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Growth factor pathways in development and progression of hepatocellular carcinoma

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1. ABSTRACT

Hepatocellular carcinoma accounts for approximately 700,000 deaths per year. This tumor displays morphologic and phenotypic heterogeneity, and heterogeneity extends to the molecular level. Nevertheless, common pathways have been identified that are variably employed by these tumors. Such pathways often include

aberrant signaling by growth factors, many of which are involved in liver development and regeneration. This review focuses on several such pathways and highlights patterns of structural expression of relevant molecules as well as effects of pathway stimulation or inhibition, both *in vitro* and *in vivo*. Specifically, the HGF/MET axis, epidermal growth factor receptors and associated ligands, insulin growth factor, vascular endothelial growth factor, fibroblast growth factor, platelet-derived growth factor and TGF-beta pathways are reviewed in the context of experimental models of HCC. Clinical-pathologic correlations are drawn for each of these, and current status of molecular targeted therapies is assessed. Review of available information indicates that redundancies and interactions among these signaling pathways must be taken into account if they are to be exploited to block and reverse HCC growth and spread.

2. INTRODUCTION

Liver cancer is the fifth most common form of malignancy and the second most frequent cause of cancer death worldwide, with 748,300 new cases and 695,900 deaths estimated for the year 2008(1). Hepatocellular carcinoma (HCC) represents 70-85% of these cases, with varying proportions associated with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, aflatoxin exposure, alcohol, or obesity related to geographic locale. Although vaccination against HBV has had some preventive effect, the absence of an effective vaccine against HCV as well as the epidemic of obesity in many developed countries makes it likely that the rising incidence of HCC will continue for some time(1).

The molecular pathogenesis of HCC is multifactorial and may even be heterogeneous among multiple tumors in the same patient. This complexity, combined with treatment intolerance due to frequent concomitant cirrhosis, may to some extent underlie the historically poor response of this tumor to conventional chemotherapy. More recently, the use of molecular agents directed against pathways active in regenerating and/or neoplastic liver has revitalized the outlook for these patients and led to an explosive increase in the number of clinical trials for HCC. In almost all cases, the drugs target one or more molecules involved in growth factor-associated signals that mediate tumor cell proliferation, protection from apoptosis or anoikis, epithelial-mesenchymal transition, or angiogenesis. We will briefly review some of these pathways and highlight clinicopathologic and therapeutic correlations specifically relevant to HCC.

3. SPECIFIC GROWTH FACTORS AND THEIR PATHWAYS

3.1. The hepatocyte growth factor (HGF)/MET axis

3.1.1. Background

Hepatocyte growth factor (HGF, scatter factor) is generated as a single chain precursor protein and is converted into its active heterodimeric form by proteolytic cleavage, particularly by HGF activator (HGFA), a factor XII-like serine protease. The resultant 85 kDa heterodimer is comprised of an alpha-chain (containing the N-terminal hairpin loop domain and four kringle domains) and beta-chain (containing the serine protease homology domain). Within the liver, HGF is normally produced by endothelial and stellate cells, with transcription upregulated by a number of molecules associated with inflammatory or neoplastic states, including IL1, IL6, tumor necrosis factor-alpha (TNF-alpha), TGF-beta, and VEGF. Norepinephrine is also a strong inducer of HGF expression in fibroblasts (2).

MET is the HGF receptor and consists of a heterodimer comprised of an extracellular -alpha-chain and transmembrane beta-chain. HGF has a constitutively active high affinity binding site for MET in the HGF -alpha-chain, and a low affinity binding site in the beta-chain that becomes functional following HGF activation. Association with -alpha6-beta4 integrin occurs and provides additional signal amplification. The hyaluronic acid receptor CD44 (variant 6, v6) forms a complex with HGF and MET, and is required for MET autophosphorylation (3, 4). HGF: MET complexes dimerize with phosphorylation of intracytoplasmic tyrosine residues that act as docking sites for proteins such as growth factor receptor (GRB)-associated binding protein 1 (GAB1), an adapter that subserves signal amplification by providing additional docking sites for v-crk sarcoma virus CT10 oncogene homolog-like protein (CrkL), p120 ras-GTPase activating protein (p120-Ras-GAP), phospholipase C gamma 1(PLC-gamma1), phosphoinositide 3-kinase (PI3K), signal transducer and activator of transcription 3 (STAT3) and SH2-containing protein-tyrosine phosphatase (SHP2). Activated MET also directly binds PI3K, v-src sarcoma viral oncogene homolog (Src), growth factor receptor-bound protein 2 (GRB2), STAT3, SHP2, SH2 domain-containing transforming protein adaptors (SHC), and PLC-gamma1.

