3.0 Review of Literature

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) accounts for nearly 85% of the primary malignant tumors of the liver (Kew, 2002). HCC is the fifth most-common malignancy in the world and is the third most-common cause of cancer-related death worldwide (Okuda, 2000; Parkin et al., 2001). The age-adjusted worldwide annual incidence is between 5.5–14.9 people per 100,000 populations and accounts for approximately 600,000 to 1,000,000 deaths annually (Parkin et al., 2001, Llovet et al., 2003 and Bosch et al., 2005). HCC is considerably more common in men than women, with an incidence ratio of approximately 3:1 (Kew, 2002; Bosch et al., 2005). It is most prevalent in regions of Eastern and South-Eastern Asia and sub-Saharan Africa (>15 per 100,000 population) (Kew, 2002; Bosch et al., 2005 and Bosch et al., 1999). Recent epidemiological analysis reports suggest a dramatic increase in developed countries in contrast to stabilization and a decreased incidence in some underdeveloped regions of the world. These increases are believed to be attributable to an increasing incidence of hepatitis C (HCV) and B virus (HBV) infection, chronic alcohol abuse and increase in nonalcoholic fatty liver disease (Kew, 2002; Davis et al., 2003 and Smart et al., 1998). Further, combinations of each of these risk factors can greatly increase a patient’s risk for the development of HCC.

Etiology

Cirrhosis and chronic liver diseases are associated with the majority (>80%) of HCC cases and represent a major underlying factor predisposing the development of HCC (Bosch et al., 2005; Davis et al., 2003; Koniaris et al., 2003 and Zhu, 2003). Approximately 75–80% of worldwide HCC cases are related to chronic infection with the hepatitis viruses HCV and HBV (Bosch et al., 2005 and Bosch et al., 1999). Similarly, exposure to dietary aflatoxin represents another common cause of hepatic cirrhosis in developing nations with
increase risk of malignant transformation (Kew, 2002 and Bosch et al., 1999). The geographic areas at the highest risk are South-East Asia and sub-Saharan Africa, where HBV infection is highly endemic and is the main cause of HCC (Fig 3.1). In areas with an intermediate incidence of HCC such as Southern Europe, HCV infection is the predominant cause, whereas in low incidence areas such as Northern Europe and the United States, HCC is often related to other factors such as alcoholic liver disease (Bosch et al., 2005).

Fig 3.1. Geographic distribution of HCC. Incidence rates % in total population. A. Female, B. Male.

HBV infection precedes HCC development and that the relative risk of development of HCC is approximately 100-fold greater for these carriers (Beasley et al., 1981). The HBX protein of HBV is a transcriptional factor that can alter the expression of many cellular genes, including a subset of oncogenes such as c-myc and c-myb, as well as tumor suppressor genes such as APC, p53, p21\textsuperscript{waf1/cip1}, and WT1 in primary human hepatocytes (Huo et al., 2001). Furthermore, the X protein has the ability to sequester the tumor suppressor p53 \textit{in vitro} and can block p53 mediated apoptosis \textit{in vivo}, which contributes to the development of preneoplastic and neoplastic hepatocytes (Huo et al., 2001).
Overall, HBV infection is believed to be the major underlying factor in \approx 80\% of HCC diagnosed globally yet, only a minority of chronic HBV carriers develop HCC during their lifetime, which indicates the possible involvement of other cofactors for the development of HCC. Such cofactors may include concomitant aflatoxin exposure, race, male sex, employment in certain job industries, and the use of tobacco (Lok, 2000). In addition, HIV coinfection, leading to increased incidence of HCC development, also has been reported (Brau, 2005).

**Natural history of HCC**

The molecular mechanisms of hepatocarcinogenesis remain poorly understood. Increasingly, malignant transformation in any cell is being recognized as a complex signaling network in which multiple interactive pathways lead to abnormal cell proliferation and resistance to apoptosis (She et al., 2005 and Bianco et al., 2003). There are currently no consistent genetic sequences of event identified that lead to HCC formation with much of the past research indicating that there are multiple pathways to its development (Feitelson et al., 2002). However, a number of molecular changes still exist that occur in high frequency within cirrhotic tissue and small tumors and that may constitute the early stages of hepatocarcinogenesis (Feitelson et al., 2002). Overall, it is apparent that hepatocarcinogenesis is a multistep process that leads to progressive loss of differentiation, loss of normal cell adhesion, degradation of the extracellular matrix, and constitutive activation of selected survival and growth-promoting pathways. Furthermore, examination of patients with advanced cirrhosis demonstrates that tumor formation may actually represent multiclonal tumor formation (Lencioni et al., 2004).

