{R1}) genes have been reported in HCC (Kim *et al.*, 1996; Murakami *et al.*, 1991), while *c-myc* and cyclin D1 are frequently overexpressed (Nishida *et al.*, 1994; Peng *et al.*, 1993). Loss of heterozygosity (LOH) involving multiple chromosomes in single tumors also strongly suggests multistep carcinogenesis (Boige *et al.*, 1997; Marchio *et al.*, 1997). A multistep process is also suggested histologically, where HCC appears within the context of chronic hepatitis and/or cirrhosis within regions of liver cell dysplasia or adenomatous hyperplasia (Lencioni *et al.*, 1994). Both of the latter are believed to be precancerous lesions. HCC also appears to evolve from adenomas in HBV encoded X antigen (HBxAg) transgenic mice (Ueda *et al.*, 1995), and from similar lesions in *c-myc*, *c-myc*/transforming growth factor alpha (TGF α), and *c-myc*/TGF β 1 transgenic mice that develop these tumors (Factor *et al.*, 1997; Murakami *et al.*, 1993). Interestingly, the *c-myc* locus at 8q24.12–13 is amplified in human HCC (Kusano *et al.*, 1999; Niketeghad *et al.*, 2001; Peng *et al.*, 1993; Wong *et al.*, 1999a), and appears to be up-regulated in microarray analysis of human tumors (Shirota *et al.*, 2001), suggesting that these transgenic models mimic events in human hepatocarcinogenesis. There is also histopathological variability in the presentation of HCC in geographically diverse regions

*Correspondence: MA Feitelson, Room 222 Alumni Hall, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107 USA; E-mail: Mark.Feitelson@mail.tju.edu

Received 3 December 2001; revised 15 February 2002; accepted 21 February 2002

Table 1 Risk factors that contribute to the development of HCC

Infectious agents:	Hepatitis B virus ^a , hepatitis C virus ^a
Pathology:	Chronic liver disease (macronodular cirrhosis) ^a , neonatal hepatitis ^b
Dietary:	Long-term aflatoxin consumption ^a , dietary iron overload ^b , low vegetable intake ^b , chronic alcoholism ^b
Hormone related:	Oral contraceptives (female) ^b , anabolic steroids ^b , elevated serum levels of testosterone ^b
Other environmental:	Vinyl chloride ^a , cigarette smoking ^b , inorganic arsenic ingestion ^b , radioactive thorium dioxide (thorotrast) exposure ^b , pesticides (?)
Genetic associations ^c :	Hereditary tyrosinemia ^a , α_1 -antitrypsin deficiency ^a , idiopathic hemochromatosis ^a , porphyria (HCV) ^b , Wilson's disease ^b , genetic polymorphisms of cytochrome P450 2E1 and 2D6 and arylamine N-acetyltransferase 2 (involved in detoxification) ^b and <i>L-myc</i> ^b , mutations in <i>GST</i> ^d , familial aggregation ^c
Other:	Elevated TGF α ^a , age (> 50) ^a , gender (male) ^b , elevated expression of <i>neu</i> oncoprotein ^b , low serum retinol (?)

^aStrong association with the development of HCC. ^bWeak or inconsistent association with the development of HCC. ^cCirrhosis, and not the underlying genetic mutation(s), appears to be the major risk factor associated with the development of HCC. ^dA dose-response relationship between AFB exposure and HCC has been documented in HBV carriers with a GST null genotype. ^eFamilial aggregation of HCC may result from environmental agents such as HBV or HCV infections, although a genetic basis for HCC susceptibility in such families has not been excluded

of the world (Okuda, 1997). For example, some slow growing, differentiated HCC nodules surrounded by a fibrous capsule are common among Japanese. In contrast, a 'febrile' form of HCC, characterized by leucocytosis, fever, and necrosis within a poorly differentiated tumor, is common in South African blacks (Okuda, 1997). While these observations suggest an underlying sequence of steps, a major problem in diagnosis and prognosis is the lack of molecular markers that characterize the pathogenic pathways that contribute to HCC (Collier and Sherman, 1998). This knowledge would help to identify patients with early HCC and to devise specific intervention strategies based upon these biochemical changes. Hence, this review presents an overview on the genetic mechanisms of HCC.

Early events in hepatocarcinogenesis

Although HCC is multistep, and its appearance in children suggest a genetic predisposition exists (Chang *et al.*, 1989), the inability to identify most of the predisposing genes, and how their altered expression relates to histological lesions that are the direct precursors to HCC, has made it difficult to identify the rate limiting steps in hepatocarcinogenesis. However, there are a number of molecular changes that occur in high frequency within cirrhotic tissue and small tumor nodules that likely represent early changes in the development of tumor. For example, in chronic HBV infection, HBxAg has been shown to bind to and functionally inactivate the tumor suppressor, p53 (Huo *et al.*, 2001; Ueda *et al.*, 1995; Wang *et al.*, 1994), and the negative growth regulator, p55^{sen}, by cytoplasmic sequestration (Feitelson *et al.*, 1999; Ueda *et al.*, 1995). Interestingly, both of these molecules appear to be part of senescence related pathways. In addition, there is also a direct correlation between cytoplasmic sequestration of wild type p53 and the development of altered foci, adenomas, and HCC in HBxAg transgenic mice (Ueda *et al.*, 1995). The finding that HBxAg relieves the p53 suppression of the alpha-fetoprotein gene (Ogden *et al.*, 2000), which is associated with the

nuclear co-localization of HBxAg and p53 (Greenblatt *et al.*, 1997), may provide an explanation for the up-regulation of alpha-fetoprotein in up to 80% of HCCs. In these and other studies (Boix-Ferrero *et al.*, 1999; Bourdon *et al.*, 1995; Buetow *et al.*, 1992), high levels of wild type p53 in HCC cells are likely to be tolerated because it is inactivated by direct binding to HBxAg, and because HBxAg has been shown to inhibit the proteasome (Sirma *et al.*, 1998; Zhang *et al.*, 2000), which is responsible for the normal degradation of p53 (Maki *et al.*, 1996). HBxAg also transcriptionally down-regulates the expression of the translation initiation factor, *sui1*, as well as the senescence factor and cyclin dependent kinase inhibitor, p21^{WAF1/CIP1/SDI1}, both of which inhibit hepatocellular growth (Feitelson *et al.*, 1999). Functional inactivation of the retinoblastoma (Rb) tumor suppressor by hyperphosphorylation has also been observed in HBxAg positive cells, resulting in the activation of E2F1 and stimulation of the cell cycle (Sirma *et al.*, 1999). The finding that these steps occur in non-tumor liver cells implies that the inactivation of these pathways represent important early steps in hepatocarcinogenesis. In addition, HBxAg appears to activate the expression of insulin-like growth factor 2 (IGF2) in premalignant proliferative nodules (D'Arville *et al.*, 1991) and IGF_{R1} in hepatoma cell lines (Kim *et al.*, 1996) implying that HBxAg may set up an autocrine loop (Aihara *et al.*, 1998) that enhances cell growth independent of other serum growth factors. Further, HBxAg stimulated cell growth is associated with constitutive activation of the *ras/raf/MAPK* and *NF κ -B* signal transduction pathways (Benn and Schneider, 1994; Lucito and Schneider, 1992). In this context, the recent finding of up-regulated expression of dynein in HCC (Shirota *et al.*, 2001), whose anti-apoptotic effects may be associated with its binding to and inactivation of the *NF κ -B* inhibitor, *I κ B*, highlights a possible role for *NF κ -B* signal transduction in tumor development. In addition to *NF κ -B*, up-regulated expression of *rhoB* has been reported in some HCCs (Shirota *et al.*, 2001). *RhoB* is in the *ras* gene family, is associated with cell transformation, and may be a common denominator to both viral and non-viral hepatocarcinogenesis. Activa-

tion of ras and NF κ -B, combined with down-regulation of multiple negative growth regulatory pathways, then, may contribute importantly to early steps in hepatocarcinogenesis.