GRB2 binding leads to rat sarcoma protein (Ras) phosphorylation via son of sevenless (SOS) activation. This initiates the raf/MEK/ERK/MAPK pathway (v-raf-1 murine leukemia viral oncogene homolog 1/mitogen-activated protein kinase kinase/mitogen-activated protein kinase) and stimulates PI3K with subsequent c-JUN N-terminal kinase (JNK) and p38 activation via MEK subtypes. The latter leads to, among other things, cyclooxygenase-2 (Cox-2) upregulation. PI3k activation also results in mammalian target of rapamycin (mTOR) activation via the Akt pathway, in addition to I kappa B kinase (IKK) activation, the latter leading to nuclear translocation of nuclear factor kappa light chain enhancer of activated B cells (NF-kappa B). Akt also separately blocks glycogen synthase kinase 3 beta (GSK3-beta), bcl-2-associated death promoter (BAD) and p53 and stimulates p21 protein-activated kinase 1(PAK1) which, together with Cox-2, inhibits apoptosis and anoikis. STAT3 is phosphorylated directly by MET, subsequently serving as a transcription factor (5). CrkL phosphorylation ultimately affects paxillin and focal adhesion kinase (FAK), which influence cell motility.

This represents only a brief overview and omits many details of the HGF: MET pathway, which are discussed in a recent review by Trusolino *et al* (5).

Inhibitors of HGFA, named HGF activator inhibitor (HAI)-1 and -2, exist as type I transmembrane serine proteinase inhibitors and further modulate activity of HGF(6) and proteins such as Sprouty2 (Spry2) can act as MET inhibitors(7).

3.1.2. HGF and MET in hepatocellular carcinoma (HCC)

HGF is typically but not universally detectable within HCC. Kiss *et al*.(8) found it over-expressed in 33% and downregulated in 21% of their tumor samples and a separate study found greater HGF mRNA levels in the peritumoral tissue relative to the tumor itself(9). Since HGF is normally secreted as an inactive precursor, its presence per se does not guarantee pathway activity. However Tung *et al*. (10) found that HAI-2, an inhibitor of HGF activator, was decreased in HCC tissue samples, as well as in a majority of HCC cell lines, due to promoter hypermethylation, thereby compatible with an environment favoring HGF activation. *In vitro* demethylation of this inhibitor led to reduced cell migration and invasiveness, further suggesting its role as a tumor suppressor gene in this setting. HGF mutations have been described in other settings (11, 12), but have not been shown to play a role in spontaneous HCC development.

MET is over-expressed in 44-70% of HCC samples relative to surrounding liver (13, 14). This may be related to ligand stimulation, most likely from HGF, but the possibility of additional stimuli such as EGF receptor crosstalk (15) or interaction with class B plexins should also be considered. Indeed, Breuhahn *et al*. (16) concluded that HGF itself was of minor significance in this regard.

The MET gene is located at 7q31.2 and although gains in 7q have been documented in a minority of HCC (17), we have not seen gene amplification as a significant source of increased MET in clinical samples (unpublished data).

MET expression may possibly be influenced by the underlying disease leading to cirrhosis. Xie *et al*(18) examined the relationship of hepatitis B virus x (HBx) protein to MET using the high MET expressing HCC cell line MHCC97-H and suggested that HBx promotes expression of MET via the ERK pathway, although the precise mechanism of this interaction was unclear.

Mutation of MET has been associated with accelerated (i.e., pediatric) onset of HCC in the setting of hepatitis B virus infection (19), but does not appear to be a significant contributor to hepatic tumorigenesis in adults.

3.1.3. HGF/MET pathway in HCC

Various components of the HGF/MET signal transduction system have been suggested as contributors to tumor promotion in some cases. Lee *et al* (7) found that the MET signal inhibitor Sprouty (Spry2) was down-regulated in conjunction with MET activation in a subset of HCC. Further, blocking this protein in a murine model of HCC facilitated MET signaling and MAPK activation, leading to enhanced hepatocarcinogenesis. MicroRNA-199a-3p, which was also shown to have a suppressive effect on MET as well as mTOR signaling *in vitro*, has been shown to be downregulated in HCC specimens(20, 21), consistent with a role as a tumor inhibitory agent.