**Pathology**

Hepatocarcinogenesis initiated by HBV, HCV, or environmental carcinogens usually follows the same sequence of necroinflammatory changes; hepatitis, fibrosis, cirrhosis, hepatocellular adenoma and, finally, HCC (Okuda, 2000 and Lim, 2002). It was first reported in 1986 that early HCC evolved in
adenomatous hyperplastic nodules within the context of chronic hepatitis and/or cirrhosis (Arakawa et al., 1986). Since then, it has been established that these dysplastic nodules will become malignant in approximately half of these patients and should be considered an absolute precursor of transformation (Takayama et al., 1990). Early histological changes that precede malignant transformation in these nodules include an increase in cellularity (nuclear crowding), an irregular, thin trabecular pattern with frequent acinus and pseudogland formation (Okuda, 2000). This early HCC is well differentiated, from which less well-differentiated HCC develops (Okuda, 2000). As the small HCC becomes dedifferentiated, the number of portal tracts decrease and the number of intratumoral arterioles increase (Nakashima et al., 1999). The tumor cells invade the neighboring portal tracts and the fibrous stroma of the cirrhotic liver (Okazaki et al., 1997). Elevated levels of matrix metalloproteinase-1 (MMP1) are seen at this stage of development, allowing the tumor to break down the extracellular matrix of the portal tract tissue and permitting further tumor growth (Okazaki et al., 1997). The result of this process is a large, poorly differentiated tumor capable of vascular invasion.

**Molecular pathways of Hepatocarcinogenesis**

Chronic HBV infection has been shown to be strongly associated with HCC (Brechot, 2004; Beasley et al., 1981 and Robinson, 1992) but the pathogenesis of HBV-induced malignant transformation is poorly understood. Much of the research on HBV hepatocarcinogenesis has been focused on the HBX gene, where several lines of evidence suggest its role in the malignant transformation process (Kim et al., 1991). The implications of these modulation effects of HBx are not fully understood, but they are likely to have wide-ranging effects on hepatocyte proliferation, apoptosis and the regulation of cell growth checkpoints (Peng et al., 2005; Ahn et al., 2002 and Arbuthnot et al., 2000).

HBx is a transactivator that up or down regulates a wide range of regulatory molecules either directly or indirectly, including, proto-oncogenes
such as c-myc and c-jun (Balsano et al., 1991 and Twu et al., 1993), transcriptional factors like NFκB, AP-1 and ATF/CREB, as well as viral promoters such as HBx and SV40 (Chirillo et al., 1996; Weil et al., 1999; Lucito and Schneider 1992; Henkler et al., 1998; Seto et al., 1990 and Choi et al., 1999) and IL-6 (Lee et al., 1998), nitric oxide synthetase (Amaro et al., 1999), Fas ligand (Shin et al., 1999), p21^WAF1/cip1(Park et al., 2000), Wnt/β-catenin (Cha et al., 2004 and Ding et al., 2005), E-cadherin (Lee et al., 2005), p53 (Ueda et al., 1995), Vascular Endothelial Growth Factor (VEGF) (Lee et al., 2000 and Moon et al., 2004). Cellular promoters of genes associated with cell proliferation, such as IL-8, Tumor Necrosis Factor (TNF), TGF-β1 and Epidermal Growth Factor Receptor (EGFR), also respond to HBx transactivation (Andrisani and Barnabas, 1999). Clinical and epidemiologic studies suggest that HCV is more hepatocarcinogenic than HBV, as the frequency of HCC development among HCV-induced cirrhosis is much higher than that of HBV-induced cirrhosis (Ikeda et al., 1993). The HCV associated hepatocarcinogenesis seems to be mediated by core, NS3 and NS5A proteins through post-transcriptional inhibition of p21^WAF1 (Kwun et al., 2001; Lan et al., 2002). Mutational inactivation of the p53 is another mechanisms involved in HCC pathogenesis, especially in geographical areas where dietary aflatoxin B1 (AFB1) exposure is prominent (Hsu et al., 1991 and Aguilar et al., 1994). The G>T mutation of the p53 gene at codon 249 has been identified as a genetic hallmark of HCC caused by AFB1 (Ozturk, 1991).