Similar studies with HCV proteins have just begun. HCV core and nonstructural protein 5A (NS5A) have been shown to have *trans*-activating properties (Kato *et al.*, 1997; Ray *et al.*, 1995). At least one strain of HCV core transgenic mice develops HCC (Moriya *et al.*, 1998) while NS3 and NS5A have been shown to transform NIH3T3 cells (Ghosh *et al.*, 1999; Sakamuro *et al.*, 1995). One way NS5A may transform cells is by binding to and inactivating the double stranded RNA protein kinase, PKR, which may act as a tumor suppressor (Gale *et al.*, 1999). Alternatively, HCV core has been shown to stimulate *c-myc* expression (Ray *et al.*, 1995), while NS5A suppresses transcription of *p21^{WAF1/CIP1/SDI1}* in HepG2 cells (Ghosh *et al.*, 1999). NS3 binds to and presumably inactivates p53 (Ishido and Hotta, 1998). It has also been reported that HCV core transcriptionally represses the p53 promoter in COS7 and HeLa cells (Ray *et al.*, 1997), suggesting that the inactivation of this tumor suppressor is an important step in HCV mediated hepatocarcinogenesis. The finding of a low percentage of p53 mutations in HCV infected patients with HCC (Pontisso *et al.*, 1998) is consistent with the possibility that in HCV (and HBV) infection, one or more viral proteins may inactivate wild type p53. The constitutive activation of the mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway by HCV core expressing HepG2 cells in the presence of a tumor promoter (Hayashi *et al.*, 2000), combined with observations that HCV core inhibits Fas and tumor necrosis factor (TNF- α)-mediated apoptosis in selected cell lines (Marusawa *et al.*, 1999; Ray *et al.*, 1998), suggests additional mechanisms of cell survival that may contribute importantly to hepatocarcinogenesis. These events suggest that one or more HCV gene products may contribute to early changes in the development of HCC, although it is not clear whether the alterations observed *in vitro* and/or in tissue culture cells accurately reflect *in vivo* mechanisms. Recently, however, the finding of elevated CD40 expression in the majority of well- or moderately differentiated HCC as well as in HCC derived cell lines, and that elevated CD40 protected cells against Fas and TNF receptor mediated apoptosis (Sugimoto *et al.*, 1999), suggests a mechanism whereby early tumor nodules survive immune mediated attack and removal. Although it is not clear whether HCV (or HBV) up-regulate CD40 expression, these observations provide important areas for future research.

Important hallmarks in the pathogenesis of HCC are changes in gene expression that underlie phenotypic changes in tumor development. As outlined above, viral proteins may alter the patterns of hepatocellular gene expression by transcriptional *trans*-regulation. Changes in gene expression associated with hyper- and hypo-methylation of cellular DNA have also been observed in preneoplastic lesions (Kanai *et al.*, 1999)

and during tumor progression (Kanai *et al.*, 1996) although the significance of these changes are not clearly understood in most cases. Recently, however, a strong correlation was reported between hypomethylation of satellite DNA sequences, chromosome 1q copy gain, and the presence of a breakpoint of 1q12 (Wong *et al.*, 2001). It was proposed that the hypomethylation altered interaction between the CpG-rich satellite DNA sequences and chromatin proteins, resulting in heterochromatin decondensation, aberrant 1q formation, and breakage. Hence, changes in the methylation patterns of preneoplastic cells may alter genetic stability and contribute to altered patterns of gene expression that are important for tumor development.

A closer look at differential gene expression using microarray analysis has shown up-regulation of the MAPK pathway in HCC, as well as many genes associated with an activated cell cycle (Okabe *et al.*, 2001; Xu *et al.*, 2001), suggesting that their constitutive overexpression was associated with the high incidence of chromosomal instability observed in HCC. This analysis also showed an up-regulated expression of several hepatocyte nuclear factors (e.g., HNF-1, -3 β , 4 α , and 4 γ) as well as components involved in protein translation (e.g., ribosomal and poly(A) binding proteins), suggesting both transcriptional and translational changes distinguish tumor from non-tumor. Independent work has also shown up-regulated expression of a locus that maps to chromosome 6p21-22 which encodes (among other things) a ubiquitin-like protein (SMT3B) that may accelerate the catabolism of selected proteins (Shirota *et al.*, 2001). The latter suggests posttranslational differences in gene expression at the level of protein stability. When down-regulated genes were examined, the majority encoded hepatocyte specific gene products (e.g., complement components, albumin and amyloid) and detoxification enzymes (e.g., cytochrome P-450), suggesting a less differentiated phenotype (Okabe *et al.*, 2001). Down-regulated genes also included several involved in retinoid metabolism (*LY6E* and *RBPI*), whose expression is associated with retinoid-induced differentiation. The independent finding that several down-regulated genes in HCC were effectors of liver enriched transcription factors, the latter of which are important in liver development and differentiation, also suggests that differentiation is altered during hepatocarcinogenesis (Xu *et al.*, 2001). These molecular changes are consistent with histopathological changes where early, differentiated HCC slowly gives rise to the moderately or undifferentiated tumors that characterize late tumors. When tumors from HBV or HCV infected patients were compared, the HCV tumor cells had increased levels of enzymes (e.g., cytochrome p450IIE) that metabolize chemical procarcinogens to active metabolites. In contrast, HBV tumor cells had decreased levels of proteins (such as glutathione peroxidase) that detoxify chemical carcinogens, suggesting that tumor cells have an increased sensitivity to the mutagenic effects of chemical carcinogens. These differences between HBV

and HCV associated HCC, however, have not been independently observed (Shirota *et al.*, 2001).

Another early event appears to involve the mutation of β -catenin, which is a component of the Wnt signal transduction pathway whose target genes include *c-myc*, *c-jun*, cyclin D1, fibronectin, the connective tissue growth factor WISP, and matrix metalloproteinases (Calvisi *et al.*, 2001). Normally, β -catenin is a submembranous protein associated with E-cadherin and participates in cell-cell adhesion. Its phosphorylation results in ubiquitination and proteasome mediated degradation. The finding of mutated β -catenin in early stages of human HCC (De La Coste *et al.*, 1998; Hsu *et al.*, 2000; Miyoshi *et al.*, 1998) and in adenomas of *c-myc* transgenic mice (Calvisi *et al.*, 2001) suggests that mutation results in alterations in normal cell-cell interactions that occur during tumor formation. In addition, the stimulated expression of extracellular matrix protein genes by constitutively active β -catenin in the nuclei suggests that β -catenin mutations may play a significant role in the development of fibrosis and cirrhosis, which are lesions that precede the development of HCC. In addition, an early role for Wnt activation in hepatocarcinogenesis comes from observations that transgenic mice expressing an oncogenic (mutated) form of β -catenin rapidly develop hepatomegaly (Cadoret *et al.*, 2001), demonstrating that constitutively activated β -catenin strongly stimulates hepatocellular growth *in vivo*. However, mutant β -catenin is not associated with metastases among HCC patients (Mao *et al.*, 2001), which also suggests that it is an early target in hepatocarcinogenesis. Interestingly, the inhibition of the proteasome by HBxAg (Huang *et al.*, 1996) may block the normal degradation of wild type β -catenin, resulting in its stabilization and accumulation at the early stages of tumor development, although there is presently no data to support this mechanism. Interestingly, the finding that p53 may down regulate Wnt signaling (Wang *et al.*, 2000) implies that the inactivation of p53 by HBxAg (Ueda *et al.*, 1995; Wang *et al.*, 1994) may increase Wnt signaling. In addition, the prevalence of β -catenin mutations in HBV negative HCCs (Laurent-Puig *et al.*, 2001) is consistent with other mechanisms of inactivation (e.g., involving HBxAg and p53) being operative in HBV positive tumors. Moreover, the observation that TGF β 1 promotes fibrogenesis and HCC development in transgenic mice (Factor *et al.*, 1997), and that TGF β 1 expression is stimulated by HBxAg (Yoo *et al.*, 1996), provides another mechanism whereby HBxAg contributes to tumor formation through the development of cirrhosis, which most often gives rise to HCC. Although HCC is resistant to TGF β 1, its up-regulated expression by tumor cells may inhibit the growth of surrounding non-tumor cells, thereby favoring tumor growth, and/or by inhibiting anti-tumor immune responses (Rossmanith and Schulte-Hermann, 2001). It is also possible that HCV core protein stimulation of *c-myc* may involve the stabilization and nuclear translocation of β -catenin, although this remains to be demonstrated.

