Review Paper

Molecular events associated with hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) causes appropriately 450,000-1,000,000 deaths a year worldwide and is the third to fourth leading cause of cancer deaths in the world. HCC is one of the most common cancers, particularly in developing countries such as certain parts of Africa and Asia. However, the incidence has recently increased remarkably in developed countries (Taylor-Robinson *et al.*, 1997; Okuda, 1987; El-Serag & Mason, 1999). The major risk factors are chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol consumption, sex hormones (both androgens and progestins), metabolic disorders of the liver (e.g. haemochromatosis, Wilson's disease) and exposure to the mycotoxin, aflatoxin B1 (Qian *et al.*, 1994; Yeh *et al.*, 1989; Lunn *et al.*, 1997).

HCC is an extremely difficult disease to manage. At present, medical therapies are unsatisfactory. Surgical resection is the only curative therapy for HCC. Unfortunately, the vast majority of HCC are unresectable since most patients present late and the associated cirrhosis restricts the extent of surgical resection (Bruix *et al.*, 1996). Moreover, the residual liver is still at extremely high risk for recurrent disease (Yamanaka *et al.*, 2000). The lack of progress in the treatment of HCC compared to other cancers in the past 10 years is alarming. Therefore, it is important to elucidate the mechanisms that control cell growth, differentiation and metastasis in hepatocarcinogenesis in order to develop new strategies to manage this difficult disease. The molecular mechanisms underlying hepatocarcinogenesis are poorly understood. A common feature of chronic hepatitis and liver cirrhosis is the long-lasting inflammation. The aim of this review is to give an update on the viral and host factors that may contribute to multistep hepatocarcinogenesis.

Hepatocarcinogenesis

Carcinogenesis is believed to be a multistage process and is currently conceptualized as occurring through a sequence of steps, termed 'initiation, promotion and progression' which need not occur either sequentially or simultaneously (Table 1). The disruption of the normal cellular mechanisms, the mutations in the growth regulatory genes and proliferation of the cells in the liver remain unresolved. Tumour initiation can be a consequence of exposure to carcinogens, microbial agents (hepatitis viruses) or necroinflammatory processes such as cellular injury due to free radical or ionising irradiation. Tumour promotion results in mutations and enhanced genetic damage. The severe, prolonged cellular injury induces a preneoplastic proliferative response that fosters secondary genetic events that program the cell for unrestrained growth (Chisari *et al.*, 1989). During tumour progression, premalignant cells continue to develop phenotypic changes and genomic instability such as chromosomal aberrations, gene amplification and altered gene expression.

Kew *et al.*(1997) has suggested that the indirect effect may be achieved from the accelerated rate of division of hepatocytes that typifies cirrhosis and chronic hepatitis. Continuous or recurring cycles of necrosis of hepatocytes followed by regeneration render the

Table 1. Events that lead to hepatocellular carcinoma

Initiation:	carcinogens, microbial agents (hepatitis B virus, hepatitis C virus)
Promotion:	chronic inflammation –> alterations in cell growth and regulation
Progression:	cellular dysplasia

DNA of these cells more susceptible to spontaneous mutation, damage to DNA by mutagens and integration by oncogenic virus. Moreover, the faster rate of cell turnover may leave insufficient time for damaged DNA to be repaired before the cell divides again, thereby fixing the altered DNA in the progeny. Thus, a series of mutations may accumulate over time.

Molecular basis of viral hepatocarcinogenesis

HBV infection

HBV is one of the major aetiological agents responsible for the appearance of chronic liver diseases and HCC (IARC, 1994). Globally, about 80% of HCC are associated with HBV. In Malaysia, 95.4% of HCC have been found to be HBV positive (Sinniah & Ooi, 1993). In Asia, 300 million people suffer from chronic HBV and they have 100-300-fold greater risk of HCC than the general population (Beasley *et al.*, 1981; Maynard, 1990).

Due to the high percentage of HCC cases associated with HBV, it is critical to explore how HBV infection can confer or accelerate transformation of hepatocytes. To date, the mechanism of HBV hepatocarcinogenesis in human HCC is poorly understood. However, recent studies suggest that there are some relevant molecular pathways that may contribute to the development of HBV-associated HCC.

Integration of HBV DNA

The integration of HBV DNA into the hepatocyte genome with subsequent chromosomal rearrangement and generation of trans-activators may be a crucial event for HBV-associated hepatocarcinogenesis (Su *et al.*, 1998). Viral DNA integration is considered a random event in most HCC (Okuda, 1992). In addition, evidence of HBV DNA insertional mutagenesis (deletion, duplication or translocation) has been demonstrated (Brechot, 1996; Paterlini *et al.*, 1994). Attempts have been made to identify the cellular sequences adjacent to the sites of HBV DNA integration that might act as HCC initiators or promoters (Brechot, 1996). Examples of cellular sequence affected are *c-myc* and cyclin A genes. Viral integration, however, is not the only mechanism responsible for the development of HCC. It has been reported that viral DNA integration resulting in alterations in cell growth account for very limited cases (Matsubara & Tokina, 1990) and p53 mutation does not require the genomic integration of HBV sequences in HBV-associated HCC (Hsu *et al.*, 1993).