A multi-step accumulation of genetic alteration has long been proposed as one of the major mechanisms underlying hepatocarcinogenesis. These genetic and epigenetic alterations combine to activate positive mediators of cellular proliferation (including cellular proto-oncogenes and their mitogenic signaling pathways) and inactivate negative mediators of cellular proliferation (including tumor suppressor genes), resulting in cells with autonomous growth potential (Pitot, 2001; Feitelson et al., 2002; Thorgeirsson and Grisham, 2002 and Chen et al., 1997). With the advances of genomic-wide analysis such as comparative
genomic hybridization (CGH), loss of heterozygosity (LOH) and fine microsatellite studies, various studies have identified ‘hotspots’ of chromosome loci that are prone to allelic gains and losses in HCC (Kusano et al., 1999; Chang et al., 2002; Guan et al., 2001 and Wong et al., 2000). There are recurrent reports of frequent deletions on chromosome arms of 17p, 8p, 16q, 16p, 13q, 1p, 4q and 9p, while gains of chromosome 1q, 6p, 8q, 17q and 20q have been widely observed (Boige et al., 1997; De Souza et al., 1995; Niketeghad et al., 2001; Yeh et al., 2001 and Wang et al., 2001). Peptidyl prolyl isomerase named PIN1, which is a novel regulator of β-catenin signaling and cyclin D1, is up-regulated in over 50% of our HCC cases. PIN1 over-expression and somatic mutations of β-catenin gene are distinct events leading to β-catenin accumulation and further underscoring the importance of PIN1 in hepatocarcinogenesis (Pang et al., 2004).

Many studies on HCC have focused on the search for putative tumor suppressor genes within these hotspots of chromosome arm deletions. p53, is responsible for the frequent losses detected in 17p (Nishida et al., 1993). AXIN1 inactivation accounts for losses detected in 16p (Taniguchi et al., 2002) whereas inactivation of insulin growth factor-2 receptor (IGF2R) and p16 accounts for the losses detected on 6q and 9p, respectively (De Souza et al., 1995; Biden et al., 1997 and Piao et al., 1998). Recent studies have identified putative tumor suppressor genes at these ‘hotspots’. Wong et al. (2003) reported that DLC-1, a putative tumor suppressor gene mapped at 8p21.3-22, encodes a GTPase-activating protein specific for RhoA and Cdc42 to exert inhibitory effects on the proliferation of HCC cells. Ching et al. (2003) identified another homologue of DLC-1, named DLC-2, which encodes a novel Rho family GTPase-activating protein. DLC-2 was reported to be significantly under-expressed in their studied HCC cases.

Nevertheless, HCCs exhibit a high degree of genetic heterogeneity, suggesting that multiple molecular pathways may be involved in the genesis of
Review of Literature

subsets of hepatocellular neoplasms. Continued investigation of the mechanisms of hepatocarcinogenesis will refine our current understanding of the molecular and cellular basis for neoplastic transformation in the liver, enabling the development of effective strategies for prevention and/or more effective treatment of HCC.

Treatment options and limitations

Surveillance programs allow doctors to identify patients at early stages of the diseases, when the tumor may be curable by radical treatments such as resection, Liver transplantation, or local ablation. Resection is effective in patients, when the tumor is less than 3 cm in size (Arii et al., 2000). Resection yields favorable results in patients with single tumors and a well-preserved liver function (5-year survival rate is 60%). However, most patients with liver tumor also have cirrhosis of the liver and would not tolerate liver resection and would look for liver transplantation surgery. Liver transplantation is the best treatment for patients with single tumors that are less than 5 cm in diameter and liver failure, but organ shortage greatly limits its applicability. Tumor recurrence complicates 70% of cases at 5 years, combining true recurrence and de novo tumors (Bismuth and Majno, 2000). Chemoembolization and local ablation are the neo-adjuvant treatments applied to patients on the waiting list to prevent tumor progression; no controlled study proving their efficacy has yet been published. For the majority of the patients (> 80%), non surgical treatment is the only alternative. In nonsurgical candidates, ultra sound guided percutaneous treatments (ethanol injection or radiofrequency ablation) are the best therapeutic approach (Sala et al., 2004). At more advanced stages, chemoembolization, a technique combining intra-arterial chemotherapy and selected ischemia, Interferon therapy has shown to slightly improve survival in a meta-analysis of randomized trials. Systemic chemotherapy of HCC using drugs like flurouracil, cisplatin and adriamycin has been of limited value in clinical practice because of their high toxicity (Okada, 1998 and Rougier et al., 1998). No survival advantages have been demonstrated with intra-arterial, hormonal compounds,
cryotherapy, immunotherapy or radiation. New agents, such as inhibitors of the tyrosine kinase receptors of growth factors and antiangiogenic agents, are currently being tested in phase II/III trials. Finally, gene therapy is an alternative approach to HCC treatment and although currently at the preclinical and experimental stage (Ferry, 1997). Universal immunisation programmes against HBV have proven to be effective in reducing HBV carrier rates by more than 10-fold (Chang, 1998).