Early nodules of HCC are often small, well differentiated, and are surrounded by a sheath of fibrotic tissue that originally formed during the development of cirrhosis. In this context, the success of tumor growth depends upon the invasion of the tumor into the neighboring portal tracts and fibrotic bands in the liver. This process is accompanied by up-regulated expression of matrix metalloproteinase-1 (MMP-1) and -7 (MMP-7) (Okazaki *et al.*, 1997; Ozaki *et al.*, 2000), which act by breaking down extracellular matrix, thereby permitting outgrowth of tumor. Elevated MMP-1 expression has been detected in early, differentiated HCC, but is absent from moderately or poorly differentiated tumor cells in late HCC. This suggests that early HCCs grow by destroying extracellular matrix, while late HCCs grow by virtue of a very high proliferative rate, and that MMP-1 expression correlates with the state of cell differentiation. MMP-7 expression is also up-regulated in early, differentiated tumor cells, promoting angiogenesis and the intrahepatic spread of tumor. The finding that β -catenin and the Ets oncoprotein stimulate of MMP-7 expression (Ozaki *et al.*, 2000), and that HBxAg trans-activation maps to Ets binding sites on cellular genes (Park *et al.*, 2000), implies that HBxAg may modulate the outgrowth of early HCC by defining the integrity of the extracellular matrix during chronic infection.

There are several mechanisms that promote the development of mutations in the pathogenesis of HCC. Some chemical carcinogens, or metabolites thereof, are directly mutagenic. AFB₁ forms adducts with DNA and protein, and is responsible for mutation of p53 at codon 249 (Liang, 1995). The ability of AFB₁ to cause mutations is partially dependent upon the genetics of the host. Individuals with mutations in one or both enzymes that detoxify AFB₁ are at a higher risk for the development of HCC than patients with wild type detoxification capabilities (McGlynn *et al.*, 1995). This may explain why there is a much higher frequency of HCC in Asian compared to African HBV carriers who are exposed to similar levels of AFB₁. In addition, the expression of O⁶-alkylguanine-DNA-alkyltransferase (ATase), which repairs the O⁶-alkylguanine lesions in DNA mediated by N-nitrosocompounds, is sequestered from the nucleus (where it is active) to the cytoplasm in the livers of HBV infected patients with cirrhosis (Lee *et al.*, 1996). Interestingly, decreased ATase activity may result in G to T transversion mutations (Abbott and Saffhill, 1977) that, for example, are frequently found in p53 gene mutations that accompany HCC (Bressac *et al.*, 1991; Hsu *et al.*, 1991). Alternatively, ethanol sensitizes liver cells to cytokine-mediated damage and promotes the peroxidation of lipids and proteins that trigger immune responses to these altered proteins. In addition, HBxAg and HCV core antigen confer a cellular phenotype that is resistant to cytokine mediated apoptosis (Elmore *et al.*, 1997; Markusawa *et al.*, 1999). The latter would protect virus-infected cells with accumulating mutations against immune elimination. This is because the inflammatory responses to chronic HBV or HCV infections, to prolonged AFB₁

exposure or chronic alcoholism result in the accumulation of reactive oxygen intermediates (ROI), which are mutagenic (Guyton and Kensler, 1993). ROIs also up-regulate signal transduction pathways and 'primary response genes' such as *c-fos*, *c-jun*, and *c-myc*, which result in altered patterns of host gene expression that, in part, regulate cell growth (Guyton and Kensler, 1993). These are some of the many paths that promote the development of mutations early on in hepatocarcinogenesis.

Integration of HBV DNA into HepG2 cells or into the host chromosomes of transgenic mice has resulted in chromosomal instability (Hino *et al.*, 1991; Livezey and Simon, 1997), which may contribute to LOH at many sites during chronic infection. In fact, LOH was widespread in tumors derived from HBV carriers compared to HBV negative patients (Laurent-Puig *et al.*, 2001), suggesting that chronic HBV infection is associated with chromosomal instability. The binding of HBxAg to p53 and to a ultraviolet light induced DNA binding protein (Becker *et al.*, 1998) suggest that HBxAg may also directly compromise DNA repair pathways that are normally operative in the presence of elevated ROI. Oxidative stress also precedes the development of HCC in transgenic mice that over-produce and accumulate intracellular HBsAg (Dunsford *et al.*, 1990), and in genetic diseases characterized by intrahepatic accumulation of a cellular protein. In these cases, where the genetic defect results in protein overexpression, is tightly linked to increased risk for the development of HCC (Table 1). Integration of HBV DNA into chromosomal DNA has also been shown to be in or near the cyclin A gene (Wang *et al.*, 1990), the retinoic acid receptor gene (Benbrook *et al.*, 1988), and several other genes that are known to regulate cell viability and growth (Gozuacik *et al.*, 2001), suggesting that HBV may act as an insertional mutagen in a small fraction of patients with HCC (Dejean and de The, 1990). The great majority of HBV DNA integration events, however, are random with respect to the sites within host DNA. Many of these events result in the integration of the X gene, which usually results in the abundant production of X mRNA (Diamantis *et al.*, 1992; Paterlini *et al.*, 1995) and protein (Haruna *et al.*, 1991; Wang *et al.*, 1991a,b) in the livers of patients with chronic liver disease. Closer examination of the HBxAg polypeptide made by these integrants has shown that it is often truncated at the carboxy-terminus (Poussin *et al.*, 1999). Interestingly, full-length HBxAg is a *trans*-activating protein (Rossner, 1992) that inhibits cell proliferation and promotes apoptosis (Elmore *et al.*, 1997; Kim *et al.*, 1998; Sirma *et al.*, 1999), while truncated HBxAg appears to have lost its *trans*-activating function, but promotes cell proliferation and transformation in cultured cells (Tu *et al.*, 2001). Hence, the place within host DNA that HBV integrates may not be nearly as important for transformation as the product(s) of the integrated viral DNA fragments and their levels of expression.

A number of studies have reported that LOH at 1p (Kuroki *et al.*, 1995), 4q (Konishi *et al.*, 1993;

Niketeghad *et al.*, 2001; Yeh *et al.*, 2001), 6q (DeSouze *et al.*, 1995; Niketeghad *et al.*, 2001), and 8p (Kishimoto *et al.*, 1996) occur early in preneoplastic liver or in small, differentiated tumors (Huang *et al.*, 1999). Most of these losses have been mapped to tumor suppressor genes that normally limit hepatocellular growth and survival, although LOH may also include genes involved in DNA repair, in carcinogen metabolism, or that protect against oxidative damage. For example, LOH at chromosome 1p includes many putative tumor suppressor genes (Knuutila *et al.*, 1999), including the p53 relative, p73. The latter, which maps to 1p36, is an imprinted gene expressed only from a maternal allele (Falls *et al.*, 1999), suggesting that loss of the single functional copy of p73 may contribute to increased tumor susceptibility. However, the finding that p73 mutations in HCC are infrequent (Peng *et al.*, 2000), and that p73 expression is up-regulated in HCC (Tannapfel *et al.*, 1999), suggest that p73 is not the target of LOH at 1p. Even so, elevated p73 expression in HCC correlates with a significantly poorer survival (Tannapfel *et al.*, 1999), suggesting that elevated p73 in tumor may be a response to physiological stresses that accompany tumor growth, such as nutrient deprivation and hypoxia. Independent evidence, however, has documented LOH at 1p36-p34 not only in HCCs, but also in cirrhotic and dysplastic nodules, indicating the presence of one or more tumor suppressors that are lost prior to the development of tumor (Sun *et al.*, 2001). One gene (designed RIZ), located within 1p36.13-p36.23, appears to be a tumor suppressor that is frequently deleted in HCC (Fang *et al.*, 2000), verifying that commonly deleted regions actually do encode negative regulators of hepatocellular growth. LOH at 6q26-27 often involves loss of the mannose 6-phosphate/IGF2 receptor (DeSouze *et al.*, 1995), which is also an imprinted gene that has been implicated as a tumor suppressor due to its ability to activate TGF- β 1 signaling (which triggers apoptosis) and degrade IGF2 (which promotes hepatocellular growth). Mutations in the M6P/IGF2R have been detected in the majority of dysplastic liver lesions, suggesting it occurs early in hepatocarcinogenesis (DeSouze *et al.*, 1995). However, mutations in the M6P/IGF2R have been observed rarely (Kawate *et al.*, 1999) or not at all in other studies (Wada *et al.*, 1999), suggesting other underlying mechanisms are operative in other patients. LOH at chromosome 4q has been reported in several tumor types, although putative tumor suppressor loci within 4q have not been identified (Huang *et al.*, 1999; Knuutila *et al.*, 1999). Interestingly, the fibronectin locus is located within 4q28, which is deleted early in some HCCs. This event may contribute to the loss of extracellular matrix surrounding tumors during tumor progression. Allelic imbalance in 4q has also been observed in cirrhotic nodules (Yeh *et al.*, 2001), confirming the early nature of these changes in tumor development. LOH at 8p has been observed in other tumors (Knuutila *et al.*, 1999), and in HCC, a putative tumor suppressor gene has been identified at 8p21.3-22 (Yuan *et al.*, 1998). The

latter encodes a protein with 80% homology to rat p122RhoGAP, which catalyzes conversion of active GTP-bound Rho proteins to inactive ones. Given that the Rho family maintains the organization of the actin stress fibers within microfilaments that are important for cell-cell contact, and that they participate in ras mediated transformation, constitutively active Rho proteins (in the absence of p122RhoGAP) could contribute to the development of HCC. Independent observations have also pointed to the existence of multiple putative tumor suppressor genes within chromosome 8p (Huang *et al.*, 1999; Piao *et al.*, 1999; Pineau *et al.*, 1999). The recent report of allelic imbalance in 8p in cirrhotic nodules (Yeh *et al.*, 2001) underscores that these genetic changes occur prior to tumor development. Hence, it is likely that multiple genes cooperate in the appearance of tumor.