Contribution by HBX

Another relevant molecular pathway that HBV may contribute to the hepatocarcinogenesis may be through one of the HBV genes, the X gene. The expression of HBV X gene, HBX, is a transcriptional trans-activator and can upregulate the activity of the HBV genome (Scaglioni *et al.*, 1996). It may interfere with the degradation of cellular proteins, alter cell cycle regulation, and disrupt functions governing DNA repair, growth arrest, and apoptosis (Poussin *et al.*, 1999). An enhanced expression of the HBX in liver provokes a progressive morphological change starting with the development of multifoci altered hepatocytes, followed by the appearance of benign adenomas and, eventually, the development of an overt HCC (Kim *et al.*, 1991). Recently, it has been demonstrated that expression of HBX enhances liver cell susceptibility to carcinogen-induced mutagenesis, possibly through alterations in the balance between DNA repair and apoptosis (Sohn *et al.*, 2000). Furthermore, it has been reported that the enhancer-X region alone is the key contributor to the malignant change of pre-malignant liver cells in HBV carriers through activation of some specific genes (Miyaki *et al.*, 2000).

At present, attention on HBX has focused on its capacity in influencing cellular apoptosis. The HBX effect on apoptosis may depend on the cell types, or other genetic issues such as the p53 status, or on external stimuli (Elmore *et al.*, 1997). HBX is noted to prevent cell apoptosis via inhibiting a common effector for apoptosis, caspase 3 (Gotllob *et al.*, 1998). It is proposed that HBX/p53 complex formation represents one of several steps whereby HBV contributes to the development of HCC (Feitelson, 1998). Recent study suggest that the shift of the reciprocal inhibitory activities at the levels of protein-protein interaction and transcription between HBX and p53 may play a decisive role in the HBV-related hepatocarcinogenesis (Lee & Rho, 2000). *In vitro*, HBX can functionally disrupt p53-mediated repression of alpha-fetoprotein transcription through protein-protein interaction and HBX modification of p53 gene regulation (Ogden *et al.*, 2000). p53 bound to a regulatory element within the HBV enhancer is able to repress transcription in the presence of viral-enhancer-bound activators (Ori *et al.*, 1998). The centrality of p53 to genomic stability, cell cycle arrest, induction of apoptosis, and in senescence related pathways (Ozlurk *et al.*, 1999), suggests that its disruption by HBX will result in genomic instability, loss of cell cycle control, a lower apoptotic rate, and an extension in the life span of HBV-infected cells.

To further support the contribution of HBX to the development of HCC, HBX can circumvent the loss of p53 functions and induce critical downstream regulatory events leading to the transcriptional activation of the cyclin-dependent kinase inhibitor p21(WAF/CIP1). In stable or transient HBV-X transformed Hep3B cells, a p53-mutant human HCC cell line, HBV-X increased protein and mRNA levels of p21(WAF/CIP1). Increased binding of p21(WAF/CIP1) with cyclin-dependent kinase 2 (CDK2) and promoter mutation analysis have shown that the HBV-X responsive site coincides with the binding sites of the factor which regulate transcriptional initiation from a variety of cellular and vital gene promoter and enhancer elements. This indicates that HBX can prolong G1 → S transition via a p53-independent pathway (Park et al., 2000). The intracellular distribution patterns of p53 and HBX has been showed to be different, with the former located within nuclei and the latter confined to the cytoplasmic compartment. HBX has been found not to co-immunoprecipitate with p53, suggesting that p53-HBX binding is infrequent in HBV-infected hepatocytes, and that it cannot be a common mechanism of HBV-associated hepatocarcinogenesis (Su *et al.*, 2000).

Another mechanism that HBX contributes to the development of HCC is via interaction with the retinoblastoma (*Rb*) gene. The *Rb* gene plays an important role in regulating the cell cycle and functions as a tumour suppressor gene. HBX is able to increase the expression level of *Rb* in Hep G2 cell line (Farshid *et al.*, 1997).

Interestingly, several reports on HCC cases showed that a substantial proportion of HBs negative with or without anti-HCV was positive for HBX, suggesting that HBV is also associated with these HCC cases (Yotsuyanagi *et al.*, 2000; Tamori *et al.*, 1999). The significance of HBV in these cases is still unclear because another study has indicated that the copy number of HBV DNA may be very low in these cases (Shintani *et al.*, 2000). To further substantiate the importance of HBX, there is evidence that overexpression of the HBV X gene in transgenic mice leads to HCC (Kim *et al.*, 1991).

