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Review

Biomarkers for novel targeted therapies of hepatocellular carcinoma

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Summary. Increasing insights into molecular alterations of signalling pathways have led to the development of specific targeted therapies for cancer. Due to the high specificity of monoclonal antibodies or small molecule inhibitors, identification of patients who will benefit from these therapeutics is crucial for treatment success. Furthermore, as classical endpoints of clinical trials are not fully applicable to targeted therapies, biomarkers for monitoring treatment response have to be identified.

The recent introduction of a multi-kinase inhibitor for the treatment of liver cancer has accelerated efforts in the field of biomarker research. As further novel targeted therapies are on the horizon for liver cancer therapy, we will here review candidate markers for new hepatocellular carcinoma therapies, with a focus on EGF- and VEGF-receptor related pathways.

Key words: Hepatocellular carcinoma, Targeted therapy, EGFR, VEGFR, Kinase inhibitors

Introduction

Primary liver cancer (hepatocellular carcinoma, HCC) belongs to the most common tumor diseases worldwide, with highly increasing incidences in industrialized nations (Llovet et al., 2003; Bosch et al., 2005). HCC usually develops on the basis of a chronic inflammation with subsequent cirrhosis, e.g. due to chronic viral hepatitis, alcohol intoxication or metabolic diseases, and curative treatment options are still limited with an unsatisfactory overall survival rate (Colombo, 1993; Omata et al., 2004).

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Therapeutic options in the treatment of HCC

Overall, differential treatment algorithms, according to the Barcelona Clinic Liver Cancer classification (Fig. 1), have been developed based on histological and morphological criteria, as well as on severity of the underlying liver disease and the performance status of the patient (Llovet et al., 2003). In short, treatment with curative intention includes liver resection, liver transplantation and percutaneous ethanol injection, as well as radiofrequency ablation. The palliative therapy of HCC still needs to be established in randomised controlled trials comparing transarterial chemotherapy and systemic chemotherapy, as well as targeted therapies (discussed in detail below). Although dramatic progress on survival is achieved through enhanced surveillance programmes, supportive care is the only therapy in about 20% of patients with end stage HCC (Llovet et al., 1999; Bruix et al., 2004). This performance could possibly be enhanced by finding better biomarkers for patient stratification and therapy response prediction.

Biomarkers

During the past decades, significant progress in cancer medicine has been achieved which has led to an overall prolongation of survival and improvement of quality of life. The increasing understanding of molecular alterations involved in tumor initiation and progression paved the way for the development of highly specific targeted therapies. This development also raised the need for markers that are (i) suitable to identify patients who will benefit from new therapeutics and that (ii) monitor treatment response and efficacy, esp. at an early stage when putative overall survival or disease progression can not be assessed with conventional diagnostic tools (Frank and Hargreaves, 2003). Therefore, three different classes of biological markers

were proposed by the NIH (Atkinson et al., 2001): 1) *Biological markers (biomarkers)* are characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes or pharmacologic responses to therapeutic interventions. 2) *Clinical endpoint* is a characteristic or variable that reflects how a patient feels, functions or survives. 3) Surrogate endpoints are expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence.

This indicates that valid new biomarkers are more complex than currently used tumor markers, as they should not only allow the monitoring of treatment response (like CEA or AFP levels) but should also provide a means to predict early and overall treatment response and benefit, preferably before treatment initiation (Bakhtiar, 2008).