Late events in hepatocarcinogenesis (tumor progression and metastases)

Genetic instability in HCC is highlighted by increases in the number of chromosomal aberrations with tumor development and progression (Kimura *et al.*, 1996). The frequency of aneuploidy also becomes more pronounced as liver lesions take on an increasing resemblance to tumor (Attallah *et al.*, 1999). In some studies, the state of tumor cell differentiation is inversely correlated with the number of chromosomal aberrations (Ohsawa *et al.*, 1996). In multifocal HCC, nodules arising *de novo* within a single liver have a different spectrum of genetic lesions (Sirivatanauksorn *et al.*, 1999). Hence, there are likely to be many paths to hepatocellular carcinoma, and this is why it has been difficult to assign specific molecular alterations to changes in hepatocellular phenotype, clinical, or histopathological changes that accompany tumor development. This genetic heterogeneity also imposes challenges for the development of specific therapeutics in patients diagnosed with multinodular HCC.

LOH at 1p, 4q, 6q, 8p, 13q, 16q, and 17p are reported to occur late or in large, undifferentiated tumors (Boige *et al.*, 1997; DeSouze *et al.*, 1995; Kishimoto *et al.*, 1996; Konishi *et al.*, 1993; Kuroki *et al.*, 1995; Kusano *et al.*, 1999; Laurent-Puig *et al.*, 2001; Marchio *et al.*, 1997; Niketeghad *et al.*, 2001; Wang *et al.*, 2001; Wong *et al.*, 1999a; Yeh *et al.*, 2001). These changes also correlate with poor prognosis after initial diagnosis and with increased risk for tumor recurrence following surgery. LOH of 8p in particular has been found increased in metastatic lesions compared to primary tumors from the same patient. Fine mapping of LOH sites in 1p, 6p, 8p and 16q showed distinct minimal deleted regions for each chromosomal arm in different tumors, suggesting the presence of up to several novel tumor suppressor genes on each chromosome. LOH at 4q11-23 and 13q13-14 correlate with moderately or poorly differentiated tumors, as does a gain in 11q13 (Kusano *et al.*, 1999), which may encode the *BCL1* oncogene and

fibroblast growth factor (Knuutila *et al.*, 1999). Allelic gains have also been reported on 1q, 8q and 17q (Wang *et al.*, 2001), although the responsible loci have not been identified. Independent observations have shown a common deletion at 4q35 in moderately or poorly differentiated HCC (Bando *et al.*, 1999). LOH at 4q has also been observed to occur in late tumors with p53 mutations (Rashid *et al.*, 1999), suggesting that chromosome 4q encodes one or more tumor suppressors that complements p53. Although some regions of LOH mapped to the same chromosomal arms deleted in preneoplastic cells or early HCC, it is likely that the extent of deletion at these sites increases with tumor progression. In addition to LOH, amplifications in 1q, 6p and 17q have been frequently seen in HCC, and presumably encode one or more oncogenes, although the only known locus on these chromosomes is the *ERBB2/NEU* oncogene located at 17q12-q21 (Knuutila *et al.*, 1999). If *ERBB2/NEU* signal transduction is upregulated in HCC, it may activate cyclin-D/CDK and constitutive cell growth, as well as promote metastasis. These observations suggest a progressive gain and loss of genetic material during tumor progression, although these changes are different in individual tumors. While this heterogeneity may be real, differences in the populations and approaches used in each study may also contribute to these findings.

LOH in late stage HCC has also been frequently detected at 13q12-14, and may encompass the known tumor suppressor genes *RBI* (13q14.3), *LEU1* (13q14) and *BRCA2* (13q12.3) (Knuutila *et al.*, 1999; Kusano *et al.*, 1999; Niketeghad *et al.*, 2001; Wong *et al.*, 1999a). Interestingly, Rb appears to be inactivated by several mechanisms other than mutation. For example, hyperphosphorylation of Rb in HBxAg positive cells (Sirma *et al.*, 1999) suggests functional inactivation during tumor development. Alternatively, Rb inactivation may involve overexpression of the newly described oncoprotein, gankyrin, in HCC. Gankyrin, which is homologous to the p28 subunit of the 26S proteasome, binds to Rb and promotes its degradation through the ubiquitin-proteasome pathway (Higashitsuji *et al.*, 2000). In addition to *RBI* inactivation, LOH at 16q12-13 (Sheu *et al.*, 1999) may be associated with the loss of RB2/p130 at 16q12.2 (Knuutila *et al.*, 1999), suggesting cell cycle deregulation. LOH at 16p13 has been mapped to one or more JAK binding proteins that negatively regulates the JAK/STAT signal transduction pathway (Koyama *et al.*, 1999; Nagai *et al.*, 2001). Constitutive activation of this pathway by HBxAg in chronic HBV infection (Lee and Yun, 1998) may also contribute to the development of HCC by stimulating cell growth by cross-talk through *src* and/or *ras* associated signal transduction. In this context, HBxAg has been shown to stimulate *src* tyrosine kinase(s) and downstream *ras* signal transduction in tissue culture cells (Klein and Schneider, 1997). Independent results have shown an increase in pp60^{c-src} protein kinase activity in HCC and surrounding non-tumor liver compared to normal liver in human and rat

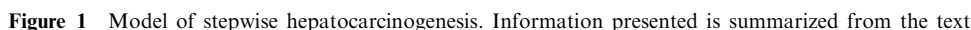
HCC (Masaki *et al.*, 1998), suggesting that constitutive activation of pp60^{c-src} is likely to play an important role in hepatocarcinogenesis. BRCA2 mutations have been rarely detected in HCC, with most being found in the germline, suggesting that such mutations may predispose to HCC development (Katagiri *et al.*, 1996). LOH has also been reported at 17p13, which encodes the tumor suppressor, p53 (Wang *et al.*, 2001). Among its many properties, p53 prevents DNA replication, so that its frequent mutation contributes importantly to genetic instability and tumor progression. There is a strong correlation between p53 mutations, large tumor size, and poor differentiation state (Ng *et al.*, 1994). Consequently, patients with p53 mutations have a poor prognosis and experience a short tumor free survival following surgery. Among AFB₁ exposed patients with p53 codon 249 mutations, LOH was also observed in chromosome 16q (Yakicier *et al.*, 2001), which among other things, encodes Rb. Hence, the integrity of Rb and p53 appear to be very important in the prevention of HCC. In addition to p53, LOH at chromosome 17p13 results in the deletion of another gene, designated HCC suppressor 1 (HCCS1), which localizes to mitochondria in non-tumor hepatocytes, but is absent from tumor cells. HCCS1 suppresses both colony and tumor formation of HCC cells in soft agar and nude mice, respectively, suggesting that it acts as a tumor suppressor (Zhao *et al.*, 2001).