Apoptosis or programmed cell death is of critical importance in normal development and homeostasis. Liver hyperplasia or regeneration may be aided by the inhibition of apoptosis; excessive apoptosis of hepatocytes can cause fulminant hepatic failure. HBX has been implicated in inducing apoptosis through sensitisation of cells to apoptotic stimuli such as tumour necrosis factor (Kim *et al*, 1998). A recent report shows that HBX may specifically activate the Bcl-2 sensitive pathway leading to apoptosis, hence suppressing the transformation of primary rat embryo fibroblasts (Schuster *et al.*, 2000). Thus, the potential role of the transactivating HBXin ttansformation by HBV is controversial. More studies are required to define the role of the X gene product in the process of malignant transformation in chronic HBV infection. Since HBX has been found to inactivate a number of key negative regulatory pathways such as p53, p21(WAF/CIP1), alter cell cycle regulation, disrupt the function of genes in DNA repair, growth arrest and apoptosis, it is worthwhile further investigating the role of HBX in the process of multistep hepatocarcinogenesis.

Contribution by other viral proteins of HBV

Another important viral protein encoded by HBV which may contribute to hepatocarcinogenesis is the precore antigen (HBe). The precore/core mutant has been observed with increasing frequency from acute hepatitis to chronic hepatitis, non-tumour and HCC. The difference in frequency was significant between HCC and acute hepatitis B groups, suggesting that the precore/core mutant or hepatocytes harbouring this mutant may be under immune selection and that such mutations may facilitate integration and subsequent tumour development (Zhong *et al.*, 2000). Another investigation also supported that precore/core mutant is related to the development of HCC (Cho *et al.*, 1999).

An earlier study has shown that transgenic mice which overproduce the HBV large envelope polypeptide within the hepatocytes develop HCC. This indicates that the inappropriate expression of a single structural viral gene is sufficient to cause HCC in this model.

Although, chronic HBV infection is one of the aetiological risk factor for the development of HCC, a direct role for HBV in liver cell transformation still remains speculative.

HCV infection

In contrast to HBV, 80 % of those exposed to HCV develop chronic HCV infection. There are approximately 200 million chronic HCV carriers worldwide. Globally, 15% of HCC cases are associated with HCV (Busutil & Farmer, 1996; Kew *et al.*, 1997; Donato *et al.*, 1998; Colombo, 1992). The prevalence of HCV antibody-positive patients with HCC was found to be 13.8% in Malaysia (Sinniah & Ooi, 1993), 29% in the USA, 62-65% in Europe, 29% in South Africa and 70-90% in Japan (Edamato *et al.*, 1996). Thus, chronic HCV infection accounts for a high number of HCC cases in

Japan, Europe, USA and South Africa. Although numerous genotypes of HCV exist, some investigators have suggested that infection with the genotype HCV-1b is associated with a higher incidence of severe liver disease (chronic active hepatitis and cirrhosis) and HCC (Mahaney *et al.*, 1994; Kato *et al.*, 1993).

Unlike HBV, HCV cannot integrate into the host genome because it is an RNA virus that lacks a reverse-transcriptase enzyme. Therefore, insertional mutagenesis can be excluded as a carcinogenic mechanism for the development of HCV-associated HCC. Despite many efforts, the molecular mechanisms by which HCV contributes to cell transformation remain unclear.

HCV core protein

HCV core, envelope and non-structural protein (NS) 3 and NS5, and replicative HCV intermediates, negative-strand HCV RNAs are detected in HCC and adjacent tissue of HCV-related HCC (Haruna *et al.*, 1994; Gerber *et al.*, 1992; Horiike *et al.*, 1993). Since the core protein of HCV has been detected in the nuclei of the infected cell (Shih *et al.*, 1993; Lo *et al.*, 1998), it has been suggested that HCV core protein may interact with p53 and modulate p53-dependent promoter activities during HCV infection (Otsuka *et al.*, 2000). The occurrence of p53 gene abnormalities in patients with HCV-associated HCC, implied that HCV might affect the pathways leading to hepatocarcinogenesis via a p53 mechanism (Teramoto *et al.*, 1994). HCV core protein may have an important biological role in the promotion of cell growth by downregulating human p53, thus weakening the cellular tumour suppressor functions. It can also exert transregulatory functions on cellular and viral promoters, and in co-operation with *H-ras*, transform primary rat embryo fibroblasts to a tumourigenic phenotype (Ray *et al.*, 1997; Shih *et al.*, 1993; Ray *et al.*, 1996a).