**Herbal medicine for HCC**

Medicinal plants are part and parcel of human society to combat diseases from the dawn of civilization. In view of the side effects of drugs used in the chemotherapy of cancers, traditional herbal medicine are being used as complementary and alternative medicine (CAM) and are becoming increasingly popular among cancer patients in the developed countries (Molassiotis et al., 2005 and Yates et al., 2005). In the traditional medicinal systems, about 150 phytoclonstituents isolated from 101 plants have been reported to possess liver protective activities and are being sold world over as a number of herbal formulations (Subramoniam and Pushpangadan, 1999). The commonly used herbal preparations in the treatment of liver diseases are - Silymarin (a lipophilic extract from the seeds of milk thistle) (Mayer et al., 2005), Glycyrrhizin (an aqueous extract of the licorice root) (Kumada, 2002), *Phyllanthus* (aqueous plant extract) (Liu et al., 2001), Sho-saiko-to or TJ-9 (a mixture of seven herbs) (Oka et al., 1995) and LIV-52 (a polyherbal Ayurvedic formulation) (Huseini et al., 2005). Curcumin is another plant-derived nontoxic polyphenol with strong anticancer effects in cell culture and animal model systems (Aggarwal et al., 2003 and Campbell and Collett, 2005). Curcumin has been quite effective against angiogenesis and metastasis in HCC (Yoysungnoen et al., 2005 and Ohashi et al., 2003). More recently, the extracts of root and latex of *Calotropis procera* have been shown to have a potent anticancer activity in cell culture as well as in animal models (Van Quaquebeke et al., 2005 and Choedon et al., 2006). Similarly, the flavonoid-rich alcoholic extracts of the aerial parts of weed
*Vicia calcarata* and flowers of *Butea monosperma* containing isobutrin and butrin are reported to protect the rat liver from chemically-induced hepatic damage (Fig 3.2.B) (Singab *et al.*, 2005 and Wanger *et al.*, 1986).

**Butea monosperma** (Flame of the Forest)

<table>
<thead>
<tr>
<th>Family</th>
<th>Fabaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>India</td>
</tr>
<tr>
<td>Type/Uses</td>
<td>flowering tree</td>
</tr>
<tr>
<td>Size</td>
<td>50 feet</td>
</tr>
<tr>
<td>Growth Rate</td>
<td>Slow growing at first</td>
</tr>
<tr>
<td>Lighter Requirements :</td>
<td>full sun</td>
</tr>
<tr>
<td>Water Requirements :</td>
<td>average, drier in the winter</td>
</tr>
<tr>
<td>Min. Temp.</td>
<td>mid 30ºs</td>
</tr>
<tr>
<td>Flower</td>
<td>late winter, spring</td>
</tr>
</tbody>
</table>

*B. monosperma* is one of the versatile medicinal plant having a wide spectrum of biological activity (Fig 3.2.A). There are several reports on the medicinal utilities, pharmacological actions and number of chemical compounds of this plant (Bhatwadekar *et al.*, 1999; Kasture *et al.*, 2002 and Prashanth *et al.*, 2001). However, the anti tumorogenic property of *B. monosperma* needs to be investigated.

**Biomarkers for HCC**

Several effective therapies are available for patients diagnosed with HCC, but still a majority of individuals are diagnosed at a too advanced stage where no active treatment is feasible. Hence, renewed effort has to be placed to further improve the efficacy of the options that are currently available and at the same time develop new strategies that ultimately will improve the survival of patients. Application of these advancements together with the implementation of health campaigns to prevent the acquisition of the risk factors for this neoplasm (viral infection, alcohol intake) will ultimately translate into the expected
Fig. 3.2  A. Butea monosperma flower (Family: Fabaceae) B. Isobutrin and Butrin compounds isolated from Butea monosperma.
reduction of the number of HCC related deaths. Therefore, early diagnosis of HCC is extremely important for the clinical outcome.

