REVIEW

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Hepatocellular carcinoma (HCC), one of the most common cancers worldwide, is often diagnosed at an advanced stage when most potentially curative therapies such as resection, transplantation or percutaneous and transarterial interventions are of limited efficacy. The fact that HCC is resistant to conventional chemotherapy, and is rarely amenable to radiotherapy, leaves this disease with no effective therapeutic options and a very poor prognosis. Therefore, the development of more effective therapeutic tools and strategies is much needed. HCCs are phenotypically and genetically heterogeneous tumors that commonly emerge on a background of chronic liver disease. However, in spite of this heterogeneity recent insights into the biology of HCC suggest that certain signaling pathways and molecular alterations are likely to play esscntial roles in HCC development by promoting cell growth and survival. The identification of such mechanisms may open new avenues for the prevention and treatment of HCC through the development of targeted therapies. In this review we will describe the new potential therapeutic targets and clinical developments that have emerged from progress in the knowledge of HCC biology, In addition, recent advances in gene therapy and combined cell and gene therapy, together with new radiotherapy techniques and immunotherapy in patients with HCC will be discussed.

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Introduction

Hepatocellular carcinoma is the fifth most common cancer worldwide and the most common form of liver cancer, being responsible for 80% of the primary malignant liver tumors in adults. The 5-year relative survival rate is about 7% and causes more than 600 000 deaths annually worldwide (Bosch *et al*., 2004). The disease is most prevalent in Eastern and Southeastern Asia, and Middle Africa, with more than half of the patients being reported from China.

Even in developed countries, potentially curative therapies are offered to only one in every four patients coming to highly committed centers (Sangro *et al*., 1993; Llovet *et al*., 1999). Tumor size, hepatic functional reserve or portal hypertension restricts indication of surgical or percutaneous ablation, and the success is laden with a high recurrence rate. Transplantation is not applicable universally and shortage of organ donation limits its indication to patients with very early tumors. For patients bearing non-ablatable tumors, transarterial embolization may be used as a palliative therapy but mostly for those asymptomatic patients with good liver function and a patent portal vein. Hepatocellular carcinoma (HCC) is a type of tumor highly resistant to available chemotherapeutic agents, administered either alone or in combination (Llovet *et al*., 2003). In addition, conventional antineoplastic drugs are typically non-selective cytotoxic molecules with significant systemic untoward effects. Thus, in many cases no effective therapy at all can be offered to patients with HCC. In this review, we will refer to new potential approaches to treat HCC. We will focus on (a) targeted therapies that have emerged from progress in the knowledge of HCC biology and (b) recent advances in gene therapy and combined cell and gene therapy. We will also make a brief reference to new radiotherapy techniques and to the role of immunotherapy in patients with HCC.

Biological therapy

Advances in the understanding of tumor biology are opening new paths to treat cancer (Figure 1). These novel therapeutics are slowly changing the prognosis of patients suffering from certain malignant tumors and this may also be the case for patients with invasive or metastatic HCC in the near future.

Potential targets for therapeutic intervention

Hepatocarcinogenesis is a multistep process, slowly unfolding on a background of chronic liver disease including chronic hepatitis and cirrhosis, which are regarded as preneoplastic stages (Coleman, 2003; Bréchot, 2004; Liang and Heller, 2004). Chronic

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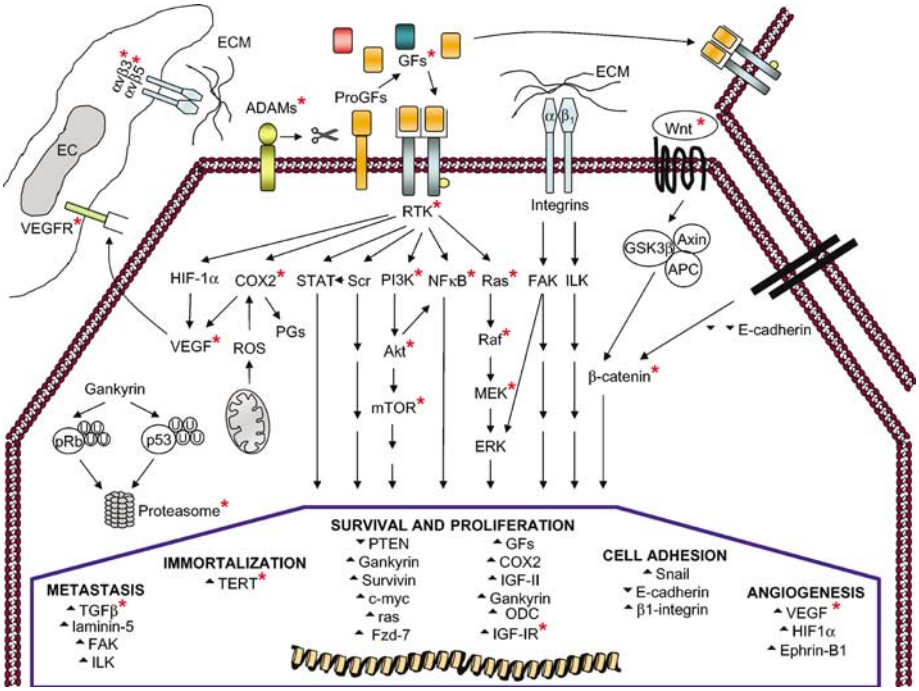

Figure 1 Relevant pathways in hepatocellular carcinoma (HCC) biology. Asterisks indicate potential targets (see main text for details). ADAM: a disintegrin and metalloproteinase; APC: adenomatous Polyposis Coli; COX2: cyclooxygenase 2; EC: endothelial cell; ECM: extracellular matrix; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; GF: growth factor; GSK3β: glycogen synthase kinase 3-beta; HIF-1α: hypoxia-inducible factor 1-alpha; IGF-II: insulin-like growth factor II; IGF-IR: insulin-like growth factor I receptor; ILK: integrin-linked kinase; MEK: mitogen-activated protein kinase kinase (extracellular signal-regulated kinase kinase); mTOR: mammalian target of rapamycin; NF-κB: nuclear factor-kappa B; ODC: ornithine decarboxylase; PGs: prostaglandins; PI3K: phosphatidylinositol 3-kinase; pRb: phospho-retinoblastoma (Rb); PTEN: phosphatase and tensin homolog; ROS: reactive oxygen species; RTK: receptor tyrosine kinase; STAT: signal transducer and activator of transcription; TERT: telomerase reverse transcriptase; TGF-β: transforming growth factor-beta; VEGF: vascular endothelial growth factor. ▲ and ▼ indicate enhanced or reduced gene expression in HCC as compared to normal liver.

hepatitis is a prolonged inflammatory process, with inflammatory cells infiltrating the liver parenchyma and triggering hepatocyte death. Persistent hepatocyte cell death is followed by a regenerative response in which viable hepatocytes are stimulated to proliferate in an attempt to restore the loss of hepatic parenchyma (Coleman, 2003; Taub, 2004). Proliferation of the stem cell compartment of the liver is also observed in experimental models of liver injury and carcinogenesis, and in various conditions of acute and chronic liver damage in humans (Libbrecht and Roskams, 2002; Sell, 2002). These stem cells, also called oval cells, possess an extensive capacity for self-renewal, and upon tissue injury expand in number and differentiate towards the biliary and hepatocytic lineages (Libbrecht and Roskams, 2002). Progression to liver cirrhosis is marked by disruption of liver architecture and, in some cases, aggregates of phenotipically altered hepatocytes emerge within regenerative nodules, being these cells considered as precursors of dysplastic nodules (Kojiro and Roskams, 2005). The presence of dysplastic hepatocytes in cirrhotic livers has been correlated with enhanced risk for HCC development (Coleman, 2003), and it has been suggested that many HCCs occurring in cirrhotic liver develop form dysplastic nodules in a multistep fashion (Kojiro and Roskams, 2005). Interestingly, the expression of markers of liver stem cells has been found in a considerable proportion of human HCCs, suggesting the possibility that human stem cells can give rise to HCC (Libbrecht and Roskams, 2002). It has been recently demonstrated that in solid tumors only a small proportion of cancer cells retain the ability to form new tumors (Al-Hajj and Clarke, 2004; Clarke, 2005; Dean *et al*., 2005). The existence of a tumor stem cell compartment in HCC, similar to that found in other solid tumors, could have strong implications for the treatment of liver cancer. The identification of the specific pathways involved in malignant stem cell survival and proliferation can provide new opportunities for targeted therapies.

Current evidences indicate that the precancerous liver and the early stages in HCC development are characterized by certain common traits governed by both epigenetic and genetic mechanisms (Thorgeirsson and Grisham, 2002; Coleman, 2003). These common features include the progressive hepatocyte dedifferentiation due to impaired liver-specific gene expression (Avila *et al*., 2000; Okabe *et al*., 2001; Berasain *et al*., 2003), and the alteration of numerous signaling pathways leading to autonomous and disregulated cell proliferation and resistance to cell death (Rust and Gores, 2000; Okabe *et al*., 2001; Feitelson *et al*., 2002; Thorgeirsson

and Grisham, 2002; Lee and Thorgeirsson, 2004; Arsura and Cavin, 2005; Kim *et al*., 2005; Roberts and Gores, 2005). Hierarchical clustering of human HCC samples based on gene expression patterns identified two subclasses of HCC with strong association with the survival of the patients, and noteworthy the expression of cell proliferation markers and antiapoptotic genes was significantly higher in the group of patients with poorer prognosis (Lee and Thorgeirsson, 2004, 2005).

Inactivation of the tumor-suppressor genes p53 and the retinoblastoma gene (Rb) through different molecular mechanisms has been observed in a significant percentage of HCCs of viral or alcoholic etiology, and in preneoplastic conditions such as chronic hepatitis and cirrhosis (Buendia, 2000; Feitelson *et al*., 2002; Coleman, 2003; Edamoto *et al*., 2003). A higher percentage of p53 genetic alterations are observed in advanced stages of HCC, suggesting that these abnormalities are associated with more advanced disease (Kim *et al*., 2005). The product of the *p53* gene is involved in many essential functions, including the regulation of DNA replication and repair, and the induction of apoptosis. Its activity is regulated by $p14^{ARF}$, a protein that indirectly stabilizes p53 by preventing mdm-2-dependent p53 ubiquitination and degradation (Zhang *et al*., 1998). Interestingly, $p14^{ARF}$ promoter hypermethylation and reduced $p14^{ARF}$ expression are also frequently detected in HCC, further contributing to the impairment of the p53 tumor-suppressor pathway (Feitelson *et al*., 2002; Edamoto *et al*., 2003). Likewise, deletions of the *Rb* gene, and *Rb* promoter hypermethylation leading to loss of expression are frequent in the cirrhotic liver and HCC (Feitelson *et al*., 2002; Coleman, 2003; Kim *et al*., 2005). The *Rb* tumor-suppressor normally inhibits cell cycle progression through its binding to the transcription factor E2F1. Phosphorylation of Rb by the cyclin-dependent kinase 4(CDK4)/cyclin D1 complex results in the release of E2F1, which in turn activates the expression of genes involved in the G_1–S transition. In addition to direct *Rb* gene alterations, impairment in the expression of the CDK4 inhibitor $p16^{INK4}$ can also lead to Rb disregulation in hepatocarcinogenesis (Feitelson *et al*., 2002; Edamoto *et al*., 2003). It is important to mention that alterations in the endogenous tumor-suppressor networks is a main contributor to the resistance of HCC to classical cancer therapies, in part because the activity of most conventional chemotherapeutic agents depends to a great extent on the same innate pro-apoptotic pathways that are disabled in HCC (Chan and Lung, 2004; Lowe *et al*., 2004; Müller *et al*., 2005).

Interestingly, gankyrin, a gene that is consistently overexpressed in human HCC, has been identified as a novel negative regulator of both p53 and Rb levels (Lozano and Zambetti, 2005). Gankyrin facilitates the phosphorylation and degradation of Rb through different pathways (Higashitsuji *et al*., 2000) and enhances the ability of the ubiquitin ligase mdm-2 to recruit p53 to the proteasome for its degradation (Higashitsuji *et al*., 2005). Downregulation of gankyrin by RNA interference increased p53 protein levels and activity, and promoted apoptosis (Higashitsuji *et al*., 2005). These actions make gankyrin a potential contributor to HCC development, and suggest that blocking its activity may prove a valuable targeted therapy for HCC.

Gankyrin was originally identified as a component of the regulatory subunit of the proteasome (Lozano and Zambetti, 2005). The ubiquitin-proteasome pathway is an essential regulator of cellular processes such as the cell cycle, signal transduction pathways and apoptosis. Given that both, tumor-suppressor proteins and oncoproteins, are targets of ubiquitination, initially it was not conceptually obvious that proteasome inhibition could result in the selective killing of cancer cells (Mani and Gelmann, 2005). However, accumulating evidences show that proteasome inhibitors can induce apoptosis of cancer cells and reduce tumor growth in mouse xenograft models (Adams, 2004). Furthermore, proteasome inhibitors such as bortezomib also sensitize tumor cells to chemotherapeutic agents, or to the activation of death signaling pathways as recently observed in human HCC cells (Ganten *et al*., 2005). Indeed, pretreatment with bortezomib sensitized HCC cells to apoptosis induced by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), while primary human hepatocytes were spared from this effect (Ganten *et al*., 2005).