Molecular pathways in HCC

The following factors are known to be essentially involved in etio-pathogenesis of HCCs (Blum, 2005): Chronic viral hepatitis (HBV, HCV), toxins such as alcohol or aflatoxins, hereditary metabolic liver disease such as hemochromatosis or α_1-antitrypsin deficiency and autoimmune hepatitis, as well as non-alcoholic steatohepatitis or non-alcoholic fatty liver disease. Based on the detailed knowledge about the underlying pathophysiologic processes, a multi-step model of HCC carcinogenesis has been proposed (see figure 2), involving changes in growth factor expression and genetic alterations from an early inflammatory stage via dysplasia to metastatic HCC (El-Serag and Rudolph, 2007; Mann et al., 2007; Saffroy et al., 2007). Commonly found genetic alterations are mutations in p53 (Bourdon et al., 1995; Lee et al., 2004), ß-catenin

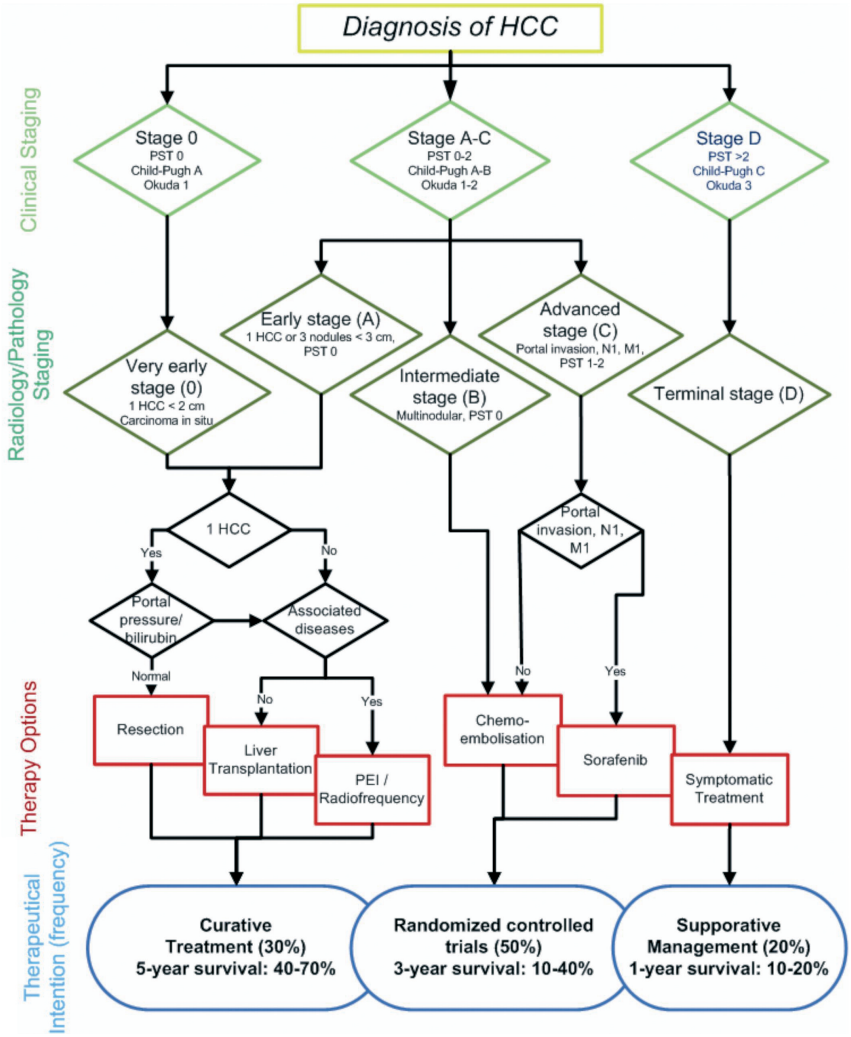

Fig. 1. Differential treatment algorithms modified according the Barcelona Clinic Liver Cancer classification (Llovet et al., 2003)

(de La Coste et al., 1998; Fujito et al., 2004), Rb (Naka et al., 1998), p16 (Chen et al., 2007),TGF-ß (Fischer et al., 2007) and other oncogenes like c-myc, ras and the ErbB family of growth factor receptor tyrosine kinases (Laurent-Puig et al., 2001; Schoniger-Hekele et al., 2005; Laurent-Puig and Zucman-Rossi, 2006; Pang et al., 2006). Yet, most of these factors (esp. p53) could not provide a significant role in prognosis or treatment outcome in HCC patients (Mann et al., 2007).