Another possible mechanism by which HCV core protein may contribute to the development of HCC is via its effects on apoptosis. Expression of HCV core protein can inhibit cisplatin-mediated apoptosis in human cervical epithelial cells and apoptosis induced by the overexpression of *c-myc* in Chinese hamster ovary cells (Ray *et al.*, 1996b). In mice transgenic for the HCV core gene, a histologic feature characteristic of chronic hepatitis C, hepatic steatosis, then HCC (Moriya *et al.*, 1998) has been observed. This evidence indicates that HCV core protein contributes to both chronic infection and the development of HCC. In contrast, another study has found that transgenic mice expressing the full-length core protein show no histologic evidence of HCC, suggesting that the HCV core protein alone is insufficient to induce HCC (Kawamura *et al.*, 1997; Pasquinelli *et al.*, 1997).

The non-coding regions of the virus are also thought to be important in viral replication. These are found at each end of the viral genome (Smith & Pontisso, 1996). There is evidence that p70, a protein encoded by the NS3 region of HCV, has helicase activity, and may be involved in DNA recombination (Takamisawa *et al.*, 1991). Truncated NS3 protease transformed murine fibroblasts, causes HCC in immunodeficient male mice (Sakamuro *et al.*, 1995)

Coinfection by HBV, HCV and HGV

Coinfection by hepatotropic viruses can occur due to the fact that HBV and HCV share similar routes of transmission. The co-occurrence of antibodies to HCV (anti-HCV) and hepatitis B core antibody (anti-HBc) was observed in 54% of HBsAg-negative HCC patients (Kew *et al.*, 1997) and co-infection of HBV and HCV was 13.8% in HCC in Malaysia using sensitive recombinant DNA second generation enzyme immunoassay test kits (Sinniah & Ooi, 1993). Co-infection with both HBV and HCV may cause a more serious liver disease than infection with either agent alone (Donato *et al.*, 1998; Colombo, 1992; Rowley, 1990; Blum, 1994). Some studies have indicated that HBV and HCV have a synergistic effect in hepatocarcinogenesis (Donato *et al.*, 1998; Chuang *et al.*, 1993; Benvegnu *et al.*, 1994). The synergistic effect mechanism of dual infection with HBV and HCV in the development of HCC is probable that HBV acts as an "initiator" of HCC through its capacity to insert into the genome and disarrange cellular genes; HCV then act as a "promoter" by causing persistent liver necroinflammation and regeneration.

Hepatitis G Virus (HGV) viremia has been found in patients with liver disease of different aetiology (hepatitis B and C). The prevalence of HGV infection has been reported to be significantly higher in patients with HCC compared to the healthy population or patients with chronic hepatitis (Kao *et al.*, 1997; Muller *et al.*, 1997). However, there are other reports that HGV infection is not associated with HCC and does not increase the severity of the liver disease (Yuan *et al.*, 2000; Chiesa *et al.*, 2000). Thus, the pathogenic capacity of HGV is questionable and findings accumulated so far suggest that HGV may induce a hepatitis-like illness only in some rare cases and under certain circumstances (Muller *et al.*, 1997).

The molecular basis of AFB1 hepatocarcinogenesis

Aflatoxin ingestion is an important risk factor in the aetiology of HCC (Yeh *et al.*, 1989). The use of experimental models and specific biomarkers for aflatoxin exposure, such as urinary metabolites or aflatoxin adducts, have validated these findings (Qian *et al.*, 1994; Lunn *et al.*, 1997). So far, the mechanism of AFB1-induced HCC bas not been fully elucidated.

It is believed that . AFB1exerts its effects through its reactive epoxide. The formation of AFB1-8, 9-expoxide and its subsequent covalent binding to DNA to form AFB1-DNA adducts are critical steps leading to its hepatocarcinogenesis (Eaton & Gallagher, 1994). This epoxide and DNA complex often result in G:C –> T:A transversions (Greenblatt *et al.*, 94). AFB1 treatment of human hepatocytes causes the same transversion in the p53 gene and occurs preferentially at the third base in codon 249 (Aguilar *et al.*, 1993). One report has shown that glutathione S-transferase M1, and microsomal epoxide hydrolase are implicated in detoxification of AFB1 and increased frequency of mutant alleles are present in patients with HCC (McGlynn *et al.*, 1995). A large fraction of the tumour tissues and adjacent non-malignant liver tissues from HCC patients in certain geographic areas where both HBV and AFB1 are risk factors also contain this mutation (Greenblatt *et al.*, 1994; Aguilar *et al.*, 1994). This contrasts dramatically with the heterogeneity of p53 mutation which occurs in low aflatoxin exposure areas (Kazachkov *et al.*, 1996; Hayashi *et al.*, 1993; Unsal *et al.*, 1994; Challen *et al.*, 1992). Thus, it is now considered that the mutation at codon 249 is a fingerprint for AFB1-induced HCC. These data also suggest that p53 mutation play an important role in hepatocarcinogenesis in human. However, codon 249 equivalent mutations were not observed in AFB1-induced HCC in non-human primates, rats, ground squirrels, woodchuck, and ducks (Fujimoto *et al.*, 1992; Hulla *et al.*, 1993; Rivkina *et al.*, 1994; Imazeki *et al.*, 1995). Moreover, this mutation has been detected only in relatively advanced HCC (Ng *et al.*, 1994; Piao *et al.*, 1997; Honda *et al.*, 1998), suggesting that although AFB1 may induce the p53 mutation, additional factors are required for the development of human HCC.