Overexpression of cell cycle regulatory genes such as *cyclins D1*, *E* and *A*, and enhanced activity of CDK4 have been widely documented in human HCC and in the cirrhotic liver (Deane *et al*., 2001; Coleman, 2003; Masaki *et al*., 2003; Huynh *et al*., 2004; Osada *et al*., 2005). Similarly, transcription factors that exert positive effects on cell proliferation, such as *c-myc*, are overexpressed in human HCC (Coleman, 2003). Another example is the *Foxm 1b* gene, a transcription factor markedly induced at the G_1/S transition during liver regeneration that is also upregulated in human HCC (Okabe *et al*., 2001; Kalinichenko *et al*., 2004). A remarkable example of the potential of manipulating a single oncogenic pathway for HCC development has been recently provided (Shachaf *et al*., 2004). Using transgenic mice that conditionally expressed the *c-myc* oncogene in the liver in an inducible manner, these authors found that switching on *c-myc* expression resulted in the development of HCC, however subsequent *c-myc* inactivation induced a sustained regression of invasive liver cancer, with tumor cells rapidly differentiating into apparently normal hepatocytes and biliary cells. Together, these observations lend further support to the hypothesis that continuous stimulation through mitogenic and survival signaling pathways is central for the development of HCC from the early stages, and that targeting one of these key pathways can reverse the neoplastic phenotype. Furthermore, most of these signaling and regulatory mechanisms are modified by infection with hepatitis B or C viruses, which are regarded as major etiologic agents of HCC (Feitelson, 2005). Among the most critical cellular signaling pathways for hepatocarcinogenesis are the Wnt/β-catenin pathway and receptor tyrosine kinase (TK)-activated

pathways, including ras/raf/MEK/ERK, PI3K/Akt/mammalian target of rapamycin (mTOR), nuclear factor-kappa B (NF-κB) and JAK/STAT (Masaki *et al*., 1998; Harada *et al*., 1999; Giles *et al*., 2003; Hu *et al*., 2003; Hwang *et al*., 2004; Pikarski *et al*., 2004; Sahin *et al*., 2004; Arsura and Cavin, 2005) (Figure 1).

Abnormal regulation of β-catenin is an early event in hepatocarcinogenesis. β-Catenin is a key intracellular effector in the Wnt signaling pathway, and also participates in homotypic cell-cell interactions through its association with E-cadherin (Giles *et al*., 2003; Harris and Peifer, 2005). Wnts are a large family of secreted glycoproteins that signal through binding to members of the frizzled (Fzd) family of seven-span transmembrane receptors (Giles *et al*., 2003). In quiescent epithelial cells β-catenin is found in a submembranous location complexed to glycogen synthase kinase 3β (GSK3β), axin and the tumor-suppressor adenomatous polyposis coli (APC). GSK3β is active in unstimulated cells and mediates the phosphorylation of β-catenin in key serine and threonine residues that mark β-catenin for ubiquitination and proteasomal degradation (Aberle *et al*., 1997; Nelson and Nusse, 2004). In the canonical Wnt pathway, Wnt ligands bind the Fz receptors and activate the downstream effector dishevelled (Dvl), which in turn prevents the phospohorylation of β-catenin by GSK3β and its subsequent degradation. Accumulation of β-catenin in the cytoplasm leads to its translocation to the cell nucleus, where it binds the transcription factors Tcf/Lef and acts as a coactivator to stimulate the transcription of many genes involved in cell proliferation, such as *c-myc*, *c-jun* and *cyclin D1*, angiogenesis, anti-apoptosis and in the formation of extracellular matrix (ECM) (Buendia, 2000; Calvisi *et al*., 2001). About 50–70% of all HCC examined presented accumulated levels of β-catenin in the cytoplasm and nucleus (Wong *et al*., 2001). Mutations in β-catenin can lead to its stabilization and intracellular accumulation, mimicking Wnt stimulation. These mutations occur in almost all stages of HCC development, suggesting that impairment of β-catenin turnover can contribute to many different aspects of the process of liver malignization, including the development of fibrosis and cirrhosis that precede tumor formation (Feitelson *et al*., 2002; Edamoto *et al*., 2003).

Another recently recognized mechanism leading to enhanced β-catenin accumulation in the absence of β-catenin mutations involves the overexpression of the Fzd type 7 receptor, which is already observed in dysplastic hepatocytes prior to HCC development (Merle *et al*., 2004). These receptors may thus constitute another target for HCC prevention. Alternatively, β-catenin accumulation in HCC cells has been correlated with impaired E-cadherin gene expression, due to LOH at the E-cadherin locus or epigenetic mechanisms such as promoter hypermethylation or binding of transcriptional repressors (Matsumura *et al*., 2001). Loss or reduced expression of E-cadherin is likely to result in redistribution of β-catenin to the cell nucleus, and is also thought to have important consequences for hepatocellular dedifferentiation, angiogenesis, tumor invasiveness and metastasis (Calvisi *et al*., 2004).

Interestingly, besides the aforementioned implications of the Wnt/β-catenin pathway in well-established tumorigenic events, Wnts are also involved in the regenerative response of the liver and in the maintenance of the stem cell pool (Monga *et al*., 2001; Dean *et al*., 2005; Reya and Clevers, 2005). These notions suggest that targeting the Wnt/β-catenin pathway may also be a strategy to quell the tumor stem cell compartment.

Although still in their early development, different strategies are being tested to interfere with this pathway. For example, blockade of Wnts with monoclonal antibodies (mAbs) (You *et al*., 2004; He *et al*., 2005), and small molecules that interfere with the interaction of β-catenin with Tcf or other transcriptional regulators such as the CREB-binding protein CBP (Lepourcelet *et al*., 2004; Emami *et al*., 2004). Additionally, due to the extensive crosstalk between signaling pathways activated in chronic liver injury and HCC, the activity of the Wnt/β-catenin axis can be interfered through the inhibition of other targets such as the enzyme cyclooxygenase (COX) and, as discussed below, the ras-/raf-/MEK-/ERK- and PI3K-signaling cascades (Ding *et al*., 2005; Beurel *et al*., 2005; Street *et al*., 2005). Wnt/β-catenin cascade is repressed by inhibitors of the inducible isoenzyme COX2 (Giles *et al*., 2003; Dihlmann and von Knebel Doeberitz, 2005), a type of drugs that hold promise for chemoprevention of HCC (Hu, 2002).

Besides the Wnt/β-catenin axis, the ras/raf/MEK/ERK pathway is one of the most critical signaling cascades for liver tumorigenesis (Chung *et al*., 2000; Coleman, 2003; Hwang *et al*., 2004; Wiessenauer *et al*., 2004; Osada *et al*., 2005; Schiffer *et al*., 2005). This pathway is central in cell growth and survival, transducing extracellular signals from ligand-bound TK receptors such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor receptor (IGFR), platelet-derived growth factor receptor (PDGFR), the stem cell growth factor receptor c-kit, and the hepatocyte growth factor receptor (MET), to the cell nucleus through a series of specific phosphorylation events that start with the activation of Ras (Alexia *et al*., 2004; Sridhar *et al*., 2005). Activated Ras turns on a family of serine threonine kinases known as Raf kinases that phosphorylate and activate MEK1/2, which in turn activates ERK1/2. Subsequently, ERK1/2 kinases phosphorylate a wide range of downstream effectors involved in many aspects of tumorigenesis, ranging from cell immortalization by telomerase induction to apoptosis resistance, ECM remodeling, cellular motility, angiogenesis and drug resistance (Wiessenauer *et al*., 2004; Sridhar *et al*., 2005).

As just mentioned, activation of the ras/raf/MEK/ERK can be achieved by ligand binding to different membrane receptors with TK activity, including the EGFR. The adult hepatocyte is the cell type in the organism where the EGFR is expressed at the highest levels, and it is thought to play a central role in liver regeneration after partial hepatectomy and tissue injury

(Taub, 2004). Although this receptor is not commonly overexpressed in HCC cells, its continuous activation by ligands such as transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF) or amphiregulin (AR), which are upregulated from the early stages of hepatocarcinogenesis, can be of significance for HCC development (Inui *et al*., 1994; Chung *et al*., 2000; Berasain *et al*., 2005a, b; Schiffer *et al*., 2005). These EGFR ligands are produced as transmembrane precursors, and need to be proteolitically released from the cell surface to signal in an autocrine or paracrine fashion (Yarden, 2001). Shedding of EGFR ligands is carried out by metalloproteinases of the ADAM family, which are also transmembrane proteins (Blobel, 2005). Interestingly, the expression of tumor necrosis factor-α-converting enzyme (TACE)/ADAM17, the metalloproteinase that cleaves the AR precursor, is upregulated in human HCC together with that of its target gene *AR* (Ding *et al*., 2004; Berasain *et al*., manuscript in preparation). Moreover, we have recently found that TACE/ADAM17 gene expression is already elevated in the cirrhotic liver (Berasain *et al*., manuscript in preparation), which further supports the early involvement of the EFGR system in the process of hepatocarcinogenesis. Among the different approaches undertaken to inhibit EGFR function are neutralizing mAbs to the EGFR, and small molecule inhibitors of the EGFR TK activity (Mosesson and Yarden, 2004; Baselga and Arteaga, 2005).

Anti-EGFR antibodies such as cetuximab, panitumumab or ABX-EGF bind to the extracellular ligand-binding domain of EGFR and completely prevent ligand binding this receptor. Small molecule EGFR inhibitors are usually quinazoline derivatives that compete with ATP binding to the TK domain of the receptor. These molecules belong to two categories: reversible inhibitors such as gefitinib and erlotinib, and irreversible inhibitors that covalently bind to cysteine residues in the ATP-binding site of EGFR, such as lapatinib, CI-1033 and EKB-569 (Baselga and Arteaga, 2005; Camp *et al*., 2005). The inhibitory activity of gefitinib and erlotinib on human HCC cell growth in culture has been recently demonstrated. These compounds induced cell cycle arrest and apoptosis, and erlotinib enhanced chemosensitivity towards cytostatics (Höpfner *et al*., 2004; Huether *et al*., 2005). In addition, *in vivo* studies have shown that gefitinib displays antitumoral effects in a rat model of chemically induced liver cirrhosis and HCC (Schiffer *et al*., 2005).

In spite of the promising results, previous experiences with anti-EGFR-targeted therapies in other types of tumors have shown that many patients show a very poor response or progressively develop resistance to the therapy (Camp *et al*., 2005). Various mechanisms account for such resistance, and some of them are present in HCC cells. For instance, alternative TK receptors can activate the same critical downstream promitogenic and antiapoptotic mechanisms triggered by EGFR, and thus compensate for EGFR blockade. This could be the case of the IGFR, its expression and that of its ligand IGFII is significantly upregulated in human cirrhotic liver and HCC (Alexia *et al*., 2004). Activation of the IGFR has been shown to stimulate the Ras/Raf/MEK/ERK, PI3K/Akt/mTOR and β-catenin pathways in HCC cells, and is considered to participate in HCC development through an autocrine mechanism (Desbois-Mouthon *et al*., 2001; Alexia *et al*., 2004). Therefore, targeting the IGFII/IGFR system can be also regarded as an antineoplastic strategy for liver cancer by itself. Inhibition of IGFR significantly reduces resistance to anti-EGFR-targeted therapies, as has been observed in human breast carcinoma cells simultaneously treated with the IGFR inhibitor AG1024 and gefitinib (Camirand *et al*., 2005), suggesting that similar combinations of targeted therapies may be effective in HCC.

Previous observations in non-small-cell lung cancer (NSCLC) patients identified somatic mutations in exons 18–21 of the EGFR that strongly correlated with robust clinical responses to gefitinib treatment (Baselga and Arteaga, 2005). However, in a recent report that examined 89 human HCC samples no such mutations were found, suggesting that human liver tumors are unlikely to show a good response to gefitinib (Su *et al*., 2005). Additionally, enhanced expression of EGFR ligands can also induce tumor cell resistance to EGFR inhibitors. This situation has been also observed in NSCLC cells, in which the overexpression of AR significantly conditions the sensitivity to gefitinib (Kakiuchi *et al*., 2004). Furthermore, increases in the circulating levels of AR and TGF-α have been proposed as predictors of poor response to gefitinib in patients with NSCLC (Ishikawa *et al*., 2005). These observations suggest that targeting EGFR ligands could be of value in overcoming resistance towards EGFR inhibitory molecules.

The ligand-independent constitutive activation of signaling pathways downstream of receptor TKs, such as the Ras/Raf/MEK/ERK pathway, can play an important role in tumor development, and is considered as another mechanism of resistance to receptor-targeted therapies (Camp *et al*., 2005). A variety of agents have been developed to interfere with these pathways at different levels. Overexpression of Ras proteins is frequently observed in HCC and preneoplastic liver (Coleman, 2003). In order to be competent for signal transduction, these guanine nucleotide-binding proteins need to be post-translationally modified by the incorporation of prenyl moieties (farnesyl and geranylgeranyl groups). Based on this, different inhibitors of the enzyme farnesyl transferase have been developed, however, clinical experience in solid tumors has shown limited efficay for these agents when used as single drugs (Graaf *et al*., 2004). Nevertheless, recent experimental evidences with ABT-100, a novel highly potent farnesyltransferase inhibitor, showed clear chemopreventive effects in chemically induced HCC in rats (Carloni *et al*., 2005). Interestinlgy, PI3K/Akt inhibition was also observed in response to ABT-100 treatment (Carloni *et al*., 2005). An alternative approach to prevent prenylation of Ras proteins is to interfere with the activity of 3-hydroxy-3-methylglutaryl coenzyme-A

reductase (HMG-CoAR), the rate-limiting enzyme in the mevalonate pathway that leads to the production of isoprenoids. Targeting of HMG-CoAR can be achieved with statins, a group of drugs widely used to treat patients with hypercholesterolemia. It has been recently reported that treatment of human HCC cells with statins blocks ERK1/2 activation and induces cell cycle arrest and apoptosis (Sutter *et al*., 2005). Dowstream of Ras, one of the most promising agents is the orally available Raf kinase inhibitor BAY 43-9006 (sorafenib), which has shown encouraging effects in several xenograft models of tumors dependent on signaling through the Ras/Raf/MEK/ERK pathway (Sridhar *et al*., 2005; Thompson and Lyons, 2005). Although originally developed as a Raf kinase inhibitor, it was subsequently appreciated that BAY 43-9006 was able to inhibit other kinases relevant to the carcinogenic and angiogenic processes such as VEGFR-2, VEGFR-3, PDGFR-β, c-kit and p38α (Sridhar *et al*., 2005). MEK is the kinase dowstream of Raf, and is also an atractive target for HCC (Wiessenauer *et al*., 2004; Osada *et al*., 2005; LoRusso *et al*., 2005). Preclinical studies have proved the potential of MEK inhibition in suppressing hepatoma cell proliferation and tumorigenicity (Wiessenauer *et al*., 2004).