Although some clinical studies used "specific" drug targets against some of these proteins involved in the molecular pathway of HCCs, the results of the studies showed no promising effects overall (Kern et al., 2007). For example, a specific antibody against c-kit/PDGF-receptor had no effect on progressive HCCs (Lin et al., 2008). COX-2 inhibition by Celocoxib, as well as application of Thalidomid, were of no evidence on regression or progression of HCCs (Kondo et al., 1999; Chuah et al., 2007). Clinical trials applying drugs against IGF-2 or EPCAM are not currently started in patients with HCC.

Nevertheless, the development of small molecular inhibitors (e.g. gefitinib, sunitinib) and monoclonal antibodies against growth factor receptors (e.g. cetuximab) or their ligands (e.g. bevacizumab) has fostered the research in pathways related to ErbB or angiogenesis in HCC (Roberts and Gores, 2005; Pang and Poon, 2007).

EGFR signalling

The Epidermal Growth Factor Receptor (EGFR, also designated as ErbB1 or Her1) is an about 170 kd transmembrane receptor tyrosine kinase which is expressed on almost all cell types without hemopoietic cells (Carpenter, 1987). EGFR transduces signals from

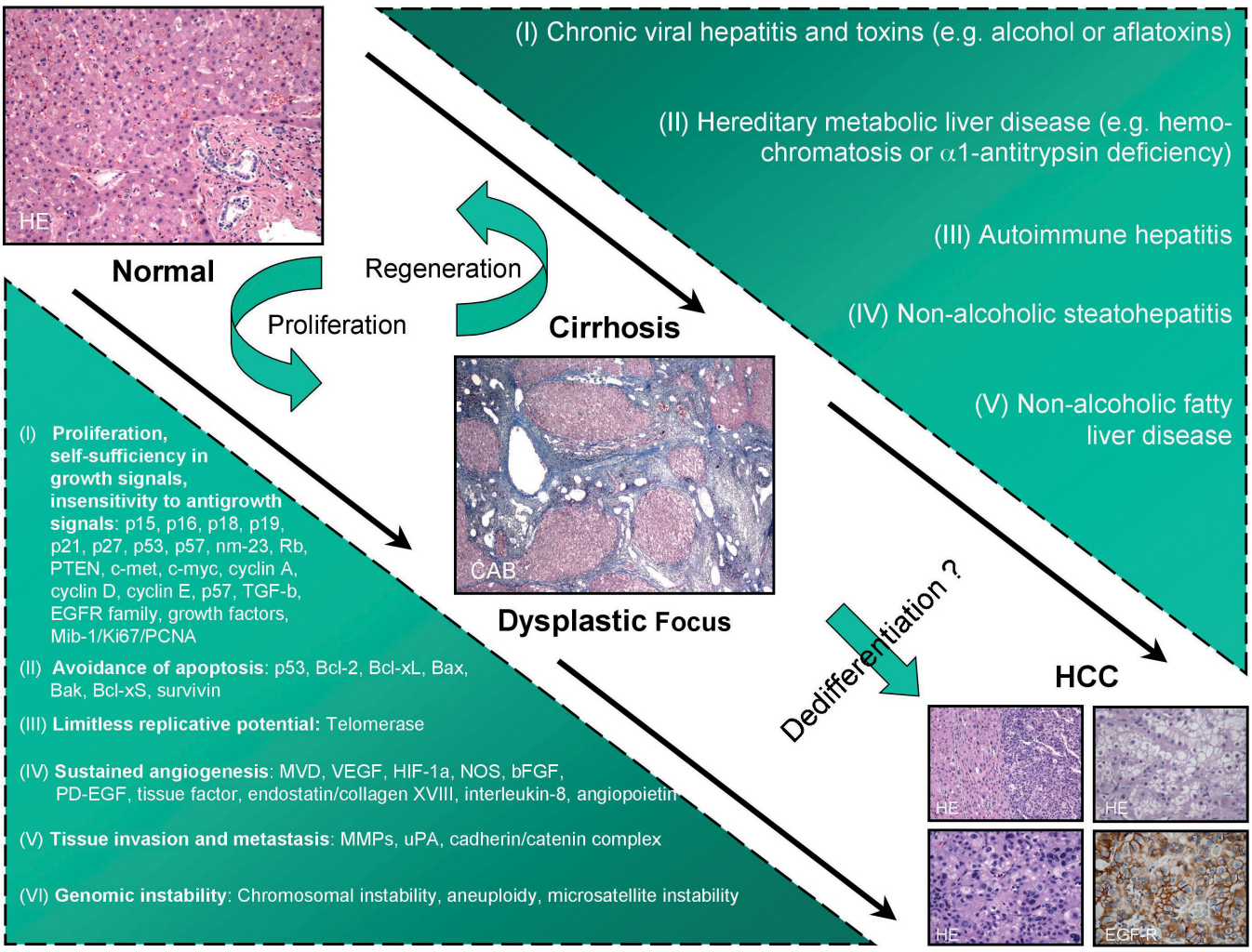

Fig. 2. Multi-step model of HCC carcinogenesis being influenced by different etiologies, as well as by different molecular events inside "hallmarks of cancer" (Hanahan and Weinberg, 2000).

extracellular ligands, such as epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), heparin-binding EGF like growth factor or betacellulin, to various intracellular signalling pathways (Fig. 3). Among the ligands, TGF-α and EGF are frequently found co-expressed with EGFR in various cancers, and they are considered to act in an autocrine/paracrine manner, leading to dysregulated EGFR activation (Zandi et al., 2007). The mRNA levels of TGF-α and EGF in HCC tissue