LOH has also been documented at a variety of other loci in some HCCs (Boige *et al.*, 1997; Ding and Habib, 1995; Kusano *et al.*, 1999; Wong *et al.*, 1999a). For example, the tumor suppressor genes *APC* (adenomatous polyposis coli), *MMC* (mutated in colorectal cancer), and a DNA mismatch repair gene (*MSH3*) are located within chromosome 5q, but their roles, if any in HCC, remain to be identified (Ding and Habib, 1995). *APC* mutations, for example, result in up-regulated wild type β -catenin levels and constitutive Wnt signaling. *MMC* is a cell cycle regulatory protein whose mutation or loss contributes to stimulated cell growth. *MSH3* is important in mediating mismatch recognition and repair, so that its inactivation may promote the persistence of mutations. Mutations in another mismatch repair gene, referred to as *MSH2*, has been detected in about one-third of HCCs examined, and correlates with malignancy and poor survival (Yano *et al.*, 1999). LOH at 10q may involve loss of the *PTEN* tumor suppressor gene at 10q23 and the *MXII* gene at 10q24-25 (Ding and Habib, 1995; Kawamura *et al.*, 1999; Kusano *et al.*, 1999). The former blocks growth stimulatory and survival signals mediated by PI3 kinase (Downward, 1998), while the latter appears to negatively regulate *c-myc* (Knuutila *et al.*, 1999), which may partially explain how *c-myc* is overexpressed in most large tumors. LOH of 16p may involve loss of the axin gene (Laurent-Puig *et al.*, 2001), which normally promotes the ubiquitination and degradation of β -catenin, resulting in the nuclear accumulation of wild type β -catenin (Satoh *et al.*, 2000). LOH of 16q also correlates with metastasis, and a candidate tumor suppressor at 16q22 is E-cadherin

(Niketeghad *et al.*, 2001; Slagle *et al.*, 1993), whose role as a receptor in adherens junctions is essential for the maintenance of tissue architecture. Since β -catenin binds to E-cadherin, mutations in the latter correlate with the accumulation of β -catenin in the cytoplasm and nucleus, resulting in constitutive Wnt activation (Barth *et al.*, 1997). Loss of E-cadherin expression is also inversely related to the state of hepatocellular differentiation (Masuda *et al.*, 2000), further suggesting its occurrence late in hepatocarcinogenesis. In addition to LOH, hypermethylation of the E-cadherin promoter has been proposed as an alternative mechanism for Wnt activation in HCC (Kanai *et al.*, 1997). Hence, genes other than β -catenin are targets for down-regulated expression or mutation during tumor progression.

Hypermethylation was also frequently observed within the promoter of the *CDKN2A* gene, which encodes p16^{INK4} located on 9p21, in both early and advanced HCC (Wong *et al.*, 1999b). LOH at 9p21, which also results in loss of p16^{INK4}, has also been observed (Wang *et al.*, 2001). Normally, p16^{INK4} inhibits the cyclin dependent kinases (CDKs) -4 and -6, which block the G1 phase of the cell cycle through dephosphorylation of Rb, the latter of which binds to and inactivates E2F1. Methylation of the same promoter also inhibits production of p14^{ARF} from an alternative reading frame of the same gene. Interestingly, p14^{ARF} indirectly stabilizes p53 by binding to and sequestering mdm-2 from the nucleolus to the nucleus and cytosol (Weber *et al.*, 1999). Given that mdm-2 binds to and triggers the degradation of p53, the tumor suppressor properties of p14^{ARF} are, in part, p53 dependent. Hence, downregulated p16^{INK4} expression may inactivate Rb, while the down-regulated p14^{ARF} expression may result in the destabilization of p53. Point mutations of the p16^{INK4} locus have not been commonly found in progressively growing HCC, but have been observed in the germline, suggesting that its inactivation may predispose to HCC (Chaubert *et al.*, 1997). Alternatively, LOH of the p16 locus has also been reported, although these findings were not associated with age, gender, size or differentiation stage of the tumor (Liew *et al.*, 1999).

Other changes have also been documented late in tumorigenesis. Inactivation of the gene that encodes the integral membrane protein, KAI-1, which appears to function in cell-cell and cell-extracellular matrix interactions, is strongly down-regulated in metastatic HCC nodules (Guo *et al.*, 1998) as well as other metastatic tumor types (Dong *et al.*, 1995), suggesting that KAI-1 is a tumor suppressor whose integrity prevents metastasis. Increased expression of other oncogenes has also been documented in large tumors. For example, hypomethylation or point mutations of the *c-myc* and *c-N-ras* oncogenes appear during tumor progression, and correlate with invasiveness and metastases (Pascale *et al.*, 1993; Shen *et al.*, 1998). Abnormally high levels of *c-met*, which encodes the receptor for hepatocyte growth factor, appears to be associated with metastases (Ueki *et al.*, 1997). Elevated levels of growth factors and



Conclusions

outlined above, and provides a partial molecular picture of stepwise hepatocarcinogenesis. It should be emphasized that tumor and non-tumor liver contain multiple changes, and that there is variability in their profile among different patients even within single studies. Variability in the number and types of genetic changes has also been observed geographically, and depends upon the etiology of the tumor (viral, chemical or both) (Wong *et al.*, 2000). Epigenetic changes, such as altered DNA methylation, have been found in non-tumor liver even in the absence of liver pathology, and may promote chromosomal instability, possibly through changes in chromosomal configuration (Kondo *et al.*, 2000). Interestingly, viral proteins inactivate tumor suppressors (such as p53 and Rb) early in carcinogenesis that are mutated much later during tumor progression. Viral oncoproteins, such as HBxAg, may constitutively activate signal transduction pathways, such as those involving *c-jun* and *ras*, and activate oncogenes, such as *c-myc*, that are otherwise

activated by β -catenin mutations. These findings suggest common molecular targets in hepatocarcinogenesis despite different mechanisms of activation or inactivation (Figure 1). For example, while viral oncoproteins and DNA methylation may be operative prior to the onset of significant liver pathology, LOH seems to occur predominantly during persistent inflammation and hepatocellular regeneration, which is why liver cell damage may be such an important risk factor for HCC. A contributing factor may involve the degree of ploidy among hepatocytes, which increases with the extent of hepatocellular turnover (Gupta, 2000). Increased polyploidy in cirrhotic livers may translate into impaired regeneration, increased apoptosis, and liver failure, while the appearance of aneuploidy (resulting from LOH) provides advantages in growth and survival that contribute importantly to tumor formation. Given that high levels of p16^{INK4a} and p21^{WAF1/CIP1/SDI1} are associated with polyploidization (Gupta, 2000), which protects against carcinogenesis, it is not surprising that they are inactivated early in tumorigenesis. Hence, common molecular changes underlie changes at the cellular level that are important to the pathogenesis of HCC. However, there is also considerable heterogeneity in the up- and down-regulated genes reported in microarray analyses, as well as the genetic loci that undergo mutation or LOH in different reports. This suggests that there are multiple pathways to HCC, and that there is redundancy in the pathways that regulate cell growth and survival. These findings also reflect that although hepatocarcinogenesis is multistep, the molecular changes that underpin histopathological changes in tumor development are likely to be different in individual tumors. This may depend upon the etiology of the tumor (Table 1) and the genetic background of the host, but may also depend upon the state of tumor differentiation used at the beginning of the analysis as well as the source of materials used in microarray studies. Overall, the consequences of these changes suggest that the pathogenesis of HCC is accompanied by a progressive loss of differentiation, loss of normal cell adhesion, loss of the extracellular matrix, and constitutive activation of selected signal transduction pathways that promote cell growth and survival (Figure 1).

Future challenges and prospects

The challenges that face the research community include identification of the most common epigenetic and genetic changes that are functionally relevant to HCC development and progression. Laboratories elucidating the cell and molecular steps in HCC must continue to have increased access to clinical samples, which include tumor, surrounding liver, and blood samples from HCC patients. It will also be important to identify germline mutations in HBV infected patients that are passed on to their children, resulting in the development of HCC in childhood (Chang *et al.*,

1989). Clinical materials will also be important for the validation of new markers with diagnostic or prognostic potential. Hence, there is an urgent need to establish simple and low cost tests based upon molecular changes that are hallmarks of HCC development to screen selected populations at high risk for tumor. This is very important for poor countries in Asia and Africa where HBV and HCV infections are endemic and AFB₁ exposure is common. Identification of patients with early HCC will also make best use of current fiscal resources and treatments, and will significantly increase survival.

Presently, the diagnosis of HCC depends upon mostly morphologic criteria. If a set of genetic alterations is found, it may permit accurate staging of lesions, proximity of such lesions to malignancy, and whether lesions with a particular genetic profile are still capable of remodeling through appropriate therapeutic intervention (Wu *et al.*, 1987). For example, the identification and further characterization of tumor suppressors that are inactivated in HCC (at 1p and 4q), will be critical in realizing new targets for therapeutic drug discovery. Antitumor gene therapy will only be successful if relevant molecules, such as p53 and/or p16^{INK4}, are targeted in the appropriate patients. Their efficient reintroduction would slow down the development of many HCCs, but the multistep nature of HCC suggests that other targets need to also be considered. The targeting of cytotoxic cytokines, such as tumor necrosis factor α , to established murine HCC cells by recombinant retroviruses, has been shown to prevent tumor growth when the treated cells were transplanted into syngeneic hosts (Cao *et al.*, 1997). Recently, the adenovirus mediated introduction of axin into hepatoma cell lines that accumulated β -catenin as a consequence of mutations in APC, β -catenin, or axin, induced apoptosis in these tumor cells (Satoh *et al.*, 2000). These observations not only underscore the importance of β -catenin activation and accumulation to hepatocellular transformation, but also suggests that axin could be used therapeutically to suppress tumor growth. Alternatively, the introduction of 'suicide genes' into HCC cells that make them highly susceptible to drug-induced killing have also been reported (Ido *et al.*, 2001). Hence, elucidating the genetic mechanisms of HCC represents both a challenge and a hope.