Activation of the PI3K/Akt/mTOR pathway is believed to play a key role in tumor development (Osaki *et al*., 2004). Besides stimulating cellular proliferation, these kinases promote cell survival through the coordinate regulation of apoptosis and cellular metabolism (Amaravadi and Thompson, 2005). PI3K is directly associated with many cell surface growth factor receptors, which upon ligand binding trigger the activation of this lipid kinase and generate the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3). Phosphatidylinositol 3,4,5-triphosphate in turn recruits and contributes to activate Akt, a serine/threonine kinase that regulates the apoptotic machinery at multiple levels, including the phosphorylation and inactivation of pro-apoptotic proteins such as Bad and caspase-9, the induction of NF-κB-dependent survival genes like *Bcl-XL* and the key metabolic regulator mTOR (Vignot *et al*., 2005). An essential regulator of this route is the PIP_3 phosphatase PTEN, a ubiquitously expressed tumor-suppressor gene frequently lost in human HCC, and that upon targeted deletion in mouse hepatocytes leads to the development of HCC (Hu *et al*., 2003; Horie *et al*., 2004). Thus, constitutive activation of this pathway in HCC can be due to enhanced stimulation of receptor TKs, such as EGFR and IGFR, but also to decreased PTEN expression. The latter condition implies that liver tumors with impaired PTEN expression can be resistant to receptor TK-targeted therapies. This has been already observed for breast cancer cells with mutated PTEN, in which sensitivity to gefitinib could be restored after adenoviral delivery of wild-type PTEN (Bianco *et al*., 2003). Together these observations make the inhibition of Akt activation an attractive strategy for targeted HCC therapy. Upstream of Akt, PI3K inhibitors such as wortmannin and LY294002 have shown some efficacy in experimental models of HCC (Nakanishi *et al*., 2002), but clinical development has been precluded due to broad specificity kinase inhibition, weak inhibitory activity and poor pharmacokinetics (Amaravadi and Thompson, 2005). The direct inhibition of Akt has been also considered for the treatment of solid tumors. The orally bioavailable alkylphospholipid perifosine, an inhibitor of Akt targeting to the cell membrane, is currently in phase II trials after showing partial responses in earlier clinical studies (Amaravadi and Thompson, 2005).

Downstream of Akt is mTOR, a serine/threonine kinase that is probably one of the most promising candidate targets within this pathway. Through a complex mechanism PI3K/Akt mediates the activation of mTOR, which in turn exerts profound effects on gene expression by the regulation of the cell translational machinery. The two main effectors of mTOR are the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), and the 40S ribosomal protein S6 kinase (p70s6k). These proteins regulate the translation of many mRNAs corresponding to genes involved in cell proliferation and angiogenesis such as *c-myc*, *cyclin D1*, *ornithine decarboxylase*, *hypoxia-inducible factor 1-*α, and indirectly the expression of VEGF (Adjei and Hidalgo, 2005). Rapamycin, a natural antibiotic, is a potent and specific inhibitor of mTOR that prevents p70s6k and 4E-BP1 phosphorylation. In the clinical setting rapamycin is well known as an immunosuppressive drug used to prevent renal graft rejection (Vignot *et al*., 2005). Convincing antineoplastic effects for rapamycin have been observed in experimental models, and have prompted the development of rapamycin analogues with more favourable pharmaceutical properties as anticancer agents (Vignot *et al*., 2005; Adjei and Hidalgo, 2005). Phase I and II studies for the rapamycin analogues CCI-779, RAD001 and AP23573 have shown some activity in solid tumors, and their use in combination with chemotherapy is being explored (Adjei and Hidalgo, 2005). In this respect, it has been recently reported that the phosphorylation of mTOR and the expression of p70s6k is upregulated in a significant number of human HCCs (45% of cases), and that rapamycin markedly inhibits the proliferation of human HCC cell lines (Sahin *et al*., 2004). Although mTOR inhibitors have not been tested yet in animal models of HCC, these observations together with the frequent loss of PTEN in human HCC, suggest a potential use for these agents in liver cancer.

As previously mentioned, the expression of the proto-oncogene *c-myc* can be activated by practically all the signaling pathways activated during HCC development, and is frequently observed in liver tumor samples. This proto-oncogene in turn stimulates the expression of many growth-related genes. Among them, telomerase is believed to play an important role in HCC progression. Its activity is upregulated in over 80% of human HCC biopsies while normal liver tissues show undetectable or very low telomerase activity (Satyanarayana *et al*., 2004). Telomerase consists of two components, an RNA (TERC) that serves as a template for the synthesis of the telomere sequence, and a reverse transcriptase (TERT). The main function of telomerase is the *de novo*

synthesis of telomeres, structures that cap the chromosome ends protecting them from fusion and triggering DNA-damage recognition (Satyanarayana *et al*., 2004). Telomere shortening limits the growth of primary human cells, and it has been proposed that repression of telomerase is a tumor-suppressor mechanism. Therefore, targeting telomerase activity may be exploited as a novel therapy for HCC. Several classes of telomerase inhibitors with different mechanisms of action have been designed and evaluated (Dikmen *et al*., 2005). In a very recent report, two thio-phosphoramidate oligonucleotides that inhibit telomerase through targeting the TERC component, GRN163 and its lipid-conjugated derivative GRN163L, have been tested on human hepatoma cells. These compounds inhibited HCC cell growth *in vitro* and in the xenograft model, and did not show any adverse effects (Djojosubroto *et al*., 2005). GRN163L has been recently approved to enter phase I/II clinical trials in chronic lymphocytic leukemia (Djojosubroto *et al*., 2005).

Increasing evidences point to the constitutive activation of the transcription factor NF-κB as one early key event in hepatic oncogenesis (Arsura and Cavin, 2005). In non-stimulated hepatocytes NF-κB is retained in the cytoplasm by a group of inhibitory factors known as inhibitor kappa B (IκB) proteins. In response to a variety of stimuli, including viral infection, pro-inflammatory cytokines and growth factors, IκB proteins are phosphorylated by the IκB kinase (IKK) complex and targeted for proteasomal degradation, which allows the NF-κB constituents (there are five NF-κB family members) to translocate to the cell nucleus and activate gene transcription (Arsura and Cavin, 2005; Luo *et al*., 2005). Nuclear factor-kappa B induces the expression of many genes that inhibit both apoptotic and necrotic cell death, representing a general defense mechanism towards hepatocellular damage of diverse etiology. However, its persistent activation during preneoplastic stages can confer a survival advantage to hepatocytes that have acquired oncogenic mutations, thus favoring tumor promotion (Pikarski *et al*., 2004). This may be one of the mechanisms linking chronic inflammation and tumorigenesis. According to these concepts NF-κB inhibitors would be useful in HCC prevention and treatment. Specific IKK inhibitors such as β-carbolin and quinazoline analogues are currently under preclinical development (Arsura and Cavin, 2005).

Angiogenesis is a crucial process for tumor progression, and is also intimately related to tumor invasion and metastasis. The formation of new blood vessels is a multistep process that involves not only the tumor cells, but also the tumor matrix, stroma and vasculature. A variety of factors stimulate the proliferation, survival, migration and assembly of vascular endothelial cells into new blood vessels that feed the tumor (Zacharoulis *et al*., 2005). Among these factors, the previously mentioned VEGF plays a predominant role, although basic fibroblast growth factor, PDGF, IGF, angiopoietin-1 and interleukin (IL)-8 are also involved in the neovascularization of tumors, including HCC (Eskens, 2004; Zacharoulis *et al*., 2005). Overexpression of VEGF has been detected in HCC, and its upregulation can be attributed to different effectors such as the hypoxic tumor environment, or the activation of EGFR and COX2 signaling (Ryan and Wedge, 2005).

In principle, targeted inhibition of angiogenesis can be achieved at different levels. These include the neutralization of growth factors with mAbs, the inhibition of the dowstream signaling from TK receptors, and the interference with the interaction between proliferating endothelial cells and matrix components (Eskens, 2004; Ryan and Wedge, 2005; Zacharoulis *et al*., 2005). Bevacizumab, a humanized recombinant mAb to VEGF, has jumped first into the clinical setting but many other small molecule VEGFR inhibitors are currently being tested in combination with conventional antineoplastics for the treatment of metastatic colorectal cancer. Among them is PTK787, an orally available VEGFR-1 and VEGFR-2 TK inhibitor that has shown promising effects on HCC xenografts in nude mice (Liu *et al*., 2005). Other compounds such as ZD6474 and CP-547,632 are able to inhibit both VEGFR and EGFR TK activity. These molecules can target tumor growth via inhibition of VEGF-mediated angiogenesis and endothelial cell survival, and through the direct inhibition of tumor cell proliferation and survival. Hepatocellular carcinoma treatment could benefit from the use of these agents, given the prominent role of the EGFR system in this type of tumor.

Another approach proposed to limit tumor-associated angiogenesis targets the interaction of the endothelial cells with the ECM. This interaction is important in mediating cell spreading and migration, and cell matrix receptors such as the transmembrane endothelial cell integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are thought to play a major role (Stupack and Cheresh, 2004). The efficacy of medi-522, a humanized mAb targeting the integrin $\alpha v\beta 3$ receptor, and that of cilengitide, a small molecule inhibitor of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, is currently being tested in clinical trials (Eskens, 2004).

Thalidomide is one of the few agents with antiangiogenic potential that has been clinically tested against HCC (Zacharoulis *et al*., 2005). Its mechanism of action is not completely known, and may involve its ability to interfere with $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors and to modulate the inflammatory response, which also contributes to neo-angiogenesis. The efficacy of thalidomide in human studies was limited to a few isolated responses, mostly manifested as disease stabilization. A second-generation thalidomide analog, lenalidomide, with much more potent immunomodulatory and antiangiogenic properties is currently being evaluated in patients with solid tumors (Zacharoulis *et al*., 2005).

Given the essential role of angiogenesis for HCC development, the identification of novel molecules and pathways to interfere with is a major issue. One of these novel targets may be represented by the ephrin/eph system. Ephrin ligands are membrane-anchored proteins that bind and activate a large family of receptor with TK activity, known as Eph receptors. The ephrin/eph signaling system was known to be an important

regulator of angiogenesis during development, however more recently has been shown to participate also in tumor neovascularization (Ogawa *et al*., 2000). Recent experimental observations clearly demonstrated that ephrin-B1, an ephrin family member specifically upregulated in human HCCs, was able to stimulate the proliferation of endothelial cells *in vitro* and to promote tumor growth and in a xenograft model (Sawai *et al*., 2003). This effect was mainly attributed to its ability to initiate tumor angiogenesis, suggesting that the inhibition of ephrin-B1 function could be a novel approach to quell HCC neovascularization.

An important event during later stages in the development of epithelial tumors, including HCC, involves the loss of cell-to-cell contacts and the acquisition of a fibroblastoid phenotype, referred to as epithelial–mesenchymal transition (EMT) (Gotzmann *et al*., 2001; Thiery, 2003; Thompson and Newgreen, 2005). Regarding tumor development, the relevance of these changes resides on the fact that EMT appears to participate in the metastatic process by enhancing the migratory and invasive properties of transformed cells. Moreover, EMT of liver cells can also result in enhanced resistance to apoptotic signals, further contributing to the neoplastic phenotype (Valdés *et al*., 2002). There are a number of molecular mechanisms and crosstalks among signaling pathways in tumor-associated EMT. As mentioned above, loss of cell–cell adhesion and cytoeskeletal rearrangements are hallmarks of EMT. The downregulation of the tumor-suppressor protein E-cadherin, a prime determinant of epithelial cell structure, is central to the EMT process. As previously indicated impaired E-cadherin expression is frequently observed in HCC tissues (Calvisi *et al*., 2004).

The molecular mechanisms of E-cadherin downregulation are not completely known, a recent report showed that in HBV-associated HCC *E-cadherin* expression is silenced through the hypermethylation of its promoter (Lee *et al*., 2005). Additionally, the inactivation of GSK3B, frequently observed in HCC, seems to be involved in the downregulation of E-cadherin expression and in the activation of transcription factors relevant to EMT such as Snail (Bachelder *et al*., 2005). However, in E-cadherin silencing, and in hepatocarcinogenesis-associated EMT as a whole, TGF-β plays a predominant role (Gotzmann *et al*., 2001). This cytokine is not detected in healthy adult hepatocytes, however, its expression is significantly induced in HCC (Bedossa *et al*., 1995). While in normal adult hepatocytes TGF-β displays growth-inhibitory and pro-apoptotic properties, in liver tumor cells these reponses are lost. The binding of TGF-β to its cell surface receptors triggers intracellular signaling mediated by the phosphorylated Smad proteins, among which Smad3 seems to play a central role in the regulation of EMT-related changes in gene expression (Zavadil and Böttinger, 2005). Additionally, extensive crosstalk exists between the TGF-β/Smad pathway and other signaling cascades in the promotion of EMT. Of special relevance is the cooperation with the Ras/Raf/MEK/ERK and PI3K/Akt pathways previously described, signaling cascades experimentally demonstrated to induce and maintain EMT in liver cells (Gotzmann *et al*., 2001; Lan *et al*., 2004; Fischer *et al*., 2005; Zavadil and Böttinger, 2005). Recent studies have identified the NF-κB signaling system as another key modulator in TGF-β-induced EMT in mammary epithelial cells, suggesting its likely implication in HCC-associated EMT (Zavadil and Böttinger, 2005). Taken together these observations suggest that the pharmacologic inhibition of TGF-β signaling could be relevant to prevent epithelial cell dedifferentiation and tumor metastasis. The identification of such inhibitors is vigorously pursued, and some promising experimental observations have been obtained with BIBU 3029, a small molecule inhibitor of TGF-β TK activity that prevents EMT *in vitro* (Eger *et al*., 2004). It would be interesting to test such compounds in combination with inhibitors of the signaling pathways that cooperate with TGF-β in the EMT of transformed liver cells.