relate to the prognosis of HCC patients (Daveau et al., 2003). It is relatively difficult to detect changes in the micro-environmental autocrine/paracrine status by whole body serum analysis and there are no former reports which showed serum levels of EGF or TGF-α in HCC patients. EGFR is activated by homo- or hetero-dimerisation with other ErbB family members ErbB2, ErbB3 and ErbB4 (Zandi et al., 2007). The dimer combination depends on which ligand interacts with EGFR and the combinations affect the function. The lifetime of dimers in the membrane depends on the ligand binding itself and the combination of EGFR-ErbB (Hynes et al., 2001). For example, no ligands for ErbB2 have been found and it is therefore only recognized as a co-receptor. EGFR-ErbB2 heterodimers appear to be the strongest inducers of cellular transformation and mitogenic signalling compared to other ErbB homo- and heterodimers. In addition, ErbB2 induces a strong ligand-independent activation of EGFR and the EGFR-ErbB2 heterodimer escapes the downregulation procedure by endocytosis, resulting in ligand-independent and continuous signal transmission to down-stream targets (Worthylake et al., 1999; Haslekas et al., 2005). The homo- and hetero EGFR dimers then induce multi-tyrosine auto-phosphorylation, which has different phosphorylating site patterns between the binding ligands (Hynes et al., 2001). However, the dimerisation can also be induced ligand independently (Jorissen et al., 2003; Pedersen et al., 2004). The auto-phosphorylated EGFR dimer sequentially activates many intracellular signalling pathways, including ras/raf/MEK/ERK1/2 (classical MAPK), PI-3K/Akt, PLC, signal transducer and activator of transcription (STAT) and c-SRC, which affect many aspects of cell functions, such as cell proliferation, differentiation and motility (Dehm and Bonham, 2004; Shien et al., 2004; Camp et al., 2005).