Acknowledgments

This work was supported by NIH grants CA48656, CA79512, and CA66971 to M Feitelson.

References

- Abbott PJ and Saffhill R. (1977). *Br. J. Cancer*, **34**, 310–315.
- Aihara T, Noguchi S, Miyoshi Y, Nakano H, Sasaki Y, Nakamura Y, Monden M and Imaoka S. (1998). *Hepatology*, **28**, 86–89.
- Attallah AM, Tabll AA, Salem SF, El-Sadany M, Ibrahim TA, Osman S and El-Dosoky IM. (1999). *Cancer Lett.*, **142**, 65–69.
- Bando K, Nagai H, Matsumoto S, Koyama M, Kawamura N, Onda M and Emi M. (1999). *Genes Chromosomes Cancer*, **25**, 284–289.
- Barth AI, Nathke IS and Nelson WJ. (1997). *Curr. Opin. Cell. Biol.*, **9**, 683–690.
- Beasley RP and Hwang LY. (1984). *Viral Hepatitis and Liver Disease*. Vyas GN, Dienstag JL and Hoofnagle JH (eds). New York: Grune & Stratton, Inc., pp. 209–224.
- Becker SA, Lee TH, Butel JS and Slagle BL. (1998). *J. Virol.*, **72**, 266–272.
- Benbrook D, Lernhardt E and Pfahl M. (1988). *Nature*, **333**, 669–672.
- Benn J and Schneider RJ. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 10350–10354.
- Boige V, Laurent-Puig P, Fouchet P, Flejou JF, Monges G, Bedossa P, Bioulac-Sage P, Capron F, Schmitz A, Olschwang S and Thomas G. (1997). *Cancer Res.*, **57**, 1986–1990.
- Boix-Ferrero J, Pellin A, Blesa R, Adrados M and Liombart-Bosch A. (1999). *Virchows Arch.*, **434**, 497–501.
- Bourdon JC, D'Errico A, Paterlini P, Grigioni W, May E and Debuire B. (1995). *Gastroenterology*, **108**, 1176–1182.
- Bressac B, Kew M, Wands J and Ozturk M. (1991). *Nature*, **350**, 429–431.
- Buetow KH, Sheffield VC, Zhu M, Zhou T, Shen FM, Hino O, Smith M, McMahon BJ, Lanier AP, London WT, Redeker AG and Govindarajan S. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 9622–9626.
- Cadoret A, Ovejero C, Saadi-Kheddouci S, Souil E, Fabre M, Romagnolo B, Kahn A and Perret C. (2001). *Cancer Res.*, **61**, 3245–3249.
- Calvisi DF, Factor VM, Loi R and Thorgeirsson SS. (2001). *Cancer Res.*, **61**, 2085–2091.
- Cao G, Kuriyama S, Du P, Sakamoto T, Kong X, Masui K and Qi Z. (1997). *Gastroenterology*, **112**, 501–510.
- Chang MH, Chen DS, Hsu HC, Hsu HY and Lee CY. (1989). *Cancer*, **64**, 2377–2380.
- Chaubert P, Gayer R, Zimmermann A, Fontollet C, Stamm B, Bosman F and Shaw P. (1997). *Hepatology*, **25**, 1376–1381.
- Chen Q, Seol DW, Carr B and Zarnegar R. (1997). *Hepatology*, **26**, 59–66.
- Collier J and Sherman M. (1998). *Hepatology*, **27**, 273–278.
- D'Arville CN, Nouri-Aria KT, Johnson P and Williams R. (1991). *Hepatology*, **13**, 310–315.
- Dejean A and de The H. (1990). *Mol. Biol. Med.*, **7**, 213–222.
- De La Costa A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A and Perret C. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 8847–8851.
- DeSouze AT, Hankins GR, Washington MK, Fine RL, Orton TC and Jirtle RL. (1995). *Oncogene*, **10**, 1725–1729.
- Diamantis ID, McGandy CE, Chen TJ, Liaw YF, Gudat F and Bianchi L. (1992). *J. Hepatol.*, **15**, 400–403.
- Ding SF and Habib NA. (1995). *J. Hepatol.*, **22**, 230–238.
- Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT and Barrett JC. (1995). *Science*, **268**, 884–886.
- Downward J. (1998). *Curr. Opin. Cell Biol.*, **10**, 262–267.
- Dunsford HA, Sell S and Chisari FV. (1990). *Cancer Res.*, **50**, 3400–3407.
- Elmore L, Hancock AR, Chang SF, Wang XW, Chang S, Callahan CP, Geller DA, Will H and Harris CC. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 14707–14712.
- Factor VM, Kao CY, Santoni-Rugiu E, Woitach JT, Jensen MR and Thorgeirsson SS. (1997). *Cancer Res.*, **57**, 2089–2095.
- Falls JG, Pulford DJ, Wylie AA and Jirtle RL. (1999). *Am. J. Pathol.*, **154**, 635–647.
- Fang W, Piao Z, Simon D, Sheu JC and Huang S. (2000). *Genes Chromosomes Cancer*, **28**, 269–275.
- Feitelson MA. (1986). *Concepts in Viral Pathogenesis II*. Notkins AL and Oldstone MBA (eds). New York: Springer-Verlag, pp. 269–275.
- Feitelson MA, Reis H, Pan J, Lian Z, Fang J, Liu J, Zhu X, Zhu M and Sun B. (1999). *Normal and Malignant Liver Cell Growth: FALK Workshop*. Fleig WE (ed). Norwell, MA: Kluwer Academic Publishers, pp. 156–170.
- Gale Jr M, Kwieciszewski B, Dossett M, Nakao H and Katze MG. (1999). *J. Virol.*, **73**, 6506–6516.
- Ghosh AK, Steele R, Meyer K, Ray R and Ray RB. (1999). *J. Gen. Virol.*, **80**, 1179–1183.
- Gozuacik D, Mrakami Y, Saigo K, Chami M, Mugnier C, Lagorce D, Okanoue T, Urashima T, Brechot C and Paterlini-Brechot P. (2001). *Oncogene*, **20**, 6233–6240.
- Greenblatt MS, Feitelson MA, Zhu M, Bennett WP, Welsh JA, Jones RA, Borkowski A and Harris CC. (1997). *Cancer Res.*, **57**, 426–432.
- Guo XZ, Friess H, DeMola FF, Heinicke JM, Abou-Shady M, Graber HU, Baer HU, Zimmermann A, Korc M and Buchler MW. (1998). *Hepatology*, **28**, 1481–1488.
- Gupta S. (2000). *Semin. Cancer Biol.*, **10**, 161–171.
- Guyton KZ and Kensler TW. (1993). *Br. Med. Bull.*, **49**, 523–544.
- Haruna Y, Hayashi N, Katayama K, Yuki N, Kasahara A, Sasaki Y, Fusamoto H and Kamada T. (1991). *Hepatology*, **13**, 417–421.
- Hayashi J, Aoki H, Kajino K, Moriyama M, Arakawa Y and Hino O. (2000). *Hepatology*, **32**, 958–961.
- Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kido T, Mayer RJ, Arii S and Fujita J. (2000). *Nat. Med.*, **6**, 96–99.
- Hino O, Tabata S and Hotta Y. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 9248–9252.
- Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL and Peng SY. (2000). *Am. J. Pathol.*, **157**, 763–770.
- Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ and Harris CC. (1991). *Nature*, **350**, 427–428.
- Huang J, Kwong J, Sun ECY and Liang TJ. (1996). *J. Virol.*, **70**, 5582–5591.
- Huang SF, Hsu HC and Fletcher JA. (1999). *Cancer Genet. Cytogenet.*, **111**, 21–27.
- Huo TI, Wang XW, Forgues M, Wu CG, Spillare EA, Giannini C, Brechot C and Harris CC. (2001). *Oncogene*, **20**, 3620–3628.
- Ido A, Uto H, Moriuchi A, Nagata K, Onaga Y, Onaga M, Hori T, Hirono S, Hayashi K, Tamaoki T and Tsubouchi H. (2001). *Cancer Res.*, **61**, 3016–3021.