So far Alpha-fetoprotein (AFP), the only serological marker commonly used in diagnosis, has failed to be a reliable marker mainly because it shows poor sensitivity, ranging from 39% to 65% and a specificity ranging from 76% to 97% (Sherman et al., 1995; Collier and Sherman, 1998; Trevisani et al., 2001 and Nguyen et al., 2002), reviewed in (Daniele et al., 2004 and Marrero and Alok, 2004). This high variability is because different cut-offs are used in the different studies and also because these data were mainly obtained from retrospective studies. AFP seems to be reliable at values over 400 IU/ml, but the percentage of patients with such high levels is very small; this represents one of the most important limits of this marker. Squamous cell carcinoma antigen (SCCA) is a member of the high molecular weight family of serine protease inhibitors (serpins) (Suminami et al., 1991). The expression of SCCA variants in HCC tissues at the protein and translational levels (Pontisso et al., 2004) has been reported. Furthermore, SCCA has been detected at higher levels in the sera of HCC than cirrhotic patients (Giannelli et al., 2005). GP73 is another protein shown to be up-regulated in HCC patients. Marrero has reported a sensitivity of 69% and a specificity of 75% in HCC versus cirrhotic patients, using 10 relative units as cut-off, calculated by densitometric scanning of immunoblotting (Marrero et al., 2005). Although this is a promising study, more investigations are required to confirm these data and establish its role in detecting early cancer. GPC3 is another oncofoetal protein that has been reported to be down-regulated in breast cancer, ovarian cancer and lung adenocarcinoma but up-regulated in HCC (Lin et al., 1999; Xiang et al., 2001; Kim et al., 2003 and Man et al., 2005). GPC3 has been mainly investigated at the tissue levels, although some studies have reported the presence of GPC3 in the sera of about 50% of HCC patients but absence in healthy subjects (Nakatsura et al., 2003 and Capurro et al., 2003). No systematic data are available concerning its sensitivity and specificity. Des-gamma carboxy prothrombin (DCP) is an abnormal prothrombin
lacking carboxylation of the 10 glutamic-acid residues in the N-terminus, and is the result of an acquired post-translational defect of the prothrombin precursor in HCC cells, and therefore, has been used as HCC marker (Ono et al., 1990). Overall, DCP is more reliable than AFP as a prognostic tool for predicting the clinical outcome of patients with HCC, rather than as a diagnostic tool for early detection of the cancer (Gotoh et al., 2003). For this reason DCP is not an adequate diagnostic marker.

Therefore, the search for new markers for HCC diagnosis has become an important quest by clinicians, as documented by the numbers of papers reported in the literature during the past 15 years (Fig 3.3) (Giannelli and Antonaci, 2006).