For example, activated EGFR stimulates Ras/MAPK pathways through adaptor proteins Grb2 or Shc (Marais and Marshall, 1996; Waters et al., 1996). Activation of the classical MAPK signal way accelerates cell proliferation, resistance to apoptosis, angiogenesis, extracellular matrix remodelling and cellular motility through activating the transcription factors in the nucleus (Wiesenauer et al., 2004; Sridhar et al., 2005).

PI-3K is activated in EGFR c-terminal docking site which contains Tyr 920 directly or indirectly through

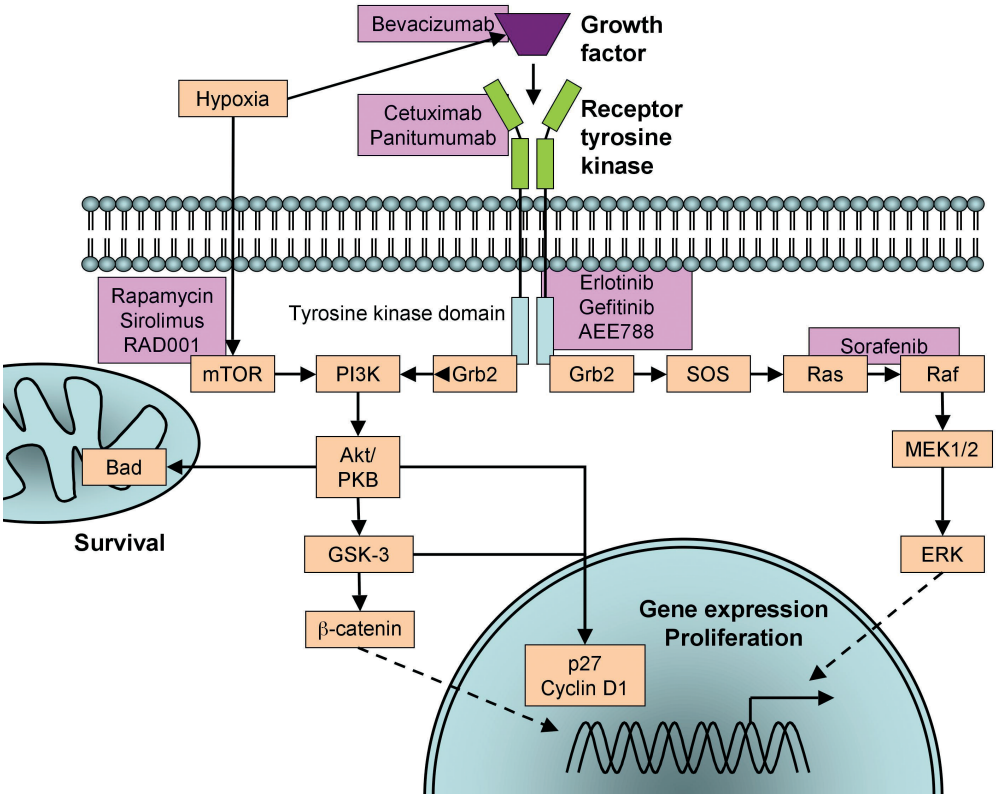

Fig. 3. Signal transduction pathways of growth factor receptor tyrosine kinases, therapeutic inhibitors and potential biomarkers. Growth factors (e.g. EGF or VEGF) bind to their cognate receptor and activate the intrinsic tyrosine kinase domain. Via several small kinase proteins, signals are transmitted to the nucleus to mediate changes in gene expression and proliferation. In parallel, pro-apoptotic pathways are inhibited. Shown are potential biomarkers (yellow boxes) as well as clinically applied inhibitors of the respective pathways or molecules (pink boxes).

binding to Grb2 (Stover et al., 1995). The PI-3K/Akt pathway also transduces the survival and proliferation signals to diverse transcriptional factors in the nucleus. Activated Akt inhibits proapoptotic factors, like Bad and caspase 9, by direct protein phosphorylation (Cardone et al., 1998; Osaki et al., 2004).