A key aspect of HCC biology is the interaction of the transformed cell with the ECM. As described before, HCC frequently develops on a fibrotic environment, a situation in which profound quantitative and qualitative alterations of the ECM have occurred (Bissell, 2001). Accumulating evidences indicate that these changes in ECM could play a role in liver tumorigenesis. For instance, it has been proposed that the extent of cirrhosis and fibrosis in HCC patients is a negative predictor of survival (Lee *et al*., 1997), and that the *de novo* expression of ECM constituents such as laminin-5, is associated with a worse prognosis of the disease and with resistance to targeted drugs like gefitinib (Giannelli *et al*., 2003, 2004). Communication between the hepatocyte and the ECM is mainly mediated by a family of trasmembrane receptor proteins collectively known as integrins (Hynes, 1992). These receptors consist of an α- and a β-subunit, and exist in at least 24 different combinations, with different binding specificities and signaling mechanisms (Schuppan and Ocker, 2003). The interaction of integrins with ECM components (i.e., collagen, laminin and fibronectin, among others) regulates essential responses like cell adhesion, migration, differentiation and survival (Hynes, 1992). The intracellular domains of integrins lack an enzymatic activity, and in order to signal they need to associate with intracellular kinases such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (Hannigan *et al*., 2005; Von Sengbusch *et al*., 2005). As occurs with the composition of liver ECM, the normal expression pattern of integrins is also altered early during hepatocarcinogenesis. The resulting abnormal combination of ECM ligands and integrin receptors has been proposed to play a role in liver tumor development and metastasis (Carloni *et al*., 2001; Nejjari *et al*., 2002; Yang *et al*., 2003; Zhang *et al*., 2003). In particular, overexpression of integrin β1 in human HCC cells has been demonstrated to enhance cell proliferation, survial, migratory capacity and resistance to chemotherapy (Carloni *et al*., 2001; Zhang *et al*., 2002, 2003, 2004). Similarly, induction of FAK gene expression has been observed in human HCC, and elevated FAK levels seem

to correlate with a more aggressive tumor behavior (Itoh *et al*., 2004; Fujii *et al*., 2004). Interestingly, the profibrogenic growth factor TGF-β, which as mentioned before participates in the EMT and induces migratory capacity in liver tumor cells, can stimulate the expression of ILK and certain integrins, and also can promote FAK tyrosine phosphorylation in human HCC cells (Nejjari *et al*., 2002; Xu *et al*., 2003). Taken together these observations suggest that inhibition of the progression of liver fibrosis and of ECM/tumor cell interactions, could represent alternative therapeutic strategies to prevent HCC development and metastasis. Various approaches acting on different targets can be envisioned. For example, interfering with TGF-β activity could have pleiotropic beneficial effects, and different strategies using synthetic peptides, soluble decoy receptors or adenovirus-mediated local expression of dominant-negative TGF-β receptors, have been attempted (Ezquerro *et al*., 2003; Pinzani *et al*., 2005). Inhibition of downstream signaling from the integrin receptors could be another possibility. The expression of the integrin-associated kinase ILK is increased in different types of tumors, and this kinase seems to participate in the malignant progression (Hannigan *et al*., 2005). These notions have prompted an active search for small molecule ILK inhibitors, and several compounds such as KP-SD-1, KP-392 and QLT0254, have proved antitumor activity in xenograft models (Hannigan *et al*., 2005; Yau *et al*., 2005). Although no experiences have been reported in HCC cells or models, liver tumors are likely to respond to ILK inihibitors, given that increased ILK activity can be due to loss of expression or inactivation of PTEN (Hannigan *et al*., 2005), which are common events in HCC.

Clinical development of targeted therapies

Unlike conventional chemotherapy, 'targeted agents' aim to block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and invasiveness. Consequently, they point towards increased efficacy and reduced toxicity. As it has been discussed, targets can be aimed at with mAbs that specifically bind to growth factors or growth-factor receptors, or can be hit with small molecules acting as signal-transduction inhibitors (Table 1). Among the latter, earlier agents such as gefitinib and erlotinib aimed at single targets (in this case EGFR), but more recently other kinase inhibitors, including sorafenib (BAY 43-9006) or SU11248, have multitarget properties. Although HCC seems to stand on the rear in the clinical research of targeted therapies, a number of targeted therapies have already moved into clinical trials on the HCC population (Table 2).

Table 1 Principal targeted agents under clinical research for the treatment of solid tumors

Name	*Target*	*Current status*
Small-molecule inhibitors		
Gefitinib (Iressa)	EGFR	Approved for NSCLC
Erlotinib (Tarceva)	EGFR	Approved for NSCLC
Imatinib (Gleevec)	bcr-abl, PDGF & c-kit	Approved for CML and GIST
Sorafenib, BAY 43-9006	Raf kinase, VEGFR, PDGFR	Approved for RCC
CI-1033	pan-erbB	Clinical development
EKB-569	EGFR	Clinical development
PKI-166	erbB1& erbB2	Clinical development
Lapatinib, GW572016	EGFR & erbB2	Clinical development
SU11248	VEGFR, PDGFR, Flt3, and c-kit	Clinical development
ZD6474	VEGFR-2, EGFR	Clinical development
SU5416	VEGFR-2, c-kit	Clinical development
Vatalanib, PTK/ZK	Multiple VEGFR	Clinical development
CI-1040	MEK-1 and MEK-2	Clinical development
Lonafarnib	Farnesyl transferase	Clinical development
Tipifarnib	Farnesyl transferase	Clinical development
Monoclonal antibodies		
Trastuzumab (Herceptin)	Humanized anti-Her2 Ab	Approved for Her-2-positive metastatic breast cancer
Cetuximab (Erbitux)	Chimeric anti-EGFR Ab	Approved for CRC in combination with irinotecan
Bevacizumab (Avastin)	Humanized anti-VEGF Ab	Approved for CRC in combination with 5-FU regimes
Panitumumab, ABX-EGF	Humanized anti-EGFR Ab	Clinical development
HuMV833	Humanized anti-VEGF Ab	Clinical development
Others		
Temsirolimus (CCI-779)	mTOR	Clinical development
AP23573	mTOR	Clinical development
VX-680	Aurora kinase	Clinical development
VEGF-Trap	Fusion protein binding VEGF in the circulation and tissues	

CML: chronic myeloid leukemia; CRC: colorectal cancer; EGFR: epidermal growth factor receptor; 5-FU: 5-fluoruracil; GIST: gastrointestinal stromal tumor; NSCLC: non-small-cell lung cancer; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; MEK: mitogen-activated protein kinase kinase; RCC: renal cell cancer; VEGFR: vascular endothelial growth factor receptor.

Table 2 Ongoing clinical trials in the USA involving new drugs specifically for the treatment of HCC (data were obtained from http://www.cancer.gov/clinicaltrials)

Agent	*Target*	*Phase*	*Observations*
Sorafenib	Multiple tyrosine kinase inhibitor (VEGF, RAF)	II/III	Randomized (DOX + SOR vs DOX)
		III	Randomized (SOR vs placebo)
		I	SOR + bevacizumab
Nolatrexed (Thymitaq)	Thymidylate synthase inhibitor	III	Randomized (NOL vs DOX)
Megestrol	Anti-estrogen	III	Randomized (megestrol vs placebo)
DENSPM	Depletion of the cellular pool of polyamines	I/II	
MB07133	Antiproliferative (precursor of araC)	I/II	
PHY906	Potentiate antitumor effect of capecitabine	I/II	Herbal remedy
Arsenic trioxide	Antineoplastic	II	
Bortezomib (Velcade)	Proteasome inhibitor	II	Approved for multiple myeloma
		I	Patients with liver dysfunction
Thalidomide	Antiangiogenic	II	In combination with epirubicin
Bevacizumab (Avastin)	anti-VEGF mAb	II	
		II	TACE vs TACE + bevacizumab
		I	SOR + bevacizumab
Erlotinib (Tarceva)	EGFR-tyrosine kinase inhibitor	II	
		I	Patients with liver dysfunction
AGI-PEG 20	Arginine depletion	II	
Thymalfasin (Zadaxin)	Immunomodulator	II	TACE vs TACE + thymalfasin
Ispinesib, SB-715992	Disrupts kinesin spindle protein	II	
Lapatinib, GW572016	ErbB-2 and EGFR tyrosine kinase inhibitor	II	

DOX: doxorubicin; NOL: nolatrexed; SOR: sorafenib; TACE: transarterial chemotherapy.

Among targeted therapies sorafenib is the agent most extensively studied (Strumberg *et al*., 2005). Sorafenib is the first oral multi-kinase inhibitor that targets serine/threonine and receptor TKs in both the tumor cell and tumor vasculature. In preclinical models, sorafenib has been shown to target members of two classes of kinases known to be involved in both tumor cell proliferation and tumor angiogenesis, including Raf kinase, VEGFR-2, VEGFR-3, PDGFR-b, c-kit, FLT-3 and ret. After having achieved promising results in a phase II trial and waiting for approval as an orphan drug for renal cell carcinoma, an international, randomized, double-blind, placebo-controlled trial is currently recruiting patients with advanced HCC. Another phase III trial is investigating if there is any advantage on adding sorafenib to doxorubicin for the treatment of HCC and a phase I trial is studying the safety of the combination of sorafenib with bevacizumab, an anti-angiogenic agent already approved for the treatment of colorectal cancer. Bevacizumab itself is on phase II trials either alone or combined with transarterial chemoembolization, an appealing combination considering that the therapeutic effect of the later could be quelled by neoangiogenesis that would be inhibited by bevacizumab.

Erlotinib, a receptor TK inhibitor with specificity for the EGFR, already approved for the treatment of NSCLC has very recently shown that warrants further research in HCC patients. In a phase II trial, 32% of the patients recruited were free of progression at 6 months (Philip *et al*., 2005). Yet, 26% of patients needed a dose reduction due to toxicity (mainly skin toxicity and diarrhea) stressing that side effect may also be important among cirrhotic patients with HCC. A phase I study is currently evaluating the pharmacokinetics of erlotinib in patients with liver dysfunction.

Other small molecules undergoing clinical research include bortezomib, a proteasome inhibitor approved for the treatment of multiple myeloma; lapatinib, an ErbB-2 and EGFR TK inhibitor; and ispinesib that acts by disrupting the function of kinesin spindle protein, a cytoskeletal protein that is essential for cell proliferation.

However, targeted agents are not the only drugs undergoing clinical development for the treatment of HCC. Thymitaq is a novel, oral thymidylate synthase inhibitor that showed promising result in a non-controlled phase II trial (Stuart *et al*., 1999) but no superiority to doxorubicin in a randomized trial in Chinese patients with HCC. An intravenous formulation is being tested in a pivotal, multinational, phase III trial among patients with unresectable HCC comparing Thymitaq to doxorubicin for which recruitment has been completed.

DENSPM is a dysfunctional polyamine analog that downregulates the synthetic enzymes of polyamine metabolism and upregulates the catabolic enzyme, rendering cells devoid of functional polyamines such as putrescine, spermidine, and spermine that are essential for cell growth and differentiation of both normal and malignant cells (Wallace and Fraser, 2003). DENSPM is now on a phase I/II trial to evaluate its potential for the treatment of HCC.

Targeting drugs to liver cells can be achieved using a series of phosphate and phosphonate prodrugs that result in liver-targeted drug delivery following a cytochrome *P*450-catalysed oxidative cleavage reaction inside hepatocytes. MB07133 is a prodrug of cytarabine that produces higher levels of the biologically active form of PMEA and araC in the liver and lower levels in the most toxicologically sensitive organs (Erion *et al*., 2005) and is currently on a phase I/II trial in HCC patients.

Pegylated arginine deiminase (ADI-PEG 20) deserves a separate mention. It has been recently found that human HCC cell lines require arginine for growth. Arginine is not an essential amino acid for human adults or infants as it can be synthesized from citrulline. Therefore, selective elimination of arginine from the circulation may be a means of treating patients with HCC. Arginine deiminase, an arginine-degrading enzyme, is active against experimental HCC (Ensor *et al*., 2002). ADI-PEG 20 partly overcomes the drawbacks of a short half-life and strong antigenicity, and it has been shown that a weekly injection of ADI-PEG 20 eliminates all detectable arginine from plasma (Izzo *et al*., 2004). Outstanding results of this phase I/II trial in which two out of 19 patients had a complete response, and seven more had a partial response have encouraged further clinical development.

Nevertheless, some issues should be born in mind regarding the development of targeted therapies for HCC. The failure of conventional antineoplastic chemotherapy in the fight against HCC is due both to tumor cell resistance and to an altered pharmacodynamics/pharmacokinetics in the setting of liver cirrhosis (Morgan and McLean, 1995) that may enhance the side effects of many 'non-selective' cytotoxic drugs. And this may be the case also for new agents. In particular, cutaneous side effects of kinase inhibitors may be very distressing since they are chronic due to the long duration of treatment and several studies have reported a link between the antitumor efficacy of EGFR inhibitors and cutaneous side effects (Robert *et al*., 2005). And it has been stressed that chronic liver disease that usually underlies HCC may in fact enhance the toxicity of agents such as erlotinib. On the other hand, many small molecules seem to work better as adjuvants to cytotoxic drugs than as single agents (antiangiogenics, inhibitors of matrix metalloproteinases and anti-EFGR antibodies) and this might obviously limit their efficacy against HCC.

Gene therapy and combined cell and gene therapy

Basic concepts

Gene therapy is based on the transfer of nucleic acid sequences to tissues to promote the local synthesis of a therapeutic protein or to block the expression of a specific gene. The transferred can be the expression cassette of a cDNA (or various expression cassettes of several cDNAs), small interfering RNAs or antisense sequences. To facilitate the entry of the genetic material into cells a variety of molecular constructs, named gene therapy vectors, are used. These can be divided into viral and non-viral vectors, being the former preferred because of their higher transduction efficiency. Viral vectors are generated by eliminating some, or all, viral genes leaving intact those sequences required in *cis* for packaging the vector genome into the viral capsid or for integration of vector DNA into the host genome. The deleted sequences can be replaced by the therapeutic gene(s) thus enabling the infected cell to express the transgene. The efficacy of gene therapy depends not only on the selection of the appropriate therapeutic gene(s), but also on efficient cell transduction (penetration and function of the transgene inside the target cell), on the duration of transgene expression in the treated tissue, on the toxicity of the vector or transgene and on the activity of regulatory elements used to control gene expression.