**Cell cycle regulators of HCC**

Tumorogenesis is a multistep process that results in uncontrolled proliferation, invasion and ultimately metastasis of tumor cells. This process frequently involves differential expression of signaling molecules. These are having great impact on cell cycle mediated cell proliferation and differentiation. The cell cycle is a highly ordered process that results in the duplication and transmission of genetic information from one cell generation to the next. During the process DNA must be accurately replicated and identical chromosomal copies distributed to two daughter cells. The cell cycle has four phases: G1 (gap
1 phase) is the interval between mitosis (M) and DNA synthesis (S) phase. During G1, the cell is subject to stimulation by extra cellular mitogens and growth factors; in response to these stimuli, the cell passes G1 and proceeds with DNA synthesis in S phase; G2 (gap2 phase) is the interval between the completion of DNA synthesis and mitosis; M phase is marked by generation of bipolar mitotic spindles, segregation of sister chromatids and cell division. The regulation of cell cycle ensures that the events in each phase are complete before moving to the next. Thus, checkpoints for monitoring the integrity of DNA are strategically placed in late G1 and at the G2/M interface to prevent progression and propagation of mutated or damaged cells. G0 refers to cells that are quiescent. Cells use a complex set of enzymes called kinases to control various steps in the cell cycle. Cyclin dependent kinases (CDKs) are a specific enzyme family that use signals to switch on cell cycle mechanism. These CDKs complex with their respective cyclins and, subsequently, are phosphorylated by an activating kinase. When functioning properly cell cycle regulatory proteins, including CDKs and cyclins, act as the body’s own tumor suppressors by inducing the death of damaged cells. Genetic mutations causing the malfunction or absence of one or more of the regulatory proteins at cell cycle checkpoints can result in the “molecular switch” being turned permanently on, permitting uncontrolled multiplication of cell, leading to carcinogenesis, or tumor development (Israels and Israels, 2001).
Progression through the G1/S phases of the cell cycle is mostly controlled by cyclin-dependent kinases (Cdk4, Cdk6 and Cdk2) and their substrates (Malumbres and Barbacid, 2001). It has been shown that most, if not all tumors, lose control of the G1/S transition through alterations in the pRb pathway. Since, the early enhanced G1/S transition usually involves a combination of high levels of cyclins and low levels of cdk inhibitors as seen in many human malignancies including HCC. Hepatitis type B virus (HBV) integration into hepatocyte DNA, which is epidemiologically related to hepatocellular carcinomas, can interrupt one of the cyclin A1 introns generating a chimeric transcript without the N terminus of cyclin A1 (Wang et al., 1990). This chimera escapes the normal degradation controls, yielding cyclin A1 overexpression through protein stabilization. Cyclin A1 has been mapped to 4q26-27, a hot spot for the HBV integration that yields HCC. AML12 cells or Chang liver cells expressing HBX also showed G1-to-S and G2-to-M progression, which has been interpreted to be due to increased mitogenic signaling coupled with activation of cyclins and CDC2 (cell division cycle 2) kinases (Lee et al., 2002 and Benn and Schneider, 1995). Overall cell cycle regulatory elements involved in human neoplasia are given in the Table 3.1.

p53 is also a tumor suppressor "gatekeeper" that prevents tumor growth by inducing apoptosis, cell cycle arrest or senescence through multiple effectors (el Deiry, 1998 and Levine, 1997). p53 upregulates p21Cip1 and induces G1 arrest (by inhibition of Cdk2 kinase) in response to stress signals. In general, p53 is the most commonly mutated gene in human cancer: about 50% of the human tumors carry mutations in this gene (Hollstein et al., 1994 and Soussi and Beroud, 2001). Furthermore, considering also the other mutations that make inoperative the p53 net, most human tumors carry a deregulated p53 pathway (Michael and Oren, 2002; Lohrum and Vousden, 2000).
Angiogenesis in HCC