- Ishido S and Hotta H. (1998). *FEBS Lett.*, **438**, 258–262.
- Kanai Y, Ushijima S, Hui AM, Ochiai A, Tsuda H, Sakamoto M and Hirohashi S. (1997). *Int. J. Cancer*, **71**, 355–359.
- Kanai Y, Ushijima S, Tsuda H, Sakamoto M, Sugimura T and Hirohashi S. (1996). *Jpn. J. Cancer Res.*, **87**, 1210–1217.
- Kanai Y, Hui AM, Sun L, Ushijima S, Sakamoto M, Tsuda H and Hirohashi S. (1999). *Hepatology*, **29**, 703–709.
- Katagiri T, Nakamura Y and Miki Y. (1996). *Cancer Res.*, **56**, 4575–4577.
- Kato N, Lan KH, Ono-Nita SK, Shiratori Y and Omata M. (1997). *J. Virol.*, **71**, 8856–8859.
- Kawamura N, Nagai H, Bando K, Koyama M, Matsumoto S, Tajiri T, Onda M, Fujimoto J, Ueki T, Konishi N, Shiba T and Emi M. (1999). *Jpn. J. Cancer Res.*, **90**, 413–418.
- Kawate S, Takenoshita S, Ohwada S, Mogi A, Fukusato T, Makita F, Kuwano H and Morishita Y. (1999). *Int. J. Oncol.*, **14**, 127–131.
- Kim H, Lee H and Yun Y. (1998). *J. Biol. Chem.*, **273**, 381–385.
- Kim SO, Park JG and Lee IK. (1996). *Cancer Res.*, **56**, 3831–3836.
- Kimura H, Kagawa K, Deguchi T, Nakajima T, Kakusui M, Ohkawara T, Katagishi T, Okanoue T, Kashima K and Ashihara T. (1996). *Cancer*, **77**, 271–277.
- Kishimoto Y, Shiota G, Wada K, Kitano M, Nakamoto K, Kamisaki Y, Suou T, Itoh T and Kawasaki H. (1996). *J. Cancer Res. Clin. Oncol.*, **122**, 585–589.
- Klein NP and Schneider RJ. (1997). *Mol. Cell. Biol.*, **17**, 6427–6436.
- Knuutila S, Aalto Y, Autio K, Bjorkqvist AM, El-Rifai W, Hemmer S, Huhta T, Kettunen E, Kiuru-Kuhlefelt S, Larramendy ML, Lushnikova T, Monni O, Pere H, Tapper J, Tarkkanen M, Varis A, Wasenius VM, Wolf M and Zhu Y. (1999). *Am. J. Pathol.*, **155**, 683–694.
- Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R and Hirohashi S. (2000). *Hepatology*, **32**, 970–979.
- Konishi M, Kikuchi-Yanoshita R, Tanaka K, Sato C, Tsuruta K, Maeda Y, Koike M, Tanaka S, Nakamura Y and Hattori N. (1993). *Jpn. J. Cancer Res.*, **84**, 893–899.
- Koyama M, Nagai H, Bando K, Ito M, Moriyama Y and Emi M. (1999). *Jpn. J. Cancer Res.*, **90**, 951–956.
- Kuroki T, Fujiwara Y, Tsuchiya E, Nakamori S, Imaoka S, Kanematsu T and Nakamura Y. (1995). *Genes Chromosomes Cancer*, **13**, 163–167.
- Kusano N, Shiraishi K, Kubo K, Oga A, Okita K and Sasaki K. (1999). *Hepatology*, **29**, 1858–1862.
- Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, Thomas G, Bioulac-Sage P and Zucman-Rossi J. (2001). *Gastroenterology*, **120**, 1763–1773.
- Lee SM, Portmann BC and Margison GP. (1996). *Hepatology*, **24**, 987–990.
- Lee YH and Yun Y. (1998). *J. Biol. Chem.*, **273**, 25510–25515.
- Lencioni R, Caramella D, Bartolozzi C and Di Coscio G. (1994). *Ital. J. Gastroenterol.*, **26**, 163–168.
- Liang TJ. (1995). *Hepatology*, **22**, 1330–1332.
- Liew CT, Li HM, Lo KW, Leow CK, Lau WY, Hin LY, Lim BK, Lai PB, Chan JY, Wang XQ, Wu S and Lee JC. (1999). *Int. J. Cancer*, **81**, 319–324.
- Livezey KW and Simon D. (1997). *Mutat. Res.*, **377**, 187–198.
- Lucito R and Schneider RJ. (1992). *J. Virol.*, **66**, 983–991.
- Maki CG, Huibregtse JM and Howley PM. (1996). *Cancer Res.*, **56**, 2649–2654.
- Mao TL, Chu JS, Jeng YM, Lai PL and Hsu HC. (2001). *J. Pathol.*, **193**, 95–101.
- Marchio A, Meddeb M, Pineau P, Danglot G, Tiollais P, Bernheim A and Dejean A. (1997). *Genes Chromosomes Cancer*, **18**, 59–65.
- Marusawa H, Hijikata M, Chiba T and Shimotohno K. (1999). *J. Virol.*, **73**, 4713–4720.
- Masaki T, Okuda M, Shiratori Y, Rengifo W, Matsumoto K, Maede S, Kato N, Kanai F, Komatsu Y, Nishioka M and Omata M. (1998). *Hepatology*, **27**, 1257–1264.
- Masuda T, Saito H, Kaneko F, Atsukawa K, Morita M, Inagaki H, Kumagai N, Tsuchimoto K and Ishii AH. (2000). *In Vitro Cell. Develop. Biol.*, **36**, 387–394.
- McGlynn KA, Rosvold EA, Lustbader ED, Hu Y, Clapper ML, Zhou T, Wild CP, Xia XL, Baffore-Bonnie A and Ofori-Adjei D. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 2384–2387.
- Miyoshi Y, Iwao K, Nagasawa Y, Aihara T, Sasaki Y, Imaoka S, Murata M, Shimano T and Nakamura Y. (1998). *Cancer Res.*, **58**, 2524–2527.
- Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T and Koike K. (1998). *Nat. Med.*, **4**, 1065–1067.
- Murakami H, Sanderson ND, Nagy P, Marino PA, Merlino G and Thorgeirsson SS. (1993). *Cancer Res.*, **53**, 1719–1723.
- Murakami Y, Hayashi K, Hirohashi S and Sekiya T. (1991). *Cancer Res.*, **51**, 5520–5525.
- Nagai H, Kim YS, Lee KT, Chu MY, Konishi N, Fujimoto J, Baba M, Matsubara K and Emi M. (2001). *J. Hepatol.*, **34**, 416–421.
- Ng IO, Srivastava G, Chung LP, Tsang SW and Ng M. (1994). *Cancer*, **74**, 30–37.
- Niketeghad F, Decker HJ, Caselmann WH, Lund P, Geissler F, Dienes HP and Schirmacher P. (2001). *Br. J. Cancer*, **85**, 697–704.
- Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, Amenomori M, Shibagaki I, Nakao K and Ikenaga M. (1994). *Cancer Res.*, **54**, 3107–3110.
- Noguchi O, Enomoto N, Ikeda T, Kobayashi F, Marumo F and Sato C. (1996). *J. Hepatol.*, **24**, 286–292.
- Ogden SK, Lee KC and Barton MC. (2000). *J. Biol. Chem.*, **275**, 27806–27814.
- Ohnawa N, Sakamoto M, Saito T, Kobayashi M and Hirohashi S. (1996). *J. Hepatol.*, **25**, 655–662.
- Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y and Nakamura Y. (2001). *Cancer Res.*, **61**, 2129–2137.
- Okazaki I, Wada N, Nakano M, Saito A, Takasaki K, Doi M, Kameyama K, Otani Y, Kubochi K, Niioka M, Watanabe T and Maruyama K. (1997). *Hepatology*, **25**, 580–584.
- Okuda K. (1997). *J. Gastroenterol. Hepatol.*, **12**, S314–S318.
- Ozaki I, Mizuta T, Zhao G, Yotsumoto H, Hara T, Kajihara S, Hisatomi A, Sakai T and Yamamoto K. (2000). *Cancer Res.*, **60**, 6519–6525.
- Park US, Park SK, Lee YI, Park JG and Lee YI. (2000). *Oncogene*, **19**, 3384–3394.
- Pascale RM, Simile MM and Feo F. (1993). *Anticancer Res.*, **13**, 1341–1356.
- Paterlini P, Poussin K, Kew M, Franco D and Brechot C. (1995). *Hepatology*, **21**, 313–321.
- Peng CY, Tsai SL, Yeh CT, Hung SP, Chen MF, Chen TC, Chu CM and Liaw YF. (2000). *Anticancer Res.*, **20**, 1487–1492.
- Peng SY, Lai PL and Hsu MC. (1993). *J. Formos. Med. Assoc.*, **92**, 866–870.