Each viral vector system is characterized by an inherent set of properties that affect its suitability for specific gene therapy applications. Thus, the choice of the vector is a critical issue since tissue tropism, duration of gene expression, number of cells that are transduced, fate of transgene (episomal vs integration into the cell genome) and toxicity differ among the various types of vectors used. For example, lentiviruses and other retroviuses as well as adeno-associated viruses (AAV) promote the integration of the transgene into the genome, while adenoviral vectors permit the transgene to remain in an episomal form. Integrating vectors and helper-dependent (HD) adenoviral vectors allow long-term expression of the transgene, while first-generation adenoviruses only enable short duration (5–8 days) of gene expression. Toxicity also varies among vectors being considerable less for HD adenoviruses than for first-generation adenoviral vectors (O'Neal *et al*., 2000). The risk of insertional mutagenesis should be considered for AAV, lentiviruses and other retroviruses and is less a concern for adenoviral vectors. Also cloning capacity, immunogenicity, as well as the feasibility and cost of large-scale production are important issues for the selection of the vector.

Gene therapy of cancer can be combined with cell therapy for two main purposes: (a) to potentiate antitumor immune responses or (b) to populate the tumor vasculature with engineered cells able to secrete a therapeutic compound inside the tumor mass (Rumpold *et al*., 2004). Thus, dendritic cells (DCs) engineered *ex vivo* with gene therapy vectors encoding immunostimulating cytokines can be pulsed with tumor lysates and injected into the lymph nodes to activate antitumor immunity. In another application endothelium progenitor cells transduced *ex vivo* with lentiviral vectors encoding therapeutic genes under the control of an inducible promoter are given intravenously for recruitement by tumors with high angiogenic activity (as is the case of HCC). This would allow the expression of the transgene inside the tumor nodules when the activator of the promoter is administered to the patient.

Strategies for hepatocellular carcinoma gene therapy

Gene therapy is a highly plastic procedure that can be used in many different ways to combat cancer. The aim of the intervention could be: (a) to induce a direct lysis of the tumor cells, (b) to stimulate antitumor immunity, (c) to block tumor growth by changing the biological conditions of the tumor environment (d) to combine various of these effects. As a result of difficulties to

transduce the tumor by intravenous route, direct injection of the vector into the tumor nodule, or in the peritumoral tissue, using echographic guidance, is the preferred way to achieve an efficient transduction in the patients with liver cancer.

Tumor-suppressor genes

It has been shown that transferring wild-type *p53* gene to *p53*-negative HCC cells inhibits tumor growth and increases the sensitivity to chemotherapy (Xu *et al*., 1996). However, only a modest therapeutic effect was obtained in clinical trials using adenovirus-mediated gene transfer of *p53* (Warren and Kirn, 2002). The limited efficacy of *p53* gene supplementation reflects the inability to transduce efficiently all the tumor cells with vectors presently available. To overcome this obstacle, a number of procedures have been proposed including the use of vectors with the ability to replicate selectively in the tumor cells and the utilization of systems enabling the transgenic product to diffuse and penetrate neighboring cells (Qian *et al*., 2002). One example of the latter strategy is the fusion of p53 to VP22, a protein from herpes simplex virus type 1 (HSV-1) with the remarkable property of being transported through cell boundaries (Zender *et al*., 2002).

Suicide genes: therapy and imaging

Genes encoding for enzymes that convert an innocuous prodrug to a toxic compound are known as suicide genes. Thymidine kinase (tk) from HSV-1 is the best-characterized prodrug activation enzyme (Fillat *et al*., 2003). It converts ganciclovir (a well-tolerated antiviral drug) into a toxic phosphorylated compound that inhibits both nuclear and mitochondrial DNA synthesis leading to cell death (Fillat *et al*., 2003; Herraiz *et al*., 2003). This strategy allows concentrating the action of the toxic metabolite in the tumoral tissue avoiding systemic toxicity. An interesting property of suicide genes is the so-called bystander effect that potentiates antitumor activity by the diffusion of the activated prodrug to non-transduced neighboring cells (Mesnil and Yamasaki, 2000). The bystander effect may also derive from the stimulation of the antitumoral immune response as a result of necrosis and apoptosis in the tumor (Kianmanesh *et al*., 1997). A synergistic antitumoral effect was observed when co-transferring tk and genes of immunostimulatory cytokines (Drozdzik *et al*., 2000).

In a recent trial (Penuelas *et al*., 2005) a first-generation adenovirus encoding HSV-tk under the control of CMV promoter (AdCMVtk) was used to treat 7 patients with HCC by intratumoral administration of the vector in a dose-escalation fashion from 2×10^{10} to 2×10^{12} v.p. in consecutive patients. Contrary to patients treated with lower doses, those who received 10^{12} v.p. or more ($n = 4$) showed estabilization of the treated tumor and in two of them wide areas of necrosis were apparent in the computed tomography scan performed at day 30 after therapy. Tolerance was good and no significant side effects were apparent. In this trial, positron emission tomography (PET) imaging was employed to monitor transgene expression using a labeled substrate of HSV-tk ([^{18}F]FHBG). With this methodology strong retention of the label was found in the treated tumor nodules in all patients that received 10^{12} v.p or more but not in those given lower vector doses (Figure 2). Interestingly the surrounding non-tumoral cirrhotic tissue was completely spared from transduction in all cases. This study demonstrated that

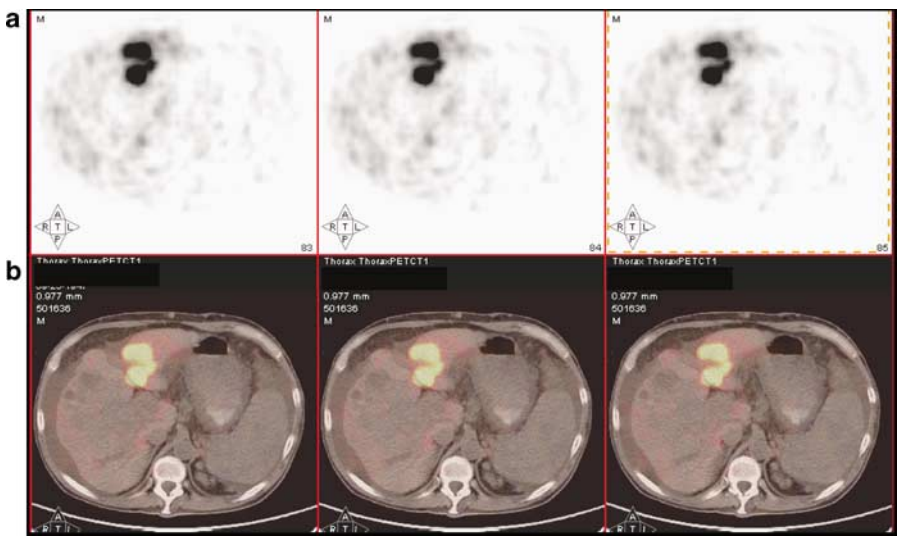

Figure 2 Imaging of adenovirus-mediated transgene (thymidine kinase) expression in hepatocellular carcinoma (HCC) tumor nodule using positron emission tomography (PET) (**a**) and combined PET plus Computed Tomography (CT) (**b**) PET imaging of a HCC tumor nodule that received an intratumor injection of 10^{12} viral particles of an adenovirus encoding thymidin kinase (HSV-tk) given 2 days before the study. The upper three panels correspond to PET images and the lower three panel to combination PET-CT. PET imaging was performed 60 min after administration of [^{18}F]FHBG (a 18-fluorine-labeled penciclovir ananlogue which is a substrate for HSV-tk). The strong retention of the label can be appretiated in the transaxial sections of PET (**a**) and PET–CT (**b**) shown in the figure.

human HCC are easily infected with adenoviral vectors and it also showed that a second dose of the vector fails to transduce the tumor possibly as result of the development of neutralizing antiadenoviral antibodies.

Antitumoral effect against HCC and metastatic liver cancer was observed using other prodrug-activating enzymes including cytosine deaminase that turns antifungal 5-fluocytosine into 5-fluoruracil (Ohwada *et al*., 1996; Humphreys *et al*., 2001), *Escherichia coli* purine nucleoside phosphorylase that converts fludarabine into a diffusible toxic compound (Mohr *et al*., 2000) and bacterial nitroreductase which converts CB1954 (5-(azidrin-1-yl)-2,4-dinitrobenzamide) to a short lived, very toxic DNA crosslinking compound. Recently, a clinical trial in patients with primary and metastatic liver cancer using intratumoral injection of escalating doses ($10^8-5\times10^{11}$ v.p.) of a first adenovirus-encoding nitroreductase showed good tolerance of the procedure and a dose-related expression of the transgene in the biopsy of the treated tumor (Palmer *et al*., 2004).

Oncolytic virotherapy

This strategy is based on the use of viruses that replicate and kill preferentially tumor cells. These cells then become cell factories of new viral particles that infect surrounding tumor cells. Adenoviruses have been extensively used for the production of oncolytic agents. The first of this kind of virus (named dl1520 or ONYX-015) is based on the deletion of the *E1B* 55K viral gene, which makes its replication dependent on the defect of the p53 pathway in the infected cells (McCormick, 2003). In clinical trials, ONYX-015 has proved to be safe after intratumoral injection in humans (Kirn, 2001). A pilot clinical trial of intratumoral injection of the virus for the treatment of primary and secondary tumors revealed that the treatment was well tolerated and some clinical responses were observed (Habib *et al*., 2001). However, a further study in which repeated intratumoral injections of this replication-selective adenovirus were given to patients with HCC failed to show clinically relevant efficacy (Habib *et al*., 2002) presumably because of generation of neutralizing antibodies. In hepatobiliary tumors, biological responses were observed, but $<10\%$ of the patients showed partial responses (Makower *et al*., 2003).

Another approach to restrict adenovirus replication in tumor cells is based on the transcriptional control of viral genes that are essential for replication (e.g. E1) using tumor-specific promoters such as AFP, the human telomerase reverse transcriptase or E2F promoters (Huang *et al*., 2003a; Jakubczak *et al*., 2003; Wirth *et al*., 2003; Irving *et al*., 2004; Zou *et al*., 2004). In addition to adenovirus, other viruses, including the rRp450 mutant of HSV-1 or the vesicular stomatitis virus, have been used as selective oncolytic agents in HCC (Pawlik *et al*., 2000; Ebert *et al*., 2003; Huang *et al*., 2003b).

The oncolytic viruses can also be used as potential vectors to convey therapeutic genes to tumor cells. Oncolytic adenovirus encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), TRAIL or second mitochondria-derived activator of caspases have been shown to exert a potent antitumor effect in experimental HCC (Bristol *et al*., 2003; Pei *et al*., 2004). Similarly, IL-12 has been incorporated into oncolytic HSV offering effective control of primary hepatic tumors and protection against microscopic residual disease after resection (Jarnagin *et al*., 2003).

Antiangiogenic gene therapy

Since HCC is a highly vascularized tumor, an attractive therapeutic strategy is based on gene transfer of antiangiogenic factors to the tumor or peritumoral tissue using long-term expression vectors. This approach would allow obtaining high concentration of the antiangiogenic molecule at the tumor site with reduced risk of systemic toxicity. Although soluble VEGF receptors (Goldman *et al*., 1998; Raskopf *et al*., 2005) and other antiangiogenic molecules, such as Tie2 receptor, angiostatin, endostatin and pigment epithelium-derived factor (PEDF) have shown antitumor activity in animal models of HCC (Lin *et al*., 1998; Griscelli *et al*., 1998; Schmitz *et al*., 2002; Folkman, 2003; Wang *et al*., 2003), clinical trials have not been initiated so far.

Immuno-gene therapy

Tumors express neoantigens that could elicit protective immunity but antitumor immune responses are in general weak or ineffective due to low expression of MHC molecules (Algarra *et al*., 2000), production of immunosuppressive factors (TGF-β, IL-10, VEGF or IL-8) (Ranges *et al*., 1987), induction of CD4+ CD25+ regulatory T cells (Treg) (Antony and Restifo, 2005) or expression of Fas ligand by neoplastic cells resulting in apoptosis of tumor-infiltrating T cells (Strand *et al*., 1996). On the other side tumour cells can exploit host-derived cytokines to increase resistance to apoptosis and to stimulate growth and dissemination (Dranoff, 2004). Gene therapy can be used to overcome these barriers and to promote antitumor responses. Cytokines (such as IL-2, IL-12, IL-15, TNF-α and IFN-γ) generated during the immune response, in inflammation and in infection, can restrict tumor growth and it has been shown that their systemic administration could elicit antitumor effects (Dranoff, 2004). However, the severe toxicity associated with these treatments limits their application.

Gene transfer of immunostimulatory cytokines to the neoplastic cells increases local levels of the cytokine without undue elevation of serum concentration thus widening the therapeutic window and reducing toxicity. A variety of immunostimulatory cytokines, chemokines or co-stimulatory molecules (IL-2, IL-7; IL-12, IL-15, IL-18, IL-21, IL-23, IFN-γ, TNF-α, GM-CSF, IP-10, CD40-L and B7.1) have been employed with efficacy in gene therapy of diverse tumor models (Prieto *et al*., 2004). Interleukin-12 is a particularly potent antitumor cytokine which induces a TH1 type of response (Trinchieri, 1998; Mazzolini *et al*., 2003a, b), activates

cytotoxic T lymphocytes and natural killer cells and displays robust antiangiogenic activities (Sgadari *et al*., 1996; Mazzolini *et al*., 2000; Barajas *et al*., 2001). Interestingly, combined gene transfer of IL-12 and the chemokine IP-10, or of IL-12 and the chemokine MIP3α, or of IL-12 and the co-stimulatory molecule B7.1 results in synergic antitumoral effect (Narvaiza *et al*., 2000; Putzer *et al*., 2001; Mazzolini *et al*., 2003a, b). In experimental HCC in rats gene therapy with either CD40-L (Schmitz *et al*., 2001) or IL-12 (Barajas *et al*., 2001) has been reported to induce tumor eradication without significant toxicity.

Of all immunogene therapeutic strategies used to treat experimental cancer models only IL-12 gene therapy has been transferred to the clinic to treat liver cancer (Sangro *et al*., 2004). The clinical trial was performed in patients with liver tumors (either primary or secondary to colorectal and pancreatic carcinomas) that received 1–3 intratumoral injections of a first-generation adenoviral vector encoding human *IL-12* genes. The treatment was without any significant side effects and in fact the maximal tolerated dose was not reached. However, the antitumor effect was weak with only one partial response in a patient with HCC. In general patients with HCC had a better outcome than other histological groups in this trial. These modest antitumor effects were probably due to the low and short-lived expression of the transgene when using first-generation adenovirus, and to the inability to repeat tumor transduction with a second vector injection due to production of neutralizing antiadenoviral antibodies after the first administration of the adenovirus.