An epidemiological investigation in parts of Africa and South-East Asia and a prospective study of HCC in China have provided evidence of a synergy between HBV and aflatoxin in hepatocarcinogenesis (Ross *et al.*, 1992; Qian *et al.*, 1994). Several animal model studies also supported this finding (Li *et al.*, 1999; Yan *et al.*, 1996; Bannasch *et al.*, 1995).

The cellular and molecular mechanisms behind these observed synergies are not well understood. One possible mechanism of interaction between HBV and chemical carcinogens is an alteration of carcinogen metabolism resulting from viral infection (De Flora *et al.*, 1989).

Using the Pekin duck model, there is evidence that AFB1 exposure leads to an increase in virus gene expression associated with intrahepatic accumulation of duck HBV large envelope protein and enhanced liver pathology (Barraud *et al.*, 1999). HBsAg expression and chemical carcinogens can also exert synergistic effects on hepatocarcinogenesis (Sell *et al.*, 1991).

The molecular alterations of host factors related to HCC

Tumour oncogenes

The ras proto-oncogene encodes a small GTP-binding protein and has a key role in controlling cell growth and differentiation through its intrinsic GTPase activity (Kjoller & Hall, 1999). Molecular switches mediate the activation of Ras. The GDP-bound inactive and the GTP-bound active forms are regulated by guanine nucleotide exchange factors (Bourne *et al.*, 1991; Bollag *et al.*, 1991; Quilliam *et al.*, 1995). Point mutations that activate the ras protein and its downstream cascade have been observed in human tumours (Serrano *et al.*, 1997). Recent studies demonstrated that the activation of Ras expression is relevant to the development of cirrhotic nodules and HCC, but that its sustained elevation is no longer required for cell proliferation or progression after tumour development (Nonomura *et al.*, 1987; Radosevich *et al.*, 1993). The expression of ras genes can interact with p53 protein (Chen & Defendi, 1992).

Besides ras, over-expression of another protocogen *c-myc* is common in most cases of HCC (Tabor, 1994). The activation of oncogenes such as ras and *c-myc*, not only induces proliferation and transformation, but also as apoptotic signal mediated by the tumour suppressor, p53.

Tumour suppressors

The p53 gene is located on human chromosome 17p13. This gene encodes a nuclear protein, p53. Because of the prolonged half-life of the mutated protein, it accumulates in the nuclei and becomes detectable with conventional immunohistochemical techniques. Recent evidence indicates that these alterations in p53 gene expression are common in human HCC. Both the frequency and the type of p53 mutations in HCC vary according to the geographical location of tumours.

Over-expression of p53 in HCC has occurred in 61% of patients from China (Hsia *et al.*, 1992), 38% of patients from Mexico (Soini *et al.*, 1996a), and 29% of Italian patients (Caruso & Valentini, 1999).

p53 mutation is more frequently detected in tumours with poorer cellular differentiation, greater tumour size and presence of giant cells, a shorter tumour-free interval and survival time in tumours (Ng *et al.*, 1995; Piao *et al.*, 1997; Honda *et al.*, 1998). These features imply that mutation of the p53 gene probably occurs after the initial stages of hepatocarcinogenesis, and it is not a prerequisite for malignant transformation. Other genomic alterations exist during an earlier phase of carcinogenesis.

HCC from countries with high aflatoxin exposure shows a characteristic mutation in p53 at codon 249 where there is an arginine to serine substitution. Most of these epidemiological studies on the p53 gene mutation have been performed on liver tissues. Recently, this mutation has been detected in cell-free DNA isolated from the serum or plasma of patients with HCC. One study in the Gambia showed that this mutation in plasma DNA is strongly associated with HCC. This mutation has also been detected in 15% of patients with cirrhosis, and 6% of control subjects (Kirk *et al.*, 2000), suggesting that this mutation can be an early genetic event in hepatocarcinogenesis. This is consistent with a previous study (Aguilar *et al.*, 1994).