The down regulation for EGFR signal pathway is mediated by dephosphorylation by phosphothyrosine phosphatase and receptor internalization (endocytosis). Among them, receptor internalization, which is followed by EGFR resolving in the lysosomes, is a robust post transcriptional regulation system. Activated EGFR is internalized through its ubiquitination by Cbl, which has E3 ubiquitin ligase activity and contains an amino-terminal phosphotyrosine binding (PTB) domain and a C3HC4 RING finger. In cytosol, internalized EGFR are sorted, and a part of them are recycled into plasma membrane, but the others are brought to lysosome for destruction (Katzmann et al., 2002; Marmor and Yarden, 2004).

Because EGFR plays a critical role in cell proliferation and survival, dysregulation of EGFR signaling pathway deeply relates to malignant neoplasm. In fact, the over-expression of EGFR and/or its ligands were observed in various carcinomas including liver, head and neck, esophageal, colorectal, pancreas, lung, breast, kidney, bladder, prostate, ovarian, and glioblastoma, and it has been shown to correlate with metastasis, apoptosis, resistance to chemotherapy and poor prognosis (Cai et al., 1999; Umekita et al., 2000; Ritter and Arteaga, 2003; Normanno et al., 2006). Furthermore, the over expression of EGFR accelerates the spontaneous dimerization following the ligand independent activation (Pedersen et al., 2004).

Some reports suggest that mutations and polymorphisms of EGFR gene correlate with the over expression of EGFR in various cancers. Some mutations of EGFR structure by point mutations, exon deletions and frame shifts result in constitutive activation of EGFR (Gebhardt et al., 1999; Willmore-Payne et al., 2006; Moutinho et al., 2008). However, Lee et al. reported that they could find neither EGFR nor ErbB2 exonic gene mutations in Asian HCC patients (Lee et al., 2006; Wong et al., 2008), while non-coding regions of the EGFR gene also affect the expression by altering the transcriptional level. For example, the p53 protein has a binding site in EGFR promoter region (Sheikh et al., 1997). Notably, the level of mutant p53 proteins is usually high in HCC (Bourdon et al., 1995; Lee et al., 2004), and it may lead to upregulation of the EGFR promoter, resulting in more EGFR expression. Furthermore, the gene polymorphism of the number of CA dinucleotide repeats in intron 1 in the EGFR gene relates to EGFR expression level and the transcriptional activity of the EGFR is in inverse proportion to the numbers of CA repeats (Amador et al., 2004; Buerger et al., 2004).

On the other hand, considerable post transcriptional modifications also strongly affect EGFR expression levels. EGFR over-expression is observed without EGFR gene mutations or increased mRNA levels (Buckley et al., 2008). Some reports reveal that escaping mechanisms of EGFR receptor down regulation, such as endocytosis inhibition, exist and promote EGFR recycling in cancer cells (Johnston et al., 1999; Worthylake et al., 1999). These EGFR expression control mechanisms in HCC may be affected by personal life styles, e.g. common food components like coffee consumption (Larsson and Wolk, 2007; Ohishi et al., 2008; Okano et al., 2008). Some unknown cross-talking systems to EGFR signaling pathways have thus been suggested that contribute to the wide-variation of EGFR expression levels in similarly differentiated HCC tissues (Ito et al., 2001; Buckley et al., 2008).

Cell signaling pathways construct complex cross-talking systems and communicate or interfere with each other. For EGFR, many cross talking effects which influence the tumor progression and sensitivity for targeted molecular therapies have been described (Eliceiri, 2001; Piedra et al., 2001; Ciardiello et al., 2003; Jones et al., 2006; Bhola and Grandis, 2008). For example, EGFR down regulation by EGFR specific RTKi leads to VEGFR over-expression, which leads to resistance against this treatment in colon cancer cells (Ciardiello et al., 2003). Furthermore, the Insulin like growth factor 1 receptor (IGF-1R) can trans-activate the EGFR signaling pathway and has been shown to be one of the main mechanisms to promote resistance to anti-EGFR treatment (Jones et al., 2006).