The HGF/MET axis modulates cell mitogenesis, morphogenesis, and motogenesis and would be expected to affect the qualities of tumor growth and invasiveness (22). Nagai *et al* (23) showed that HGF-associated *in vitro* changes resembling epithelial-mesenchymal transition were associated with upregulation of N-cadherin and the transcription factor SNAI1. Wang *et al* (24) demonstrated in an experimental rat model of HCC that metastatic disease could be diminished by treatment with estrogen, and this was associated with decreased levels of tumoral IL6 and HGF.

3.1.4. HGF/MET clinicopathologic correlations

Although occasional studies have not found a correlation between HGF/MET expression and clinicopathologic features of HCC (25), others have found that activity of this signaling pathway is associated with more aggressive tumors and poor patient outcome. Daveau *et al* (26) found increased mRNA for both MET and EGFR in poorly differentiated HCC with a tendency for early recurrence and Ueki *et al* (27) found an association between MET expression and tumor size, along with an inverse correlation to survival. Interestingly, they did not find similar correlations for HGF. However, a more recent study showed a statistically significant inverse association between HGF level and survival(28), and a similar correlation was found in the large Phase III sorafenib HCC assessment randomized protocol (SHARP) Trial examining the therapeutic efficacy of this drug (unpublished observations).

HGF is also currently being investigated as a potential biomarker for HCC. Liu *et al* (29) found fucosylated HGF to represent one of several marker candidates for diagnosing early HCC. Larger studies are needed, however, since HGF has also been found to be a specific marker of liver cirrhosis (30).

The experimental compound SU11274 has been modified by radiomethylation to serve as a positron emission tomography (PET) visualization agent to localize MET (31). This is currently used experimentally in the xenograft model to evaluate drug efficacy, but raises the possibility of future use of specific radiographic molecular markers to help guide therapy.

3.1.5. HGF/MET Therapeutic Correlations

Several agents capable of interfering with the HGF/MET pathway are undergoing investigation at this time. SU11274 acts as a MET tyrosine kinase inhibitor and has been shown to decrease tumor cell proliferation and migration in an experimental system utilizing MET-positive melanoma (32). Of note, MET showed constitutive

activity in the tumor despite absence of HGF ligation or observable mutations.

ARQ197 (ArQule, Woburn MA) is a MET tyrosine kinase inhibitor (TKI) (33) that has shown activity (combined with erlotinib) with increased progression-free survival in a phase 2 study of patients with non-small cell lung cancer. A phase I study of this drug in patients with HCC and cirrhosis is active at this time (NCT00802555).

INC280 (INCB028060) (Novartis Oncology licensed from Incyte) is a selective MET TKI with a Phase I trial for patient with solid tumors, including MET-dependent HCC, in the early stages (NCT01324479).

Foretinib (GSK1363089, GlaxoSmithKine) is a small molecule orally available TKI of both MET and VEGF (34) that is currently undergoing Phase I/II testing for advanced hepatocellular carcinoma (NCT00920192). Since HGF and VEGF cooperate to facilitate angiogenesis under hypoxic conditions (35, 36), this dual targeting effect may in theory provide an enhanced anti-tumor effect.

Cabozantinib (XL184, Exelixis, San Francisco, CA) is an oral small molecule multi-TKI that targets c-met, VEGFR-2, v-kit feline sarcoma viral oncogene homolog protein (c-kit), rearranged during transfection (RET), AXL receptor tyrosine kinase (AXL), fms-related tyrosine kinase 3 (FLT3) and Tie family angiopoietin 1 receptor (Tie-2). It has shown activity against HCC, among other tumors, and may have activity against bone metastases, based on company preclinical and early clinical data (unpublished observations). It is currently undergoing Phase II trial for patients with solid tumors, including HCC (NCT00940225).

While early results of these compounds are encouraging, additional hurdles doubtless remain. Qi *et al* (37) recently investigated acquired resistance to MET inhibitors using a gastric carcinoma cell line and observed at least two tumor adaptations. The first resulted from spontaneous mutation in the MET activation loop, and the second resulted from increased expression of TGF-alpha with consequent upregulation of the EGFR pathway. Although this second mechanism responded to anti-EGFR therapy, the observation highlighted the crosstalk that is present among the growth factor pathways and underscores the need to account for this possibility in formulating optimal molecular targeted therapy.