Angiogenesis & Anti-angiogenesis

The aberrant formation of sprouting new blood vessels (angiogenesis) also associated with a number of pathological conditions and diseases, including cancer (Folkman, 1995 and Ferrara and Alitalo, 1999). Tumors, like many normal tissues, use the vasculature, as a means to obtain oxygen and nutrients and to remove waste products. Based on numerous animal studies, the vascular endothelial growth factor (VEGF) pathway is the only well-defined signaling pathway known to be required for normal development of the vasculature as well as for the pathologic angiogenesis that accompanies cancer and other disease states (Eriksson and Alitalo, 1999; Yancopoulos et al., 2000). Angiogenesis plays an important role in the aggressive biological behavior of HCC, which is one of the most vascular human cancers. VEGF appears to be the most critical angiogenic factor regulating angiogenesis in HCC. However, the exact
regulatory mechanisms of HCC remain to be clarified. Mise et al. (1996) first reported overexpression of VEGF by Northern blot analysis and immunohistochemical staining in 12 of 20 human HCC specimens compared with non-tumorous liver tissue, and the level of VEGF mRNA was found to be significantly correlated with the intensity of angiographic tumor staining. A subsequent study also demonstrated a strong association between VEGF immunostaining and angiographic vascularity, which suggested an important role of VEGF in the development of neovascularization in HCC (Torimura et al., 1998). Using the technique of in situ hybridization, the expression of VEGF mRNA has been confirmed in HCC tumor cells, especially in areas adjacent to tumor necrosis, where the VEGF expression may be stimulated by local hypoxia (Suzuki et al., 1996). The expression of VEGF in the hypoxic area of HCC may be upregulated through the hypoxia inducible factor 1-alpha (HIF1-α) pathway (Yasuda et al., 2004). An et al. demonstrated that seven times more endothelial cells were positive for VEGF antibody in carcinoma areas than in non-carcinoma areas in HCC, suggesting that VEGF is an important angiogenesis factor for HCC (An et al., 2000). VEGF appears to play a significant role in the early stage of hepatocarcinogenesis. Its expression increases gradually from low-grade dysplastic nodules to high-grade dysplastic nodules to early HCC (Park et al., 2000). The role of VEGF in hepatocarcinogenesis has also been demonstrated in a study using a murine HCC model (Yoshiji et al., 2004). Recent studies have shown that HBx protein, which is an important oncogenic protein of HBV, activates VEGF through the HIF1-α pathway and may play a significant role in inducing angiogenesis in HBV related hepatocarcinogenesis (Lee et al., 2000). Yoo et al. (2003) demonstrated that HBx enhanced transcriptional activity of HIF-1alpha in the reporter genes encoding hypoxia response element or VEGF promoter, and the expression of HIF-1alpha and VEGF was increased in the liver of HBx-transgenic mice. Further studies to elucidate the mechanism involved in angiogenesis of HCC will not be only crucial to our understanding of the tumor biology of HCC, but they also help provide guidance to the use of appropriate antiangiogenic agents for HCC.
In addition to its role in hepatocarcinogenesis, VEGF also plays an important role in tumor progression and metastasis. Torimura et al. (1998) reported a significant association between high VEGF immunostaining and poorly differentiated HCCs. Pathological correlation studies have demonstrated that high VEGF expression in HCC is associated with a high proliferative index, poor encapsulation of tumors, and venous tumor emboli or portal vein thrombosis (Chow et al., 1997 and Li et al., 1998). The direct correlation between overexpression of VEGF in tumor cells and tumor angiogenesis in HCC has also been demonstrated (Moon et al., 2003). The critical role of VEGF in angiogenesis has been demonstrated in an experimental model in which the VEGF gene expression was manipulated in murine HCC cells transfected with a retroviral vector system carrying the gene (Yoshiji et al., 1998). In this experiment, the growth and neovascularization of the HCC tumor were shown to correspond directly to the level of VEGF gene expression. HCC is unique among various human cancers in the aspect that it is associated with cirrhosis in the non-cancerous liver in the majority of cases. VEGF has been shown to be overexpressed in cirrhosis and appears to play a role in angiogenesis, which is also an important pathological process in cirrhosis (El-Assal et al., 1998; Deli et al., 2005 and Medina et al., 2005). The significance of angiogenesis in cirrhosis and its relationship with hepatocarcinogenesis in cirrhotic liver are areas worthwhile of further investigation. Fig 3.4 summarizes the role of angiogenesis in the various steps of tumor growth and metastasis (Poon et al., 2001).
A switch to an angiogenic phenotype is a prerequisite for the development from a premalignant stage to an invasive tumor. The neovascularization provides not only nutrients for tumor growth but also a large surface area of leaky vessels with incomplete basement membrane that facilitate intravasation of tumor cells. Tumor cells seeding in distant organs develop initially as avascular dormant micrometastases. Angiogenic switch leads to secondary angiogenesis and tumor growth, resulting in overt metastases.

**Anti-angiogenesis in HCC**

Antiangiogenic therapy is one of the most promising novel strategies for treating cancers, and the results of the on-going clinical trials in patients with HCC will help clarify the role of antiangiogenic therapy in this highly vascular and aggressive malignancy. In 1971, Folkman advanced the view that tumor growth depends on angiogenesis and that targeting such angiogenesis could be a new way of preventing tumor progression. Three strategies block tumor growth in experimental models through regression of angiogenesis: vascular targeting, gene therapy and direct inhibition of proliferating and migrating endothelial cells. Alternatively, indirect anti-angiogenic drugs prevent the expression or block the activity of tumor proangiogenic factors by interfering with their endothelial cell receptors.
Table 3.2 Antiangiogenic agents applied for treatment of HCC