VEGFR signalling

Angiogenesis is another indispensable target for cancer therapy. Most cancers produce some angiogenic factors to resolve intra-tumor hypoxia and nutrient deficiency (Ferrara and Davis-Smyth, 1997; Liekens et al., 2001). Among many of these factors, VEGF is one of the most potent angiogenic cytokines, which mainly binds to two distinct receptors on endothelial cells, fms-like tyrosine kinase 1 (flt-1: VEGFR-1) and fetal liver kinase 1 (flk-1 / KDR: VEGFR-2). VEGFR-1 has higher affinity for VEGF, but former reports showed that VEGFR-2 plays a major role as a VEGF signal mediator (Kroll and Waltenberger, 1997; Kanno et al., 2000). VEGF promotes proliferation, migration and tube formation of endothelial cells. It also induces expression of bcl-2 in endothelial cells and protects these cells from apoptosis and stimulates extracellular matrix (ECM) degradation (Wang and Keiser, 1998; Liekens et al., 2001). Activation of VEGFRs does not only accelerate the tumor vascularization but also has a direct effect on growth and invasion of tumor cells. These functions of VEGF relate to aggressive tumor growth, metastatic potential and poorer prognosis of substantial carcinomas (Ruggeri et al., 2003; Amaoka et al., 2006). VEGFR transduces the signal through many intracellular pathways, and MAPK pathway is one of the main signal ways to induce angiogenesis (Doanes et al., 1999;

Schlessinger, 2000; Meadows et al., 2004).

Gene polymorphisms of VEGFRs have been analyzed but no significant relationship between VEGFR-1 and VEGFR-2 and cancer development has been shown so far (Menendez et al., 2006; Forsti et al., 2007).

Different from EGFR ligands, some reports suggested that serum VEGF level is a significant independent predictor of tumor recurrence, disease-free survival and overall survival in HCC patients (Chao et al., 2003; Poon et al., 2004). This fact may reflect the difference of the role in HCC development mechanism between EGFR and VEGFR, and also reflects the different biologic and morphologic features of HCC: although it is an epithelial tumor (thus depending on EGFR signaling), the hypervascularised (VEGF-driven) phenotype is predominant. However, there are still some controversial issues about serum VEGF assays and a large diversity of VEGF values between individuals has been observed. In addition, some other reports showed no relationships between the serum VEGF level and high response rate to anti-VEGF agents (Bertolini et al., 2007; Golshayan et al., 2008). Although serum VEGF may be a relatively simple biomarker for predicting HCC prognosis, further large-scale and well-designed studies are required to clarify its availability as a prediction factor for anti-VEGF therapies.

The inhibition of both EGFR and VEGFR might be favorable to prevent resistance development in HCC, and several preclinical and clinical trials have been investigating this approach.

Targeted therapy in HCC

Based on this knowledge, several novel targeted therapy approaches, using small molecule inhibitors of receptor function of monoclonal antibodies against receptors or ligands, have been applied to HCC in clinical trials (Fig. 3).

Different anti-EGFR and anti-VEGFR agents have been applied to HCC models in vitro and several phase I - III trials are currently ongoing (Hopfner et al., 2008), and some encouraging results have been obtained in early clinical trials for single agents like erlotinib (Philip et al., 2005; Zhu et al., 2007; Zhu, 2008) or bevacizumab (Schwartz et al., 2006). Recently, the multi-kinase inhibitor sorafenib has shown good efficacy and tolerability in patients with advanced HCC as a single agent, and has received approval as the first-line therapy for HCC in Europe and the US (Abou-Alfa et al., 2006; Furuse et al., 2008; Llovet et al., 2008). While sorafenib prolongs time to progression and overall survival compared to placebo, it is still unclear which patients benefit most from this treatment. So far, it is only safe to say that patients with elevated transaminases or low Child B cirrhosis show the same response as patients with regular liver function (Bolondi et al., 2008; Cabrera et al., 2008; Greten et al., 2008), although a marked increase in side effects has to be noted in these patients. From these studies, it is known that the pre-treatment phosphorylation levels of ERK correlate with time to progression, and a panel of 18 genes was identified that might distinguish responders from non-responders to sorafenib treatment (Abou-Alfa et al., 2006). However, these genes have not been validated in larger studies and can therefore not be used as biomarkers for HCC.