3.2. Epidermal Growth Factor Receptors (EGFRs) and Associated Ligands

3.2.1. Background

The epidermal growth factor receptor (EGFR) family is comprised of four closely related members: EGFR/ErbB1/Her1, ErbB2/HER2/c-neu, ErbB3/Her3, and ErbB4/Her4. These single pass transmembrane glycoproteins contain an extracellular ligand-binding domain (except for ErbB2), carboxy-terminal autophosphorylation domain and, with the exception of ErbB3, an intracytoplasmic tyrosine kinase domain.

EGFR is expressed in hepatocytes, biliary epithelial cells, and hepatic stellate cells, but not in Kupffer or normal endothelial cells (expression is reported in tumor-associated endothelial cells (38)). Synthesis in hepatocytes is approximately similar in normal vs. cirrhotic liver as estimated by *in situ* mRNA hybridization (39). ErbB2 is not detectable in the quiescent liver, but is upregulated in some conditions such as liver regeneration. ErbB3 and especially ErbB4 are also expressed in nonneoplastic hepatocytes (40).

There are seven main categories of ligands for EGFRs. Epidermal growth factor (EGF) and transforming growth factor alpha (TGF-alpha), two major direct hepatocyte mitogens, are ligands for ErbB1/EGFR. Other ligands for this receptor include amphiregulin, epigen, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and epiregulin, with the last three ligands also able to bind ErbB4. ErbB3 binds neuregulin 1/heregulin (which itself has 6 isoforms) and neuregulin 2, which are found in but not limited to the central nervous system. These ligands also bind to ErbB4, which additionally binds neuregulins 3 and 4. As noted earlier, ErbB2 has no known ligands.

These growth factors are produced as transmembrane precursor proteins that are most commonly cleaved by proximate membrane-based ADAM (A Disintegrin And Metalloprotease) proteins. Members of the ADAM family are activated by partially understood mechanisms consequent to signals from cytokines such as IL8, IL1-beta, IFN-gamma or TNF-alpha and thus these molecules, by extension, may also influence the EGFR pathway.

Upon ligand: receptor binding, homo- or heterodimerization occurs with autophosphorylation of tyrosine kinase domains and subsequent docking of a number of adapter and signaling proteins, with many similarities to pathways highlighted in the discussion of HGF: MET, above. For example, GAB1 serves as a docking protein, and GRB2 is also recruited to activate ras via SOS. PLC-gamma activation eventuates in the Akt pathway as well as IP3 signaling and nuclear translocation of NF-κB. The Janus kinase (JAK)/STAT pathway is activated, and both MAPK and JNK pathways are operative. Additionally, EGFR activation stimulates several proteins such as focal adhesion kinase (FAK), caveolin, E-cadherin and beta-catenin which play roles in cell migration and cytoskeletal rearrangement.

The action of EGFR appears to be non-redundant with that of MET, despite several similarities in pathway usage.

3.2.2. EGFRs and their Ligands in HCC

EGFR expression is estimated to occur in up to 80% of HCC (41). Lee *et al* (42), examining EGFR in a diethylnitrosamine-induced HCC rat model, found ErbB1 to be present in 75% of HCC nodules, with ErbB2 in 89% and ErbB4 in 62% of tumors. Expression of ErbB1 increased, and ErbB4 decreased, as nodules progressed

from hyperplasias to fully developed HCC. Komuves *et al* (39) found no increase in EGFR mRNA expression in clinical HCC samples compared to non-neoplastic liver, as estimated by *in situ* hybridization. Although these studies employ different systems, increased receptor expression in the setting of unaltered mRNA level raises the possibility of reduced receptor turnover. Any such change may depend upon factors other than the receptor itself, since the frequency of EGFR mutations is 1% or less in this tumor type (41). Recently, Schroeder *et al* (43) identified a novel interaction of several proteins that function late in the endocytic degradation process of EGFR and result in the buildup and continued signaling of this receptor. The relationship of this to HCC remains unexplored.

In addition to alterations in level of EGFR expression, qualitative changes in distribution may also exist. Moon *et al*. (44) found EGFR present in tumor-associated endothelial cells in almost half of their cases.

EGF expression is also known to be increased in HCC and EGF mRNA is reportedly increased in HCC samples relative to background liver (39).