Engineered dendritic cells

Genetic manipulation of DCs is a promising alternative to activate antitumor immunity. Dendritic cells transduced with specific antigens together with genes encoding cytokines or co-stimulatory molecules exhibit augmented antigen-presenting function (Tirapu *et al*., 2002) and it has been shown that intratumoral injection of DC engineered to produce IL-12 can induce tumor regression in animal models of digestive cancer (Melero *et al*., 1999). Moreover, the antitumor efficacy of this strategy can be reinforced by administration of the anti-CD137 mAb which is endowed with potent immunostimulatory activity (Tirapu *et al*., 2004). On the basis of these preclinical findings a clinical trial was performed in patients with primary or metastatic liver cancer using intratumoral injections of monocyte-derived DC transduced with first-generation adenoviral vectors encoding IL-12 (Mazzolini *et al*., 2005). The patients received up to three equal doses of cells (doses were escalated from 10 to 50 millions of cells in three cohorts of patients) at 21 days intervals. The expectation was that the modified DCs would take up tumor antigens and migrate to lymph nodes to activate a specific TH1 antitumoral response by locally secreting IL-12. However, although the procedure was well tolerated, the elicited antitumor effect was weak. It was found that the tumor, by secreting IL-8, sequesters the injected DC and prevents their migration to regional lymph nodes rendering the therapy inefficient (Feijoo *et al*., 2005).

Clinical trials are needed to test whether autologous mature DC (genetically engineered or not) pulsed with tumor lysate or tumor RNA (obtained from the surgical specimen) and injected inside lymph nodes (not within the tumor mass) could efficiently stimulate specific antitumor responses in HCC patients. This therapy might be useful to eliminate minimal residual disease after surgery rather than to control growth of advanced cancer.

Perspectives of hepatocellular carcinoma gene therapy

Until present, first-generation adenoviruses are the vectors most frequently used in clinical trials of cancer. With this type of vectors, HCC tumor transduction is good but the expression of the transgene is short lived and repeated transduction of the tumor is not feasible due to neutralizing antiadenoviral antibodies. The short duration of transgene expression seems to be the cause of the limited therapeutic effect. From the accumulated experience it seems that long-term expression vectors should be used to enhance effectiveness and appropriate promoters should be developed to ensure prolonged and regulable expression of the therapeutic transgene and vector technology should be improved to generate sophisticated vectors at high titers for clinical use.

With the progress in the knowledge of tumor biology novel targets have been identified. This creates new opportunities for gene therapy which can rely not only on immuno-gene strategies, oncolytic virotherapy or suicide genes but also on the expression inside the proper tumor mass of humanized mAbs or decoy molecules directed to block those factors that are crucial for tumor progression such as growth factors, growth factors receptors, metalloproteases or integrins. In other words, gene therapy can be used to generate within the proper tumor mass those substances that can disrupt the biologic microenvironment that the tumor needs to grow. The recruitment of circulating progenitor cells by the tumor can be exploited by genetic engineering of these cells which can then be administered to the patient as vehicle of therapeutic genes. This combination of cell and gene therapy represents an attractive strategy to target all metastatic tumor lesions.

Radiotherapy

Once neglected because of the low tolerance of the liver to irradiation, radiotherapy has emerged as a promising tool for the treatment of HCC as new technologies allow the selective delivery of tumoricidal doses of radiation to liver tumors (Geschwind *et al*., 2004). External conformal radiation therapy using linear accelerator (Park *et al*., 2005) or proton beam (Kawashima *et al*., 2005) result in response rates above 60% with doses higher than 50 Gy showing that HCC is a radiosensitive neoplasm. Selective radiation can also be accomplished with the use of carriers, and this is particularly valuable

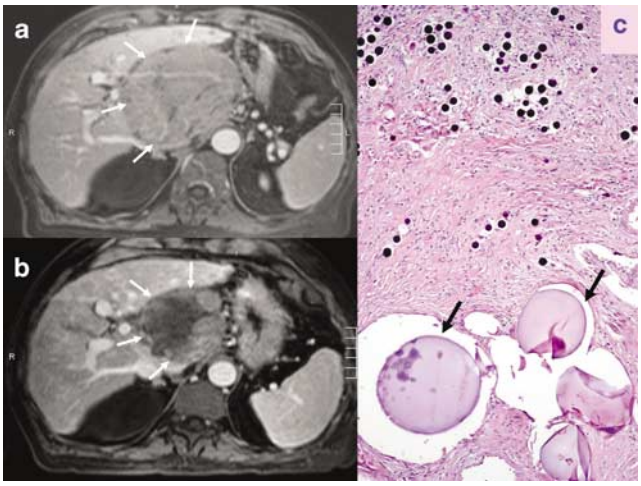

Figure 3 Selective internal radiation therapy (SIRT) for hepatocellular carcinoma. The left panel shows the appearance of a large liver tumor (white arrows) before (**a**) and 3 months after the injection of microspheres loaded with Yttrium-90 into the common hepatic artery (**b**). The right panel shows the size and lodgement of these microspheres relative to the large (150–300 μm) spheres commonly used for transarterial embolization (black arrows) (**c**).

for multinodular tumors (Table 1). Selective Internal Radiation Therapy (SIRT) can be achieved using ^{90}Y-loaded microspheres made of either resin (SIR-Spheres®, Figure 3) or glass (Therasphere®). They can be delivered as selectively as possible (from subsegmental to whole liver), and provides millions of scattered point sources of radioactivity, as opposed to the uniform fields of external beam radiotherapy. This difference in field properties for a given measure of radiation-absorbed dose results in different biological effects (Kennedy *et al*., 2004). Results from non-randomized trials or retrospective series show that tumor control can be achieved in a significant proportion of patients with good tolerance and suggest that survival may be similar to that obtained with transcatheter arterial embolization (Liu *et al*., 2004; Kulik *et al*., 2005; Goin *et al*., 2005a, b). Yet, large randomized trials should be conducted to compare SIRT vs TACE for the treatment of advanced HCC. SIRT also merits clinical investigation as a method to prevent tumor progression while on the waiting list for liver transplantation. Lipiodol can also be used as a carrier of radionuclides including ^{131}I (Raoul *et al*., 1997) and ^{188}Re (Sundram *et al*., 2004), the latter having several assets from the radioprotection perspective. Yet, there is little evidence that therapy is indeed selective and toxicity may be a significant problem, particularly for patients with Child-B functional status (Lambert *et al*., 2005).

Immunotherapy

A thorough review of new immunological therapies for HCC is beyond the scope of this paper. However, a number of strategies aiming at stimulating immunity against liver tumors can be outlined. High-dose interferon-alpha (50 MU/m^2, tiw) has significantly prolonged survival of HCC patients (Lai *et al*., 1993) suggesting that it might be worth stimulating the immune system of patients with HCC if the immune stimulating agent has a good toxic profile. Interleukin 2 was also able to produce objective remissions among HCC patients (Palmieri *et al*., 2002). But then again, toxicity limits widespread application of IL-2 to cirrhotics. Other cytokines such as IL-12, TNF-α or TRAIL have still not been tested for human liver cancer but there are preclinical data in rodents that suggest a potential interest.

Monoclonal antibodies can be designed that bind molecules on the surface of lymphocytes or antigen-presenting cells and provide activating signals. Alternatively, they can be used to block the action of surface receptors that normally downregulate immune responses. In combined regimes of immunotherapy, these mAbs are expected to improve therapeutic immunizations against tumors as it has been observed in preclinical models. Anti-CTLA-4 mAb that block the inhibitory function of CTLA-4 on T cells have already started clinical trials against prostate cancer (Kuhns *et al*., 2000), and the preclinical effects described for anti-CD40, anti-CD137 (4-1BB), anti-CD102 (intercellular adhesion molecule-2), and Treg-depleting mAbs should lead to their prompt clinical development. (Murillo *et al*., 2003).

Dendritic cells, the most potent antigen-presenting cells *in vivo*, can be used to elicit antitumor immunity. In a recent trial autologous DCs derived from peripheral monocytes and pulsed with tumor lysates were injected intravenously in 31 patients with advanced HCC. A partial response was observed in 13% of cases and survival was better in those that received boost vaccinations after the initial pulsed therapy than in those treated by pulsed therapy alone (Lee *et al*., 2005).

Adoptive T-cell therapy consists in rising and infusing *in vitro* cultured T cells that mediate specific destruction of tumor cells. The main hurdle in its application is the difficulty to obtain such T-cell cultures against epithelial tumors. The use of dendritic cells to prime T-cell cultures *in vitro* can be very helpful for this purpose. In a randomized clinical trial, Takayama *et al*. (2000) showed that adoptive immunotherapy consisting of autologous lymphocytes activated *in vitro* and administered to patients who had received curative resection for HCC is a safe therapeutic approach that reduces post-surgical recurrence at 3 (48 vs 33%) and 5 years (38 vs 22%). New techniques of selection and culture of tumor-specific cells as well as combination therapy with tumor vaccination protocols are expected to improve the outcome.

Corollary

Non-resectable cancer can be treated either by directly targeting the tumor cells or by increasing the defense of

the host against tumor growth. Direct attack to tumor cells can be executed using non-specific cytotoxic therapies such as conventional chemotherapy or radiotherapy or by targeted therapy with substances able to block specific biological processes conferring tumor cells the ability to grow inappropriately, to resist apoptosis, to invade and to metastatize. Host defense against cancer can be boosted by vaccination with tumor antigens, inhibition of Treg or adoptive immunization. Host resistance to neoplastic growth can also be enhanced by disrupting the tumor biologic microenvironment blocking signals emanating from the neoplastic cells that promote angiogenesis and the formation of tumor stroma.

Hepatocellular carcinoma is highly resistant to conventional chemotherapy. This tumor is also a poorly immunogenic neoplasm although immunotherapy may be used to prevent recurrences after surgical resection or percutaneous ablation. On the other hand HCC is rarely amenable to radiotherapy because of multinodularity and underlying liver disease although SIRT and external conformal radiation may have a role in specific indications. All these features make advanced HCC a very difficult tumor to treat. However, the impressive advances in the knowledge of tumor biology taken place during the last years and the remarkable success of targeted therapy in other tumors (either alone or in combination with cytostatics) has opened promising avenues for HCC therapy. In HCC multiple molecular alterations ensure the incessant growth of the neoplastic cells. Since the blockade of a specific target can be overcome by other molecular abnormalities, it is likely that HCC could easily develop resistance to compounds that hit a single molecule. Drugs designed to block different growth-promoting pathways (promiscuous drugs such as multiple kinase inhibitors) or combination of different targeted therapies might however attain success in the control of liver cancer. As shown in the case of breast cancer, targeted therapy against molecules conveying growth-promoting signals may render HCC sensitive to specific chemotherapeutic protocols. In the future methods to analyse the molecular signature of each HCC might make possible to select the appropriate combination of targeted therapies that should be used in the particular patient. However toxic side effects of drug combinations will be a risk specially in patients with underlying liver cirrhosis and/or poor liver function.

Gene therapy is still at its infancy. Transduction of the neoplasm with viral vectors leads to expression of the transgene within the tumor mass enabling a high concentration of the therapeutic substance inside the tumor with low systemic levels thus increasing the therapeutic window. Although, until present, the use of first-generation viral vectors did not achieve clinical efficacy, the lessons from pioneer clinical trials, the utilization of PET for imaging of gene expression and the development of new vectors enabling long duration and regulatable transgene expression, make gene therapy a promising approach to treat HCC.

Cell therapy represents an additional promise for the future of HCC therapy. The possibility of isolating, culturing and genetically engineering progenitor cells that are recruited by the tumor after their intravenous injection affords the possibility to direct the expression of therapeutic molecules to both the primary and metastatic tumor lesions.

To conclude, although at present the prognosis of advanced HCC is dismal, new horizons with the promise of better times are now at sight.

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References

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. (1997). *EMBO J* **16**: 3797–3804.
Adams J. (2004). *Cancer Cell* **5**: 417–421.
Adjei AA, Hidalgo M. (2005). *J Clin Oncol* **23**: 5386–5403.
Alexia C, Fallot G, Lasfer M, Schweizer-Groyer G, Groyer A. (2004). *Biochem Pharmacol* **68**: 1003–1015.
Algarra I, Cabrera T, Garrido F. (2000). *Hum Immunol* **61**: 65–73.
Al-Hajj M, Clarke MF. (2004). *Oncogene* **23**: 7274–7282.
Amaravadi R, Thompson CB. (2005). *J Clin Invest* **115**: 2618–2624.
Antony PA, Restifo NP. (2005). *J Immunother* **28**: 120–128.
Arsura M, Cavin LG. (2005). *Cancer Lett* **229**: 157–169.
Avila MA, Berasain C, Torres L, Martín-Duce A, Corrales FJ, Yang H *et al*. (2000). *J Hepatol* **33**: 907–914.
Bachelder RE, Yoon SO, Franci C, García de Herreros A, Mercurio AM. (2005). *J Cell Biol* **168**: 29–33.
Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V *et al*. (2001). *Hepatology* **33**: 52–61.
Baselga J, Arteaga C. (2005). *J Clin Oncol* **23**: 2445–2459.
Bedossa P, Peltier E, Terris B, Franco D, Poynard T. (1995). *Hepatology* **21**: 760–766.
Berasain C, García-Trevijano ER, Castillo J, Erroba E, Lee DC, Prieto J *et al*. (2005a). *Gastroenterology* **128**: 424–432.
Berasain C, García-Trevijano ER, Castillo J, Erroba E, Santamaria M, Lee DC *et al*. (2005b). *J Biol Chem* **280**: 19012–19020.
Berasain C, Herrero JI, García-Trevijano ER, Avila MA, Esteban JI, Mato JM *et al*. (2003). *Hepatology* **38**: 148–157.
Beurel E, Kornprobst M, Blivet-Van Eggelpoel MJ, Cadoret A, Capeau J, Desbois-Mouthon C. (2005). *Int J Oncol* **27**: 215–222.
Bianco R, Shin I, Ritter CA, Yakes FM, Basso A, Rosen N *et al*. (2003). *Oncogene* **22**: 2812–2822.
Bissell DM. (2001). *Exp Mol Med* **33**: 179–190.
Blobel CP. (2005). *Nat Rev Mol Cell Biol* **6**: 32–43.
Bosch FX, Ribes J, Diaz M, Cleries R. (2004). *Gastroenterology* **127**: S5–S16.