Experiences with receptor targeting therapies in other cancer diseases have shown the urgent need for predictive biomarkers. Especially, for the use of monoclonal antibodies like cetuximab, panitumumab or herceptin it is essential to determine activating mutations of downstream signalling molecules (e.g. ras) that would render the upstream inhibition of ligand binding ineffective or mediate resistance to this treatment (Amado et al., 2008; Wheeler et al., 2008). Furthermore, differences between expression levels as determined by immunohistochemistry and gene copy numbers (by fluorescence in situ hybridization) have to be considered (Eberhard et al., 2008). As a surrogate endpoint, rash has recently been defined as a response marker for anti-EGFR therapies (Bianchini et al., 2008). As these markers have to be determined from tissues, which is sometimes difficult to obtain repeatedly in patients with advanced HCC in cirrhotic livers, serum markers are preferred.

We recently reported about the effect of the dual receptor tyrosine kinase inhibitor AEE788 in preclinical models of HCC. Here, a marked suppression of HCC growth was observed, mediated by inhibition of tumor and endothelial cell proliferation with reduced microvessel density in a mouse model (Okamoto et al., 2008). Notably, responsive animals developed an acne-like skin reaction which correlated with the extent of tumor growth inhibition as was observed for other anti-EGFR therapies.

Therapy responses in oncology have usually been measured with standardized parameters, such as radiologic tumor assessment (Schima et al., 2007; Julka et al., 2008). However, several clinical trials with targeted therapies against various solid tumors have shown that radiologic size assessment may not be sufficient to measure response rates, and that novel markers are needed (Curran et al., 2006; Hahn and Stadler, 2006). For anti-angiogenic therapies, reduction of microvessel density (MVD) has been broadly used as a surrogate endpoint (Pang and Poon, 2006). Yet, with various different markers available (CD34, CD105, vWF, VEGFR, etc.), results cannot be compared and still need to be standardized for clinical trials (Li et al., 2006; Yao et al., 2007; Nico et al., 2008). As already described, serum markers of angiogenesis (e.g. sVEGF) are more easily accessible for repeated measurements, although these markers are not yet suitable as predictive markers for cancer treatment (Bertolini et al., 2007; Golshayan et al., 2008). Recently, circulating endothelial cells and endothelial progenitor cells have also been proposed as sensitive biomarkers for measuring response rates of anti-angiogenesis therapies (Bertolini et al., 2005, 2006;

Shaked et al., 2006). In breast cancer, these markers have been shown to be predictive for overall survival and need now to be verified in HCC (Mancuso et al., 2006).

Summary and conclusion

In the past years, substantial progress has been made in identifying molecular changes (genetic alterations and epigenetic events) during HCC development, which has improved diagnosis of HCC and led to the development of novel targeted therapies. Despite this increasing knowledge, only a few markers have been identified so far that fulfil the criteria to be used as biomarkers. However, these markers have not yet been validated in larger cohorts of HCC patients. Future efforts are therefore needed to determine these and novel array based data (genomic, proteomic, miRNA) to identify prognostic and predictive biomarkers for HCC. With new agents being brought to clinical development (e.g. inhibitors of histone deacetylases or the mTOR/PI3K/Akt signalling pathway), validated biomarkers for HCC are urgently needed.

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