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Stage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab</td>
<td>Inhibition of VEGF</td>
<td>Phase II</td>
<td>Schwartz et al., 2004 and 2005</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Inhibition of endothelial cell proliferation</td>
<td>Phase II</td>
<td>Lin et al., 2005</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Inhibition of endothelial cell migration</td>
<td>Phase II</td>
<td>Patt et al., 2005</td>
</tr>
<tr>
<td>IL-12</td>
<td>Inhibition of angiogenesis</td>
<td>Murine model</td>
<td>Peron et al., 2004</td>
</tr>
<tr>
<td>PTK 787</td>
<td>Inhibition of tyrosine kinase</td>
<td>Murine model</td>
<td>Liu et al., 2005</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Inhibition of EGFR</td>
<td>Murine model</td>
<td>Matsuo et al., 2003</td>
</tr>
<tr>
<td>TNP-470</td>
<td>Inhibition of angiogenesis</td>
<td>Murine model</td>
<td>Kin et al., 2000</td>
</tr>
<tr>
<td>Batimastat (BB-94)</td>
<td>Inhibition of MMP</td>
<td>Murine model</td>
<td>Bu et al., 1998</td>
</tr>
<tr>
<td>Endostatin</td>
<td>Inhibition of tumor cell growth</td>
<td>Phase I</td>
<td>Eder Jr et al., 2002</td>
</tr>
</tbody>
</table>

Direct angiogenesis inhibitors target the microvascular endothelial cells recruited to the tumor and prevent them from responding to mitogens and motogens. Indirect angiogenesis inhibitors generally prevent the expression of or block the activity of a tumor protein that activates angiogenesis, such as FGF-2 and VEGF, or block the expression of its receptors on endothelial cells. Approximately, 75 anti-angiogenic compounds have been developed and are being tested clinically. An overview of ongoing studies on anti-angiogenic treatment is provided by the National Cancer Institute (http://www.cancer.gov) (Table 3.2).
Transcriptomic analysis of HCC

A transcriptome is a collection of all the gene transcripts present in a given cell. A transcriptome represents the very small percentage of the genetic code that is transcribed into RNA molecules, which is estimated to be much less than 5 percent of the genome in humans and other mammals. In addition, one of the lessons of the Human Genome Project is that each gene may produce many different types of mRNA molecules, so the transcriptome is much more complex than the genome that encodes. By collecting and comparing transcriptomes of different types of cells, researchers can gain a deeper understanding of what constitutes a specific cell type and how changes in cell activity may reflect or contribute to disease. Search of a transcriptome database can give researchers a list of all the tissues in which a gene is expressed, thus giving clues to its functions. For example, if the transcriptome database shows a gene's expression levels are dramatically higher in cancer cells than in healthy cells, it is possible that the unknown gene may play a role in promoting tumor growth. The National Human Genome Research Institute (NHGRI) is participating in two projects that will create transcriptome resources that will be made available to researchers around the world. Those projects are: the Mammalian Gene Collection and the Mouse Transcriptome Project.

The Mouse Transcriptome Project is an NIH initiative that is generating a free, public database of gene transcripts for many mouse tissues. Currently, transcriptome data are available on more than 90 tissue samples. These tissue-specific expression data, which are mapped to the mouse genome, are available in a searchable format in the mouse reference transcriptome database [ncbi.nlm.nih.gov] and at the mouse reference transcriptome [sgbpub.lynxgen.com]. The mouse was chosen for this effort because its genome has been sequenced, because its tissues can be obtained under rigorous quality control conditions, and because of its importance as a model for the study of human biology and disease. The transcriptome analysis of human cancer by using EST strategy, cDNA microarray, oligonucleotide microarray, as well as
serial analysis of gene expression (Adams et al., 1991), known as the cancer genome anatomy project, thus may provide important clues for understanding oncogenesis.

The differences in gene expression profiles between HCC and noncancerous liver were characterized in large scale by transcriptome level to explore the potential HCC molecular pathogenesis showed 6-phosphofructokinase-1 was increased, which is in agreement with increased glycolysis as seen in many cancers, a large number of genes participating in the metabolism of glucose, lipids, and amino acids and those responsible for the liver-synthesized proteins were down-regulated. In addition to the overexpression of, α-fetal protein and low expression level of the hepatocyte nuclear factor 4γ, a relatively hepatic-specific transcription factor also found among most HCC patients (Xiang-Ru et al., 2001). Over-expression of the ribosomal protein L36A (also referred to as RPL44) gene associated with cellular proliferation in HCC also identified using messenger RNA (mRNA) differential display method (Kim et al., 2004).

In short, an overview of the transcriptome status of hepatocellular carcinogenesis may lay a foundation for the further research on the mechanisms of this cancer of utmost clinical and biological significance. Moreover, recognition of pathognomonic alterations in gene expression might provide a basis for improved diagnosis and therefore allowing selection of the most appropriate therapeutic strategies.