Hisaka *et al* (38) examined several HCC cell lines by flow cytometry and found that expression of TGF-alpha exceeded that of EGF, and also exceeded HB-EGF and AR expression. Several studies co-localized TGF-alpha and EGFR in HCC tumors and/or adjacent hepatocytes and biliary epithelium (45-47). These analyses generally support the concept of autocrine/juxtacrine/paracrine signaling, but since the immunoreactive TGF-alpha protein normally exists in inactive form, and antibodies did not assess the spatial relationship of the ligand to or phosphorylation status of the EGFR, such studies do not rigorously prove this point.

In some cases, the underlying disease may affect this pathway. For example, co-localization of TGF-alpha and HBV within individual hepatocytes or HCC has been demonstrated in several studies (48-51). Kim *et al*(52) showed TGF-alpha mRNA upregulation in HCC cell lines via viral HBx protein transactivation of AP-2 binding sites and Ono *et al* (53) showed transactivation of the proximal TGF-alpha promoter (-373 to -59) by HBV pre-S1 protein, suggesting direct viral interference with this pathway.

Other EGFR ligands are also described in HCC. Moon *et al*. (44) found betacellulin to be increased to a greater extent than TGF-alpha and to be present more frequently in a series of HCC samples. HB-EGF was immunolocalized to HCC cells by Inui *et al* (54), who also showed that these cells produced this protein in increased amounts relative to surrounding liver. Miyoshi *et al*, (55) using the LEC rat spontaneous hepatoma model, showed upregulation of HB-EGF at the time of HCC emergence from background hepatitis. They were also able to induce HB-EGF mRNA in the rat AH66t HCC cell line, but not in non-neoplastic hepatocytes, by exposure to TGF-alpha and, to a lesser extent, EGF.

The presence of neuregulin 1 in human HCC was demonstrated by Hsieh *et al*. (56). In this case phosphorylation of ErbB3 was shown to follow neuregulin binding and to be dependent upon the presence of ErbB2.

In sum, these and other studies confirm the presence of this signaling axis in both clinical and experimental HCC, with general concurrence of TGF-alpha as a major, but not solitary, signaling molecule.

3.2.3. EGFR pathway in HCC

Transgenic mice constitutively expressing TGF-alpha develop both hepatomegaly and HCC (57), and this model has been modified to incorporate other aspects of hepatocyte neoplasia. For example, Baek *et al* (58) found that combining TGF-alpha overexpression with TGF-beta receptor inactivation resulted in higher HCC proliferative rate, with increased phospho-ERK1/2 relative to tumors arising in TGF-alpha over-expressing mice alone. (Isolated knockout of TGF-beta receptor did not lead to HCC development). These changes simulate those found in human HCC and suggest interaction between the two pathways. A separate study (59) of combined TGF-alpha c-myc overexpressing murine HCC (60), in which tumors developed at a more rapid rate than in c-myc expressing mice alone, also found disturbance of the TGF-beta pathway. Preneoplastic foci lacking TGF-beta receptor II were seen in the combined c-myc/TGF-alpha mice, but not in c-myc mice. The combination mice also showed down-regulation of the cell cycle inhibitor p27, which the authors suggested represented post-transcriptional regulation by TGF-alpha. Cavin *et al* (61) examined the combined TGF-alpha /c-myc over-expressing murine model and concluded that TGF-alpha stimulated PI3K/Akt-related NF-κB production, which led to upregulation of Bcl-X(L) and X-linked inhibitor of apoptosis (XIAP), thereby inhibiting c-myc-associated apoptosis. Since these effects are mediated via the EGFR pathway, it is possible that related ligands may have a similar effect.

Liu *et al* (62) used a human HCC line to demonstrate that both EGF treatment and transfection of ErbB2 caused downregulation of the metastasis suppressor gene nm23-H1. Other EGFR ligands were not tested. Since ErbB2 gene amplification is infrequent in human HCC (63), the extent to which this model mimics the clinical situation may be limited.

Other EGFR ligands are also documented within HCC. Nakamura *et al* (64) showed that HCV core protein expression led to upregulation of HB-EGF with autocrine signaling via the ERK/MAPK and Ras/PI3K/AKT circuits in HepG2 cells, thereby linking this viral protein with both mitogenic and anti-apoptotic signaling through the EGFR pathway. Caja *et al*(65) showed that TGF-beta signaling could lead to increased HB-EGF in FaO rat hepatoma cells, reflecting the survival side of this dual-natured cytokine (see below). The tetraspanin CD9 is also able to upregulate the activity of HB-EGF (66), but CD9 mRNA is reportedly downregulated in HCC (67), rendering this association of unknown relevance.