- Piao Z, Kim NG, Kim H and Park C. (1999). *Cancer Lett.*, **138**, 227–232.
- Pineau P, Nagai H, Prigent S, Wei Y, Gyapay G, Weissenbach J, Tiollais P, Buendia MA and Dejean A. (1999). *Oncogene*, **18**, 3127–3134.
- Pontisso P, Belluco C, Bertorelle R, Demoliner L, Chieco-bianchi L, Nitti D, Lise M and Alberti A. (1998). *Cancer*, **83**, 1489–1494.
- Poussin K, Kienes H, Sirma H, Urban S, Beaugrand M, Franco D, Schirmacher P, Brechot C and Brechot PP. (1999). *Int. J. Cancer*, **80**, 497–505.
- Rashid A, Wang JS, Qian GS, Lu BX, Hamilton SR and Groopman JD. (1999). *Br. J. Cancer*, **80**, 59–66.
- Ray RB, Lagging LM, Meyer K, Steele R and Ray R. (1995). *Virus Res.*, **37**, 209–220.
- Ray RB, Meyer K, Steele R, Shrivastava A, Aggarwal BB and Ray R. (1998). *J. Biol. Chem.*, **273**, 2256–2259.
- Ray RB, Steele R, Meyer K and Ray R. (1997). *J. Biol. Chem.*, **272**, 10983–10986.
- Rossmann W and Schulte-Hermann R. (2001). *Microscopy Res. Tech.*, **52**, 430–436.
- Rossner MT. (1992). *J. Med. Virol.*, **36**, 101–117.
- Sakamuro D, Furukawa T and Takegami T. (1995). *J. Virol.*, **69**, 3893–3896.
- Sato S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y and Nakamura Y. (2000). *Nat. Genet.*, **24**, 245–250.
- Shen L, Fang J, Qiu D, Zhang T, Yang J, Chen S and Xiao S. (1998). *Hepato-Gastroenterol.*, **45**, 1753–1759.
- Sheu JC, Lin YW, Chou HC, Huang GT, Lee HS, Lin YH, Huang SY, Chen CH, Wang JT, Lee PH, Lin JT, Lu FJ and Chen DS. (1999). *Br. J. Cancer*, **80**, 468–476.
- Shirota Y, Keneko S, Honda M, Kawai HF and Kobayashi K. (2001). *Hepatology*, **33**, 832–840.
- Sirivatanauksorn Y, Sirivatanauksorn V, Bhattacharya S, Davidson BR, Dhillon AP, Kakkar AK, Williamson RC and Lemoine NR. (1999). *Gut*, **45**, 761–765.
- Sirma H, Giannini C, Poussin K, Paterlini P, Kremsdorf D and Brechot C. (1999). *Normal and Malignant Liver Cell Growth: FALK Workshop* Fleig WE (ed). Norwell, MA: Kluwer Academic Publishers, pp. 171–186.
- Sirma H, Weil R, Rosmorduc O, Urban S, Israel A, Kremsdorf D and Brechot C. (1998). *Oncogene*, **16**, 2051–2063.
- Slagle BL, Zhou YZ, Birchmeier W and Scorsone KA. (1993). *Hepatology*, **18**, 757–762.
- Sugimoto K, Shiraki K, Ito T, Fujikawa K, Takase K, Tameda Y, Moriyama M and Nakano T. (1999). *Hepatology*, **30**, 920–926.
- Sun M, Eshleman JR, Ferrell LD, Jacobs G, Sudilovsky EC, Tuthill R, Hussein MR and Sudilovsky O. (2001). *Hepatology*, **33**, 1415–1424.
- Tannapfel A, Wasner M, Krause K, Geissler F, Katalinic A, Hauss J, Mossner J, Engeland K and Wittekind C. (1999). *J. Natl. Cancer Inst.*, **91**, 1154–1158.
- Tu H, Bonura C, Giannini C, Mouly H, Soussan P, Kew M, Paterlini-Brechot P, Brechot C and Kremsdorf D. (2001). *Cancer Res.*, **61**, 7803–7810.
- Ueda H, Ullrich SJ, Gangemi JD, Kappel CA, Ngo L, Fingleton MA and Jay G. (1995). *Nat. Genet.*, **9**, 41–47.
- Ueki T, Fujimoto J, Suzuki T, Yamamoto H and Okamoto E. (1997). *Hepatology*, **25**, 862–866.
- Wada I, Kanada H, Nomura K, Kato Y, Machinami R and Kitagawa T. (1999). *Hepatology*, **29**, 1718–1721.
- Wang G, Zhao Y, Liu X, Wang L, Wu C, Zhang W, Liu W, Zhang P, Cong W, Zhu Y, Zhang L, Chen S, Wan D, Zhao X, Huang W and Gu J. (2001). *Genes Chromosomes Cancer*, **31**, 221–227.
- Wang J, Chenivresse X, Henglein B and Brechot C. (1990). *Nature*, **343**, 555–557.
- Wang J, Shou J and Chen X. (2000). *Oncogene*, **19**, 1843–1848.
- Wang W, London WT and Fingleton MA. (1991a). *Cancer Res.*, **51**, 4971–4977.
- Wang W, London WT, Lega L and Fingleton MA. (1991b). *Hepatology*, **14**, 29–37.
- Wang XW, Forrester K, Yeh H, Fingleton MA, Gu JR and Harris CC. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 2230–2234.
- Weber JD, Taylor LJ, Roussel MF, Sherr CJ and Bar-Sagi D. (1999). *Nat. Cell Biol.*, **1**, 20–26.
- Wong N, Lai P, Lee SW, Fan S, Pang E, Liew CT, Sheng Z, Lau JW and Johnson PJ. (1999a). *Am. J. Pathol.*, **154**, 37–43.
- Wong N, Lai P, Pang E, Fung LF, Sheng Z, Wong V, Wang W, Hayashi Y, Perlman E, Yuna S, Lau JW and Johnson PJ. (2000). *Clin. Cancer Res.*, **6**, 4000–4009.
- Wong N, Lam WC, Lai PBS, Pang E, Lau WY and Johnson PJ. (2001). *Am. J. Pathol.*, **159**, 465–471.
- Wong IH, Lo YM, Zhang J, Liew CT, Ng MH, Wong N, Lai PB, Lau WY, Hjelm NM and Johnson PJ. (1999b). *Cancer Res.*, **59**, 71–73.
- Wu TC, Tong MJ, Hwang B, Lee SD and Hu MM. (1987). *Hepatology*, **7**, 46–48.
- Xu L, Hui L, Wang S, Gong J, Jin Y, Wang Y, Ji Y, Wu X, Han Z and Hu G. (2001). *Cancer Res.*, **61**, 3176–3181.
- Yakicic MC, Legoix P, Vaury C, Greslin L, Tubacher E, Capron F, Bayer J, Degott C, Balabaud C and Zucman-Rossi J. (2001). *Oncogene*, **20**, 5232–5238.
- Yano M, Asahara T, Dohi K, Mizuno T, Iwamoto KS and Seyama T. (1999). *Int. J. Oncol.*, **14**, 447–451.
- Yeh SH, Chen PJ, Shau WY, Chen YW, Lee PH, Chen JT and Chen DS. (2001). *Gastroenterology*, **121**, 699–709.
- Yoo YD, Ueda H, Park K, Flanders KC, Lee YI, Jay G and Kim SJ. (1996). *J. Clin. Invest.*, **97**, 388–395.
- Yuan BZ, Miller MJ, Keck CL, Zimonjic DB, Thorgeirsson SS and Popescu NC. (1998). *Cancer Res.*, **58**, 2196–2199.
- Zhang Z, Torii N, Furusaka A, Malayaman N, Hu Z and Liang TJ. (2000). *J. Biol. Chem.*, **275**, 15157–15165.
- Zhao X, Li J, He Y, Lan F, Fu L, Guo J, Zhao R, Ye Y, He M, Chong W, Chen J, Zhang L, Yang N, Xu B, Wu M, Wan D and Gu J-r. (2001). *Cancer Res.*, **61**, 7383–7387.