Although p53 mutation is related to a later stage of the disease, reports on the prognostic significance of over-expression of p53 gene in HCC are few (Soini *et al.*, 1996; Hsu *et al.*, 1993; Ng *et al.*, 1995). It has been reported that p53 gene mutation is an independent risk factor for recurrence (Hayashi *et al.*, 1995), but this is inconsistent with another study (Ng *et al.*, 1995).

p53 gene controls cell cycle, apoptosis, and cellular differentiation (Oztürk, 1999). Accumulation of p53 can lead to cell cycle arrest (at G1)(Levine, 1997). It can mediate the transcriptional activation of FasL leading to p53-dependent apoptosis.

p53 can also induce the transcription of downstream genes, such as the cyclin-dependent kinase inhibitor p21 (Boulaire *et al.*, 2000). The p21 also can bind to proliferating cell nuclear antigen to inhibit DNA replication (Boulaire *et al.*, 2000), thus leading to a quiescent state. It has also been reported that expression of the cell cycle regulatory transcription factor DP1 is strongly inhibited by p53, at the level of transcription (Gopalkrishnan *et al.*, 1998).

As mentioned above, there is interaction between HBX and p53. The normal p53 gene is able to regulate X gene expression negatively (Takada *et al.*, 1996). Thus, the interaction between HBX and p53 may be one of mechanisms that p53 contributes to the development of HBV-associated HCC.

Several other tumour suppressor genes have also been described in HCC, such as *Rb* gene (Ashida et al., 1997) and p16 (INK4A) (Liew *et al.*, 1999). The *Rb* gene plays an important role in regulating the cell cycle and functions as a tumour suppressor gene. Mutation, deletion or altered expression of Rb have been reported in HCC (Asluda *et al.*, 1997). It has been found that 70% of HCC showed p16 INK4 gene alterations suggesting a role for p16 in HBV-mediated tumour progression (Liew *et al.*, 1999). The cyclin A gene is important in both S and G2-M phases of the cell cycle. It has been identified as the site for HBV integration (Paterlini *et al.*, 1995). The deregulation of cyclin A expression could affect the modulation of cell proliferation and regulation of oncogene/tumour suppressor proteins in progression of hepatocarcinogenesis. Mutation or loss of the cyclin-dependent kinase inhibitor, p16 will allow the cancer cells to enter the cycle, to maintain its progress and to growth-arrest stimulus.

Telomerase activity

The telomere is the structure at the end of the chromosome whose length is continuously reduced because of incomplete replication at the time of cell division, which eventually leads to cellular senescence when the telomere length is reduced beyond the critical level (Sedivy, 1998). Telomerase is an enzyme which binds and extends telomere ends (Greider & Blackburn, 1989). Normal human somatic cells express low or undetectable telomerase activity, but the activity of telomerase is detected in germ cell, stem cells, and cancer cells (Greider, 1998; Breslow *et al.*, 1997; Shay & Gazdar, 1997), suggesting that telomerase activity correlates with the unlimited proliferation of cancer cell. Telomerase activity is also extremely high in HCC (Tahara *et al.*, 1995; Ide *et al.*, 1996; Huang *et al.*, 1998).

Recently, three telomerase component genes: human telomerase reverse human telomerase reverse transcriptase (hTERT), human telomerase RNA component (hTERC), and telomerase-associated protein 1 (TEP1) have been identified (Nugent *et al.*, 1998). Telomerase reaction during hepatocarcinogenesis may be regulated by hTERT (Takahashi *et al.*, 2000, Toshikuni *et al.*, 2000) and an increase in telomerase activity level in tumour progression may be regulated by both hTERT and hTERC (Takahashi *et al.*, 2000). Down regulation of cyclin D1, cdk2, cdk4 protein is

correlated with telomerase activity (Hsieh *et al.*, 2000). The mechanism of activation of telomerase in hepatocarcinogenesis remains unknown. Further investigation on the control of telomerase expression and activity will be beneficial for the prevention of HCC.

Fas/FasL

Fas (Apo-1, CD95) is a type I transmembrane protein, a member of the nerve growth factor/tumour necrosis factor receptor family. It is expressed in a variety of cell types including hepatocytes, activated B and T cells (Nagata *et al.*, 1995; Krammer *et al.*, 1994; Nagata & Golstein, 1995). Fas ligand (FasL, CD95L) is a type II transmembrane protein, a member of the tumour necrosis factor family and induces cells to send an apoptotic signal to cells expressing Fas (Nagata *et al.*, 1995; Krammer *et al.*, 1994).

Previous investigations have demonstrated that Fas and FasL mediated apoptosis are involved in organ development and in pathological changes including cancer (Nagata & Golstein, 1995; Thompson, 1995). Non-cancerous hepatocytes, well or moderately differentiated HCC express Fas antigen, but lose this ability when they become poorly differentiated HCC (Ito *et al.*, 1998). Accordingly, another study also indicated that hepatocytes can co-express Fas and FasL in the areas of interface hepatitis and adjacent to HCC, but expression of Fas/FasL was significantly decreased within the tumour (Roskams *et al.*, 2000). It has been proposed that hepatoma cells may eliminate Fas expression and allow the hepatocytes and infiltrating mononuclear cells to generate soluble Fas in order to escape from the immune surveillance system (Nagao *et al.*, 1999).