Amphiregulin plays an important role as a paracrine signal pathway for estrogen-associated mammary

gland development (68), and Miceli *et al* (69) found that levels of this ligand correlated with aromatase activity in HCC cell lines, aromatase being an enzyme involved in estrogen biosynthesis. They speculated that estrogen exposure and perhaps local aromatase activity might affect HCC growth through amphiregulin signaling. This suggestion is of interest in light of the known association of sex steroids with both benign and malignant hepatocellular neoplasms. Castillo *et al* (70) showed that amphiregulin interaction with EGFR led to alternative splicing of the tumor suppressor gene TP73 into the truncated dominant negative isoform delta Ex2p73, which is known to be present in HCC. It is not known whether other EGFR ligands perform a similar function.

EGFR ligands may have effects beyond those related to the tumor cell itself. Lee *et al* (71) used a mouse Matrigel plug assay to show synergism between EGF and IGF-II for angiogenesis. EGF down-regulated hypoxia-induced IGH-II binding protein, an IGF-II antagonist, and also upregulated mRNA of the angiogenic beta-FGF protein. The authors suggest these as two potential mechanisms of synergy in this system.

Factors other than specific ligands may influence the EGFR pathway. A correlation between EGFR and cyclooxygenase-2 (COX-2) has been observed in human HCC (72), and overexpression of COX-2 in a HCC cell line is associated with increased EGFR, c-MET phosphorylation and invasiveness in a src-dependent manner. Conversely, EGF: EGFR binding was associated with increased COX-2 promoter activity and protein expression, suggesting crosstalk between these pathways.

EGFR also appears to be a target of Wnt/beta-catenin activity, as shown in Wnt-overexpressing transgenic mice, and the liver enlargement that occurs in these mice could be inhibited by EGFR inhibition (73). This same study showed coordinate upregulation of beta-catenin and EGFR in 7 of 10 hepatoblastomas, suggesting relevance to this pediatric hepatocellular malignancy.

3.2.4. EGFR/ligand clinicopathologic correlations

Several features of EGFR ligands have been associated with HCC risk, phenotype, or prognosis. Tanabe *et al* (74) found the EGF61*G polymorphism to be associated with greater mRNA stability, increased protein secretion, and higher levels of serum and liver EGF in comparison to EGF61*A. Retrospective evaluation showed 4-fold odds (CI 1.6-9.6) of G/G vs. A/A genotype in cirrhotic patients with HCC, compared to those patients without tumor, independent of age, sex, and type and severity of cirrhosis. Li et al (75) also found higher levels of serum EGF in G/G compared to G/A or A/A patients with higher frequencies of the G allele in hepatitis B cirrhotic patients with HCC versus those without tumor. Chen et al (76) found increased risk for HCC in G/G but not G/A patients with HBV infection and cirrhosis. Qi et al (77), were unable to find this association in patients with chronic HBV infection without cirrhosis, and also noted ethnic heterogeneity in EGF polymorphisms, suggesting the need for additional studies.

EGF over-expression may also relate to certain phenotypic alterations within HCC. Yoneda and coworkers (78) induced the biliary cytokeratin CK19 in several HCC cell lines following stimulation with EGF. This effect, which was accompanied by c-Jun-N-terminal kinase activity, was not seen with stimulation by HGF, beta-FGF, or TGF-beta 1. Patients with CK19-positive HCC had higher frequency of vascular involvement and metastatic disease as well as reduced survival, consistent with the experience of others studying this phenotype.

In an immunohistochemical study, Ito *et al* (79) found HB-EGF to be more frequently expressed in small HCC with low proliferative activity and without vascular invasion, and suggested this as an indicator of disease-free survival by both univariate and multivariate analysis.

3.2.5. EGFRs/ligands therapeutic correlations

Agents directed specifically toward the EGFR are of two types, monoclonal antibodies and tyrosine kinase inhibitors (TKI). Antibodies include cetuximab (Erbitux, Bristol-Myers Squibb) and panitumumab (Vectibix, Amgen). TKI include gefitinib (Iressa, AstraZeneca), erlotinib (Tarceva, Genentech), and lapatinib (Tykerb, GlaxoSmithKline).