Bréchot C. (2004). *Gastroenterology* **127**: S56–S61.
Bristol JA, Zhu M, Ji H, Mina M, Xie Y, Clarke L *et al*. (2003). *Mol Ther* **7**: 755–764.
Buendia MA. (2000). *Semin Cancer Biol* **10**: 185–200.
Calvisi DF, Factor V, Loi R, Thorgeirsson SS. (2001). *Cancer Res* **61**: 2085–2091.
Calvisi DF, Ladu S, Conner EA, Factor V, Thorgeirsson SS. (2004). *Lab Invest* **84**: 1137–1147.
Camirand A, Zakikhani M, Young F, Pollak M. (2005). *Breast Cancer Res* **7**: R570–R579.
Camp ER, Summy J, Bauer T, Liu W, Gallick GE, Ellis LM. (2005). *Clin Cancer Res* **11**: 397–405.
Carloni V, Mazzocca A, Pantaleo P, Cordella C, Laffi G, Gentilini P. (2001). *Hepatology* **34**: 42–49.
Carloni V, Vizzuti F, Pantaleo P. (2005). *Clin Cancer Res* **11**: 4266–4274.
Chan KT, Lung ML. (2004). *Cancer Chemother Pharmacol* **53**: 519–526.
Chung YH, Kim JA, Song BC, Lee GC, Koh MS, Lee SG *et al*. (2000). *Cancer* **89**: 977–982.
Clarke MF. (2005). *Biol Blood Marrow Transplant* **11**: 14–16.
Coleman WB. (2003). *Curr Mol Med* **3**: 573–588.
Dean M, Fojo T, Bates S. (2005). *Nat Rev Cancer* **5**: 275–284.
Deane NG, Parker MA, Aramandla R, Diehl L, Lee W-J, Washington MK *et al*. (2001). *Cancer Res* **61**: 5389–5395.
Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoël M-J, Bertrand F, Cherqui G, Perret C *et al*. (2001). *Oncogene* **20**: 252–259.
Dihlmann S, von Knebel Doeberitz M. (2005). *Int J Cancer* **113**: 515–524.
Dikmen ZG, Gellert GC, Jackson S, Gryaznov S, Tressler R, Dogan P *et al*. (2005). *Cancer Res* **65**: 7866–7873.
Ding Q, Xia W, Liu J-C, Yang J-Y, Lee D-F, Xia J *et al*. (2005). *Mol Cell* **19**: 159–170.
Ding X, Yang LY, Huang GW, Wang W, Lu WQ. (2004). *World J Gastroenterol* **10**: 2735–2739.
Djojosubroto MW, Chin AC, Go N, Schaetzlein S, Manns MP, Gryaznov S *et al*. (2005). *Hepatology* **42**: 1127–1136.
Dranoff G. (2004). *Nat Rev Cancer* **4**: 11–22.
Drozdzik M, Qian C, Xie X, Peng D, Bilbao R, Mazzolini G *et al*. (2000). *J Hepatol* **32**: 279–286.
Ebert O, Shinozaki K, Huang TG, Savontaus MJ, Garcia-Sastre A, Woo SL. (2003). *Cancer Res* **63**: 3605–3611.
Edamoto Y, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle H-M *et al*. (2003). *Int J Cancer* **106**: 334–341.
Eger A, Stockinger A, park J, Langkopf E, Mikula M, Gotzmann J *et al*. (2004). *Oncogene* **23**: 2672–2680.
Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M *et al*. (2004). *Proc Natl Acad Sci USA* **101**: 12682–12687.
Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. (2002). *Cancer Res* **62**: 5443–5450.
Erion MD, van Poelje PD, Mackenna DA, Colby TJ, Montag AC, Fujitaki JM *et al*. (2005). *J Pharmacol Exp Ther* **312**: 554–560.
Eskens FA. (2004). *Br J Cancer* **90**: 1–7.
Ezquerro IJ, Lasarte JJ, Dotor J, Castilla-Cortazar I, Bustos M, Penuelas I *et al*. (2003). *Cytokine* **22**: 12–20.
Feijoo E, Alfaro C, Mazzolini G, Serra P, Penuelas I, Arina A *et al*. (2005). *Int J Cancer* **116**: 275–281.
Feitelson MA. (2005). *Cancer Lett* [E-pub ahead of print].
Feitelson MA, Sun B, Satiroglu Tufan NL, Liu J, Pan J, Lian Z. (2002). *Oncogene* **21**: 2593–2604.
Fillat C, Carrio M, Cascante A, Sangro B. (2003). *Curr Gene Ther* **3**: 13–26.
Fischer AN, Herrera B, Mikula M, Proell V, Fuchs E, Gotzmann J *et al*. (2005). *Carcino genesis* **26**: 931–942.
Folkman J. (2003). *Cancer Biol Ther* **2**: S127–S133.
Fujii T, Koshikawa K, Nomoto S, Okochi O, Kaneko T, Inoue S *et al*. (2004). *J Hepatol* **41**: 104–111.
Ganten TM, Koschny R, Haas TL, Sykora J, Li-Webber M, Herzer K *et al*. (2005). *Hepatology* **42**: 588–597.
Geschwind JF, Salem R, Carr BI, Soulen MC, Thurston KG, Goin KA *et al*. (2004). *Gastroenterology* **127**: S194–S205.
Giannelli G, Azzariti A, Fransvea E, Porcelli L, Antonaci S, Paradiso A. (2004). *Br J Cancer* **91**: 1964–1969.
Giannelli G, Fransvea E, Bergamini C, Marinosci F, Antonaci S. (2003). *Clin Cancer Res* **9**: 3684–3691.
Giles RH, van Es JH, Clevers H. (2003). *Biochim Biophys Acta* **1653**: 1–24.
Goin JE, Salem R, Carr BI, Dancey JE, Soulen MC, Geschwind JF *et al*. (2005a). *J Vasc Interv Radiol* **16**: 195–203.
Goin JE, Salem R, Carr BI, Dancey JE, Soulen MC, Geschwind JF *et al*. (2005b). *J Vasc Interv Radiol* **16**: 205–213.
Goldman CK, Kendall RL, Cabrera G, Soroceanu L, Heike Y, Gillespie GY *et al*. (1998). *Proc Natl Acad Sci USA* **95**: 8795–8800.
Gotzmann J, Huber H, Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R *et al*. (2001). *J Cell Sci* **115**: 1189–1202.
Graaf MR, Richel DJ, Van Noorden CJF, Guchelaar H-J. (2004). *Cancer Treat Rev* **30**: 609–641.
Griscelli F, Li H, Bennaceur-Griscelli A, Soria J, Opolon P, Soria C *et al*. (1998). *Proc Natl Acad Sci USA* **95**: 6367–6372.
Habib N, Salama H, Abd El Latif Abu Median A, Isac Anis I, Abd Al Aziz RA, Sarraf C *et al*. (2002). *Cancer Gene Ther* **9**: 254–259.
Habib NA, Sarraf CE, Mitry RR, Havlik R, Nicholls J, Kelly M *et al*. (2001). *Hum Gene Ther* **12**: 219–226.
Hannigan G, Troussard AA, Dedahr S. (2005). *Nat Rev Cancer* **5**: 51–63.
Harada K, Shiota G, Kawasaki H. (1999). *Liver* **19**: 318–325.
Harris TJC, Peifer M. (2005). *Trends in Cell Biol* **15**: 234–237.
He B, Reguart N, You L, Mazieres J, Xu Z, Lee AY *et al*. (2005). *Oncogene* **24**: 3054–3058.
Herraiz M, Beraza N, Solano A, Sangro B, Montoya J, Qian C *et al*. (2003). *Hum Gene Ther* **14**: 463–472.
Higashitsuji H, Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo H *et al*. (2005). *Cancer Cell* **8**: 75–87.
Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kido T *et al*. (2000). *Nat Med* **6**: 96–99.
Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J *et al*. (2004). *J Clin Invest* **113**: 1774–1783.
Höpfner m, Sutter AP, Huether A, Schuppan D, Zeitz M, Scherübl H. (2004). *J Hepatol* **41**: 1008–1016.
Hu KQ. (2002). *J Lab Clin Med* **139**: 234–243.
Hu TH, Huang CC, Lin PR, Chang HW, Ger LP, Lin YW *et al*. (2003). *Cancer* **97**: 1929–1940.
Huang TB, Ebert O, Shinozaki K, García-Sastre A, Woo SL. (2003b). *Mol Ther* **8**: 434–440.
Huang TG, Savontaus MJ, Shinozaki K, Sauter BV, Woo SL. (2003a). *Gene Therapy* **10**: 1241–1247.
Huether A, Höpfner M, Sutter AP, Schuppan D, Scherübl H. (2005). *J Hepatol* **43**: 661–669.
Humphreys MJ, Ghaneh P, Greenhalf W, Campbell F, Clayton TM, Everett P *et al*. (2001). *Gene Therapy* **8**: 1241–1247.
Huynh H, Do PT, Nguyen TH, Chow P, Tan PH, Quach TH *et al*. (2004). *Int J Oncol* **25**: 1839–1847.
Hwang YH, Choi JY, Kim S, Chung ES, Kim T, Koh SS *et al*. (2004). *Hepatol Res* **29**: 113–121.

Hynes RO. (1992). *Cell* **69**: 11–25.
Inui Y, Higashiyama S, Kawata S, Tamura S, Miyagawa J, Taniguchi N *et al*. (1994). *Gastroenterology* **107**: 1799–1804.
Irving J, Wang Z, Powell S, O'Sullivan C, Mok M, Murphy B *et al*. (2004). *Cancer Gene Ther* **11**: 174–185.
Ishikawa N, Daigo Y, Takano A, Taniwaki M, Kato T, Hayama S *et al*. (2005). *Cancer Res* **65**: 9176–9184.
Itoh S, Maeda T, Shimada M, Aishima S, Shirabe K, Tanaka S *et al*. (2004). *Clin Cancer Res* **10**: 2812–2817.
Izzo F, Marra P, Beneduce G, Castello G, Vallone P, De Rosa V *et al*. (2004). *J Clin Oncol* **22**: 1815–1822.
Jakubczak JL, Ryan P, Gorziglia M, Clarke L, Hawkins LK, Hay C *et al*. (2003). *Cancer Res* **63**: 1490–1499.
Jarnagin WR, Zager JS, Klimstra D, Delman KA, Malhotra S, Ebright M *et al*. (2003). *Cancer Gene Ther* **10**: 215–223.
Kakiuchi S, Daigo Y, Ishikawa N, Furukawa C, Tsunoda T, Yano S *et al*. (2004). *Hum Mol Genet* **13**: 3029–3043.
Kalinichenko VV, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM *et al*. (2004). *Genes Dev* **18**: 830–850.
Kawashima M, Furuse J, Nishio T, Konishi M, Ishii H, Kinoshita T *et al*. (2005). *J Clin Oncol* **23**: 1839–1846.
Kennedy AS, Nutting C, Coldwell D, Gaiser J, Drachenberg C. (2004). *Int J Radiat Oncol Biol Phys* **60**: 1552–1563.
Kianmanesh AR, Perrin H, Panis Y, Fabre M, Nagy HJ, Houssin D *et al*. (1997). *Hum Gene Ther* **8**: 1807–1814.
Kim Y, Sills RC, Houle CD. (2005). *Toxicol Pathol* **33**: 175–180.
Kirn D. (2001). *Expert Opin Biol Ther* **1**: 525–538.
Kojiro M, Roskams T. (2005). *Semin Liver Dis* **25**: 133–142.
Kuhns MS, Epshteyn V, Sobel RA, Allison JP. (2000). *Proc Natl Acad Sci USA* **97**: 12711–12716.
Kulik LM, Mulcahy MF, Hunter RD, Nemcek Jr AA, Abecassis MM, Salem R. (2005). *Liver Transpl* **11**: 1127–1131.
Lai CL, Lau JY, Wu PC, Ngan H, Chung HT, Mitchell SJ *et al*. (1993). *Hepatology* **17**: 389–394.
Lambert B, Bacher K, De Keukeleire K, Smeets P, Colle I, Jeong JM *et al*. (2005). *J Nucl Med* **46**: 1326–1332.
Lan M, Kojima T, Osanai M, Chiba H, Sawada N. (2004). *Carcinogenesis* **25**: 2385–2395.
Lee JO, Kwun HJ, Jung JK, Choi KH, Min DS, Jang KL. (2005). *Oncogene* **24**: 6617–6625.
Lee JS, Thorgeirsson SS. (2004). *Gastroenterology* **127**: S51–S55.
Lee JS, Thorgeirsson SS. (2005). *Semin Liver Dis* **25**: 125–132.
Lee RG, Tsamandas AC, Demetris AJ. (1997). *Hepatology* **26**: 1415–1422.
Lee WC, Wang HC, Hung CF, Huang PF, Lia CR, Chen MF. (2005). *J Immunother* **28**: 496–504.
Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F *et al*. (2004). *Cancer Cell* **5**: 91–102.
Liang TJ, Heller T. (2004). *Gastroenterology* **127**: S62–S71.
Libbrecht L, Roskams T. (2002). *Semin Cell Dev Biol* **13**: 389–396.
Lin P, Buxton JA, Acheson A, Radziejewski C, Maisonpierre PC, Yancopoulos GD *et al*. (1998). *Proc Natl Acad Sci USA* **95**: 8829–8834.
Liu MD, Uaje MB, Al-Ghazi MS, Fields D, Herman J, Kuo JV *et al*. (2004). *Am Surg* **70**: 947–953.
Liu Y, Poon RT, Li Q, Kok TW, Lau C, Fan ST. (2005). *Cancer Res* **65**: 3691–3699.
Llovet JM, Burroughs A, Bruix J. (2003). *Lancet* **362**: 1907–1917.
Llovet JM, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M *et al*. (1999). *Hepatology* **29**: 62–67.
LoRusso PM, Adjei AA, Varterasian M, Gadgeel S, Reid J, Mitchell DY *et al*. (2005). *J Clin Oncol* **23**: 5281–5293.
Lowe SW, Cepero E, Evan G. (2004). *Nature* **432**: 307–315.
Lozano G, Zambetti GP. (2005). *Cancer Cell* **8**: 3–4.
Luo J-L, Kamata H, Karin M. (2005). *J Clin Invest* **115**: 2625–2632.
Makower D, Rozenblit A, Kaufman H, Edelman M, Lane ME, Zwiebel J *et al*. (2003). *Clin Cancer Res* **9**: 693–702.
Mani A, Gelmann EP. (2005). *J Clin Oncol* **23**: 4776–4789.
Masaki T, Okada M, Shiratori Y, Rengifo W, Matsumoto K, Maeda S *et al*. (1998). *Hepatology* **27**: 1257–1264.
Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K *et al*. (2003). *Hepatology* **37**: 534–543.
Matsumura T, Makino R, Mitamura K. (2001). *Clin Cancer Res* **7**: 594–599.
Mazzolini G, Alfaro C, Sangro B, Feijoo E, Ruiz J, Benito A *et al*. (2005). *J Clin Oncol* **23**: 999–1010.
Mazzolini G, Narvaiza I, Martinez-Cruz LA, Arina A, Barajas M, Galofre JC *et al*. (2003a). *Gene Therapy* **10**: 1067–1078.
Mazzolini G, Prieto J, Melero I. (2003b). *Curr Pharm Des* **9**: 1981–1991.
Mazzolini G, Qian C, Narvaiza I, Barajas M, Borras-Cuesta F, Xie X *et al*. (2000). *Hum Gene Ther* **11**: 113–125.
McCormick F. (2003). *Cancer Biol Ther* **2**: S157–S160.
Melero I, Duarte M, Ruiz J, Sangro B, Galofre J, Mazzolini G *et al*. (1999). *Gene Therapy* **6**: 1779–1784.
Merle P, de la Monte SM, Kim M, Herrmann M, Tanaka S, von dem Bussche A *et al*. (2004). *Gastroenterology* **127**: 1110–1122.
Mesnil M, Yamasaki H. (2000). *Cancer Res* **60**: 3989–3999.
Mohr L, Shankara S, Yoon SK, Krohne TU, Geissler M, Roberts B *et al*. (2000). *Hepatology* **31**: 606–614.
Monga SP, Pediaditakis P, Mule K, Stolz DB, Michalopoulos GK. (2001). *Hepatology* **33**: 1098–1109.
Morgan D, McLean AJ. (1995). *Clin Pharmacokinet* **29**: 370–391.
Mosesson Y, Yarden Y. (2004). *Semin Cancer Biol* **14**: 262–270.
Müller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M *et al*. (2005). *J Exp Med* **188**: 2033–2045.
Murillo O, Arina A, Tirapu I, Alfaro C, Mazzolini G, Palencia B *et al*. (2003). *Clin Cancer Res* **9**: 5454–5464.
Nakanishi K, Sakamoto M, Yasuda J, Takamura M, Fujita N, Tsuruo T *et al*. (2002). *Cancer Res* **62**: 2971–2975.
Narvaiza I, Mazzolini G, Barajas M, Duarte M, Zaratiegui M, Qian C *et al*. (2000). *J Immunol* **164**: 3112–3122.
Nejjari M, Hafdi Z, Gouysse G, Fiorentino M, Bëatrix O, Dumortier J *et al*. (2002). *Hepatology* **36**: 418–426.
Nelson WJ, Nusse R. (2004). *Science* **303**: 1483–1487.
O'Neal WK, Zhou H, Morral N, Langston C, Parks RJ, Graham FL *et al*. (2000). *Mol Med* **6**: 179–195.
Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB. (2000). *Oncogene* **19**: 6043–6052.
Ohwada A, Hirschowitz EA, Crystal RG. (1996). *Hum Gene Ther* **7**: 1567–1576.
Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y *et al*. (2001). *Cancer Res* **61**: 2129–2137.
Osada S, Kanematsu M, Imai H, Goshima S, Suguiyama Y. (2005). *J Am Coll Surg* **201**: 405–411.
Osaki M, Oshimura M, Ito H. (2004). *Apoptosis* **9**: 667–676.
Palmer DH, Mautner V, Mirza D, Oliff S, Gerritsen W, van der Sijp JR *et al*. (2004). *J Clin Oncol* **22**: 1546–1552.
Palmieri G, Montella L, Milo M, Fiore R, Biondi E, Bianco AR *et al*. (2002). *Am J Clin Oncol* **25**: 224–226.
Park W, Lim do H, Paik SW, Koh KC, Choi MS, Park CK *et al*. (2005). *Int J Radiat Oncol Biol Phys* **61**: 1143–1150.