Insulin growth factor II (IGF-II)

IGF-II is a 7.5 kDa polypeptide known to be involved in the regulation of growth and differentiation (Baker *et al.*, 1993). IGF-II has been reported to play a significant role in liver regeneration and hepatocarcinogenesis. IGF-II gene expression level is significantly higher in the dysplastic nodules compared to the control livers, but a significant increase in IGF-II gene expression has not been observed in well- and moderately differentiated HCCs as compared with the control livers, suggesting that IGF-II may play a role in the early stage of hepatocarcinogenesis (Aihara *et al.*, 1998). Using a cell line and chick embryos, Bae *et al.* reported that cell culture containing IGF-II from the human HCC cells, HepG, induced angiogenesis on the chorioallantoic membrane (CAM) of chick embryos, but the normal human liver cells, Chang liver cells did not induce angiogenesis in the CAM (Bae *et al.*, 1998). This result indicated that IGF-II may act as an angiogenic factor for the hypervascularization of HCC. Recently, it has been reported that IGF-II transcription may be activated by p53 mutant 249 which induced by AFB1 (Lee *et al.*, 2000). This may provide an explanation for the mechanism of hepatocarcinogenesis induced by AFB1.

EGF and ErbB-2

Epidermal growth factor (EGF) is a hormone that stimulates the intrinsic tyrosine kinase activity of the epidermal growth factor receptor (EGFR) (Ullich *et al.*, 1984). The EGFR (erbB-1) is a transmembrane glycoprotein with intrinsic tyrosine kinase activity in its cytoplasmic domain and is a member of a receptor family that includes the structurally related erbB-2, erbB-3, and erbB-4. Highly metastatic HCC cells derived from surgical specimens of late stage HCCs or liver metastases expressed 10 - 20-fold higher steady-state mRNA levels for EGF-R compared with low metastatic cell lines derived from early-stage HCCs. Immunohistochemical staining of these highly metastatic KM12SM cells demonstrated uniform and intense staining for total EGF-R. In contrast, low metastatic parental KM12C cells showed heterogeneous EGF-R staining, with less than 10% of cells staining positive (Radinsky *et al.*, 1995). HCC cells expressing increased EGF-R levels have a selective advantage in the production of liver metastases in athymic nude mice (Radinsky *et al.* 1995; Singh *et al.* 1997). These indicate that the production of liver metastases by HCC may depend, in part, on the activation status of EGF-R.

Amplification of erbB-2 has a high degree of correlation with disease recurrence and poor survival (Mulcahy *et al.*, 1985). Both clinical and basic research studies indicate a role for erbB-2 amplification in initial transformation events and in progression to metastases (Satoh *et al.*, 1990; Satoh *et al.*, 1992).

Transforming growth factor alpha and beta

Transforming growth factor-α (TGF-α) is thought to be important for growth and development of normal cell. It was reported that TGF-α mRNA levels were extremely higher in patients with HCC compared with patients with chronic viral hepatitis and normal controls, and the levels in patients with chronic viral hepatitis also were elevated compared with normal controls. The levels of TGF-α mRNA were over-expressed in the underlying livers of patients with HCC compared with patients with chronic viral hepatitis, although they

were lower than those found in HCC tissues. The levels of TGF-α mRNA were higher in samples from patients with chronic hepatitis B than in samples from patients with chronic hepatitis C. The over-expression of TGF-α mRNA in the liver seems to be associated with the regeneration of hepatocytes rather than hepatic necrosis or viral replication. Also, it may be related closely to the development or progression of HCC, especially in the livers of patients with chronic hepatitis B (Chung *et al.*, 2000).

TGF-β, related functionally to activins and inhibins, also regulate cell proliferation and differentiation. Increased levels of TGF-β have also been demonstrated in HCC tissue and serum of patients with HCC (Abou-Shady *et al.*, 1999; Sacco *et al.*, 2000). It has been suggested that increased secretion of TGF-β by cells may lead to loss of their responsiveness to the growth-inhibitory activity of TGF-β, facilitating tumour progression (Factor *et al.*, 1997). The antimitogenic activity of TGF-β has been shown to be partly mediated through induction of the tumour suppressor INK4b and partly through downregulation of the protooncogene Myc in Mv1Lu lung epithelial cell lines that conditionally express levels of human c-Myc (Warner *et al.*, 1999). These events appear to be linked to the cell-cycle arrest response to TGF-β. Whether these events occur in HCC is unknown at this stage.

The function of TGF-β is dependent on the interaction with surface receptor types I, II and III (TGF-βRsI-III). It is proposed that TβR III is involved in regulating the access of ligand to TGF-βRs I and II. The activation of TGF-βR II leads to the phosphorylation of TGF-βR I which, in turn, phosphorylates smad proteins which transduce the signal to the nucleus (Nakao *et al.*, 1997; Inagaki *et al.*, 1993; Bassing *et al.*, 1994).