Pawlik TM, Nakamura H, Yoon SS, Mullen JT, Chandrasekhar S, Chiocca EA *et al*. (2000). *Cancer Res* **60**: 2790–2795.
Pei Z, Chu L, Zou W, Zhang Z, Qiu S, Qi R *et al*. (2004). *Hepatology* **39**: 1371–1381.
Penuelas I, Mazzolini G, Boan JF, Sangro B, Marti-Climent J, Ruiz M *et al*. (2005). *Gastroenterology* **128**: 1787–1795.
Philip PA, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G *et al*. (2005). *J Clin Oncol* **23**: 6657–6663.
Pikarski E, Porat RM, Stein h, Abramovitch R, Amit S, Kasem S *et al*. (2004). *Nature* **431**: 461–466.
Pinzani M, Rombutus K, Colagrande S. (2005). *J Hepatol* **42**: S22–S36.
Prieto J, Qian C, Hernandez-Alcoceba R, Gonzalez-Aseguinolaza G, Mazzolini G, Sangro B *et al*. (2004). *Expert Opin Biol Ther* **4**: 1073–1091.
Putzer BM, Stiewe T, Rodicker F, Schildgen O, Ruhm S, Dirsch O *et al*. (2001). *J Natl Cancer Inst* **93**: 472–479.
Qian C, Sangro B, Prieto J. (2002). *Gastroenterology* **123**: 639–642.
Ranges GE, Figari IS, Espevik T, Palladino Jr MA. (1987). *J Exp Med* **166**: 991–998.
Raoul JL, Guyader D, Bretagne JF, Heautot JF, Duvauferrier R, Bourguet P *et al*. (1997). *Hepatology* **26**: 1156–1161.
Raskopf E, Dzienisowicz C, Hilbert T, Rabe C, Leifeld L, Wernert N *et al*. (2005). *Hepatology* **41**: 1233–1240.
Reya T, Clevers H. (2005). *Nature* **434**: 843–850.
Robert C, Soria JC, Spatz A, Le Cesne A, Malka D, Pautier P *et al*. (2005). *Lancet Oncol* **6**: 491–500.
Roberts LR, Gores GJ. (2005). *Semin Liver Dis* **25**: 212–225.
Rumpold H, Wolf D, Koeck R, Gunsilius E. (2004). *J Cell Mol Med* **8**: 509–518.
Rust C, Gores GJ. (2000). *Am J Med* **108**: 567–574.
Ryan AJ, Wedge SR. (2005). *Br J Cancer* **92**(Suppl. 1): S6–S13.
Sahin F, Kannangai R, Adegbola O, Wang J, Su G, Torbenson M. (2004). *Clin Cancer Res* **10**: 8421–8425.
Sangro B, Bilbao I, Herrero I, Corella C, Longo J, Beloqui O *et al*. (1993). *Hepatology* **18**: 309–414.
Sangro B, Herraiz M, Martinez-Gonzalez MA, Bilbao I, Herrero I, Beloqui O *et al*. (1998). *Surgery* **124**: 575–583.
Sangro B, Mazzolini G, Ruiz J, Herraiz M, Quiroga J, Herrero I *et al*. (2004). *J Clin Oncol* **22**: 1389–1397.
Satyanarayana A, Manns MP, Rudolph KL. (2004). *Hepatology* **40**: 276–283.
Sawai Y, Tamura S, Fukui K, Ito N, Imanaka K, Saeki A *et al*. (2003). *J Hepatol* **39**: 991–996.
Schiffer E, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C *et al*. (2005). *Hepatology* **41**: 307–314.
Schmitz V, Barajas M, Wang L, Peng D, Duarte M, Prieto J *et al*. (2001). *Hepatology* **34**: 72–81.
Schmitz V, Wang L, Barajas M, Peng D, Prieto J, Qian C. (2002). *Gene Therapy* **9**: 1600–1606.
Schuppan D, Ocker M. (2003). *Hepatology* **38**: 289–291.
Sell S. (2002). *Semin Cell Dev Biol* **13**: 419–424.
Sgadari C, Angiolillo AL, Tosato G. (1996). *Blood* **87**: 3877–3882.
Shachaf CM, Kopelman A, Arvanitis C, Karlsson A, Beer S, Mandl S *et al*. (2004). *Nature* **431**: 1112–1117.
Sridhar SS, Hedley D, Siu LL. (2005). *Mol Cancer Ther* **4**: 677–685.
Strand S, Hofmann WJ, Hug H, Muller M, Otto G, Strand D *et al*. (1996). *Nat Med* **2**: 1361–1366.
Street A, Macdonald A, McCormick C, Harris M. (2005). *J Virol* **79**: 5006–5016.
Strumberg D, Richly H, Hilger RA, Schleucher N, Korfee S, Tewes M *et al*. (2005). *J Clin Oncol* **23**: 965–972.
Stuart K, Tessitore J, Rudy J, Clendennin N, Johnston A. (1999). *Cancer* **86**: 410–414.
Stupack DG, Cheresh DA. (2004). *Curr Top Dev Biol* **64**: 207–308.
Su M-C, Lien H-C, Jeng Y-M. (2005). *Cancer Lett* **224**: 117–121.
Sundram F, Chau TC, Onkhuudai P, Bernal P, Padhy AK. (2004). *Eur J Nucl Med Mol Imaging* **31**: 250–257.
Sutter AP, Maaser K, Höpfner M, Huether A, Schuppan D, Scherübl H. (2005). *J Hepatol* **43**: 808–816.
Takayama T, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J *et al*. (2000). *Lancet* **356**: 802–807.Erratum in: *Lancet* (2000) **356**; 1690.
Taub R. (2004). *Nat Rev Mol Cell Biol* **5**: 836–847.
Thiery IP. (2003). *Curr Opin Cell Biol* **15**: 740–746.
Thompson N, Lyons J. (2005). *Curr Opin Pharmacol* **5**: 350–356.
Thompson EW, Newgreen DF, Tarin D. (2005). *Cancer Res* **65**: 5991–5995.
Thorgeirsson SS, Grisham JW. (2002). *Nat Genet* **31**: 339–346.
Tirapu I, Arina A, Mazzolini G, Duarte M, Alfaro C, Feijoo E *et al*. (2004). *Int J Cancer* **110**: 51–60.
Tirapu I, Rodriguez-Calvillo M, Qian C, Duarte M, Smerdou C, Palencia B *et al*. (2002). *Curr Gene Ther* **2**: 79–89.
Trinchieri G. (1998). *Adv Immunol* **70**: 83–243.
Valdés F, Alvarez AM, Locascio A, Vega S, Herrera B, Fernández M *et al*. (2002). *Mol Cancer Res* **1**: 68–78.
Vignot S, Faivre S, Aguirre D, Raymond E. (2005). *Ann Oncol* **16**: 525–537.
Von Sengbusch A, Gassmann P, Fisch KM, Enns A, Nicolson GL, Haier J. (2005). *Am J Pathol* **166**: 585–596.
Wallace HM, Fraser AV. (2003). *Biochem Soc Trans* **31**: 393–396.
Wang L, Schmitz V, Perez-Mediavilla A, Izal I, Prieto J, Qian C. (2003). *Mol Ther* **8**: 72–79.
Warren RS, Kirn DH. (2002). *Surg Oncol Clin N Am* **11**: 571–588.
Wiessenauer CA, Yip-Schneider MT, Wang Y, Schmidt M. (2004). *J Am Coll Surg* **198**: 410–421.
Wirth T, Zender L, Schulte B, Mundt B, Plentz R, Rudolph KL *et al*. (2003). *Cancer Res* **63**: 3181–3188.
Wong CM, Fan ST, Ng IO. (2001). *Cancer* **92**: 136–145.
Xu GW, Sun ZT, Forrester K, Wang XW, Coursen J, Harris CC. (1996). *Hepatology* **24**: 1264–1268.
Xu Z, Ma D, Wang L, Su J, Zha X. (2003). *Biochem Biophys Res Commun* **312**: 388–396.
Yang C, Zeisberg M, Lively JC, Nyberg P, Afdhal N, Kalluri R. (2003). *Cancer Res* **63**: 8312–8317.
Yarden Y. (2001). *Eur J Cancer* **37**: S3–S8.
Yau CYF, Wheeler JJ, Sutton KL, Hedley DW. (2005). *Cancer Res* **65**: 1497–1504.
You L, He B, Xu Z, Uematsu K, Mazieres J, Fujii N *et al*. (2004). *Cancer Res* **64**: 5385–5389.
Zacharoulis D, Hatzitheofilou C, Athanasiou E, Zacharoulis S. (2005). *Expert Rev Anticancer Ther* **5**: 645–656.
Zavadil J, Böttinger EP. (2005). *Oncogene* **24**: 5764–5774.
Zender L, Kock R, Eckhard M, Frericks B, Gosling T, Gebhardt T *et al*. (2002). *Gastroenterology* **123**: 608–618.
Zhang H, Ozaki I, Mizuta T, Matsuhashi S, Yoshimura T, Hisatomi A *et al*. (2002). *Cancer* **95**: 896–906.
Zhang H, Ozaki I, Mizuta T, Yoshimura T, Matsuhashi S, Eguchi Y *et al*. (2004). *Cancer Sci* **95**: 878–886.
Zhang H, Ozaki I, Mizuta T, Yoshimura T, Matsuhasi S, Hisatomi A *et al*. (2003). *Hepatology* **38**: 305–313.
Zhang Y, Xiong Y, Yarbrough WG. (1998). *Cell* **92**: 725–734.
Zou W, Luo C, Zhang Z, Liu J, Gu J, Pei Z *et al*. (2004). *Oncogene* **23**: 457–464.