Catenins

In recent years, there has been increasing interest in catenins, a series of undercoat protein that interact with the intracellular domain of E-cadherin, a cell adhesion receptor. These include α-catenin, β-catenin and ε-catenin. The β-catenin is anchored to the carboxy-terminus of E-cadherin via the α-catenin linked to the actin cytoskeleton. Besides its function as a protein that supports the cell-cell junction, β-catenin can also function as a co-activator for the transcription factors of the lymphocyte enhancer binding factor (LEF) family. Thus, β-catenin provides a molecular mechanism for the transmission of signals, from cell-adhesion components to the nucleus (Behrens *et al.*, 1996). The selective loss of E-cadherin expression can generate dedifferentiation and invasiveness of human carcinoma cells (Frixen *et al.*, 1991). These studies have been shown in cancers such as breast and colorectal cancers (Hazan *et al.*, 2000; Gofuku *et al.*, 1999). It is most likely that β-catenin is also involved in the development of HCC as β-catenin mutations have been detected in 41% of HCC associated with HCV (Huang *et al.*, 1999). Another study has shown that HCC patients with overexpression of β-catenin had poor survival rates (Endo *et al.*, 2000). Further studies on E-cadherin and catenins need to be performed to better understand the mechanisms associated with hepatocarcinogenesis.

Alpha-foetoprotein

The serum alpha-foetoprotein (AFP) concentration in man falls rapidly after birth and increased level of AFP has been detected in greater than 70% of patients with HCC. There is a slight elevation of serum AFP in patients with other malignant diseases such as those of the gastrointestinal tract and benign liver diseases (chronic hepatitis, fulminant hepatitis and cirrhosis). Therefore, the measurement of serum AFP has been extensively used for the detection of HCC and for screening populations at high risk of HCC such as those with cirrhosis or HBV carriers.

An early study has suggested that AFP is indeed subjected to hormonal, nutritional, and haematological regulation (Belanger *et al.*, 1975). There is also evidence that AFP may serve to modulate growth factor-mediated proliferation during development and neoplasia (Leal *et al.*, 1980; Keel *et al.*, 1991). AFP has also been implicated in regulation of immune responses of the humoral and cell-mediated types (Murgita & Wigzell, 1979; Hoskin & Murgita, 1989). The role of AFP in the aetiology of HCC is unclear.

Summary

As reviewed above, hepatocarcinogenesis is believed to be a multistage process and some genes are related to the development of HCC. HBX, the viral protein encoded by HBV, has the ability to inactivate a number of key negative growth regulatory pathway such as p53, p21WAF/CIP1, alter cell cycle regulation and disrupt functions governing DNA repair, growth arrest and apoptosis. Mutations in tumour suppressor genes and other molecules such as the growth factors and adhesion molecules such as the catenins have also been implicated to contribute to hepatocarcinogenesis. The frequencies of mutations in oncogenes and tumour suppressor genes and growth factor/receptors are

summarised in Table 2. Although abnormal genetic changes have been gradually unveiled in HCC, molecular mechanisms leading to the development of HCC is still fragmentary. The functions of HCC-associated genes including viral oncogenes are mostly unknown. There are still some issues which need to be explored, such as how many gene mutations are required, which gene plays a key role; which factor(s) serve(s) as promoter(s), how these factors interact between the host genes, chemicals and viruses in hepatocarcinogenesis. Whether there are other genes relevant to hepatocarcinogenesis and the reasons for the great variation of HCC development from patient to patient requires further investigation. Further elucidation of the genetic and molecular mechanisms of hepatocarcinogenesis will aid in the prevention and treatment of HCC.

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Table 2. Oncogenes and tumour suppressor genes associated with HCC

Genes	Functions	Incidence (%) in HCC
Cell cycle genes		
P53	transcription factor; checkpoint control; apoptosis	29-61 (Carus, *et al.*, 1999; Hsia *et al.*, 1992)
P16	negative regulators of cell cycle	70-96 (Liew *et al.*, 1999; Ito *et al.*, 1999)
p21	GTPase	62 (Nonomura *et al.*,1987)
p27	regulation of the cdk inhibitor	
Rb	negative regulators of cell cycle	85 (Ito *et al.*, 1999)
Cyclin D1	regulation of cell cycle	32 (Ito *et al.*, 1999)
Signal transduction pathway		
Ras	control of proliferation	
TGF-βRII	regulation of growth and differentiation	
IGF-II		4-100 (Fiorentino *et al.*, 1994, Takeda *et al.*, 1996)
Growth factor receptor		
erb-b2	growth factor receptor	92 (Tang *et al.*, 1998)
EGFR	tyrosine kinase	47 (Tang *et al.*, 1998)

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