Cetuximab is a chimeric mouse/human IgG1κ monoclonal antibody directed against the N-terminal portion of human EGFR with a half-life of 114 hours. *In vitro* studies showed variable cell cycle arrest in HCC cell lines with synergy in combination with erlotinib, doxorubicin, or fluvastatin (80). Fuchs *et al*. (81) showed that *in vitro* epithelial to mesenchymal transition (EMT), independent of EGFR presence, was associated with decreased sensitivity to cetuximab, and this resistance could be overcome by blocking integrin-linked kinase activity. Other mechanisms of resistance to anti-EGFR antibodies exist as summarized by Wheeler *et al* (82). Included among these are over-expression of EGFR or its ligand(s), altered EGFR processing in conjunction with enhanced src family kinase (SFK) activity, nuclear translocation of EGFR with possible transcription factor activity, mutations in PI3K, phosphatase and tensin homolog (PTEN) or k-ras, and increased VEGF or VEGFR activity. These have not all been described in HCC and some changes, such as k-ras mutation, are known to be rare in this tumor type.

Clinically, no anti-tumor response was seen in one phase II study of cetuximab alone in patients with advanced HCC (83), but a 20% response rate was found in another study of patients with advanced HCC treated with cetuximab combined with gemcitabine and oxaliplatin (84). At present, a phase II study of cetuximab in combination with oxaliplatin and capecitabine in patients with advanced HCC is ongoing (NCT00483405).

Panitumumab is a recombinant human IgG2κ monoclonal antibody that binds to the extracellular domain of EGFR and has a modeled half-life of approximately 8 days. We are not aware of any HCC-related trials using this agent at present.

Gefitinib is an oral quinazoline with a 48 hour half-life that binds to the ATP-binding site of EGFR, inhibiting tyrosine kinase activity. Early studies showed *in vitro* cell cycle arrest and apoptosis of HCC lines, with EMT as one source of drug resistance, similar to the situation for monoclonal antibody therapy. A phase II study (NCT00071994) found no significant activity when used as a single agent in advanced HCC (85).

Erlotinib is also an oral quinazoline inhibitor of EGFR that attaches to the ATP binding site and has a half-life of 36.2 hours. It additionally has activity against mutant JAK found in polycythemia vera (86), but this mutation is not known to occur in HCC. A phase II study of erlotinib alone for HCC did not show response (87), but in a separate study, combination with bevacizumab showed an overall 25% response rate in advanced HCC (88). At present, several phase II studies of these two agents are active (NCT01180959, NCT00242502, NCT00287222, NCT00881751), the last comparing the combination to sorafenib as first-line therapy. A separate study to assess sorafenib alone vs. combination sorafenib/erlotinib is also active (NCT00901901), as is a study combining erlotinib with gemcitabine and oxaliplatin (NCT00832637).

Lapatinib is a third quinazoline TKI with a 24 hour half-life and dual specificity against both EGFR and ErbB2, thus making it attractive for tumors such as Her2/neu positive breast cancer. However, two recent phase II studies of this agent alone in advanced HCC found little to no anti-tumor response, although they suggested that a subset of patients may benefit from this agent (89, 90).

3.3. The insulin-like growth factor (IGF) axis

3.3.1. Background

Insulin-like growth factors (IGF) comprise IGF-1 (formerly somatomedin C), a 70 amino acid polypeptide (mw 7.6 kDa) and IGF-II, containing 67 amino acids (mw 7.5 kDa). These members of the ancient insulin superfamily have 76% homology with each other (91). Seven binding proteins (IGF-BP1-7) exist and approximately 75% of circulating IGF is bound, primarily to IGF-BP3 complexed with an 88kDa glycoprotein known as acid labile subunit. The IGF-II maternal gene is thought to be imprinted, with expression normally restricted to the paternal gene (92). IGF are produced by a number of cells, including hepatocytes and fibroblasts.

Two IGF receptors (IGFR) are known (IGFR1 and 2). These transmembrane molecules have similarity to the insulin receptor and exist as dimers which, indeed, may also incorporate the insulin receptor, resulting in IGFR1/1, IGFR2/2, IGFR1/2, IGFR1/IR, and IGFR2/IR combinations. The IGFR-2 lacks intracytoplasmic tyrosine kinase activity and is thought to serve a "sump" function, sequestering and facilitating degradation of interstitial IGF. IGF does have a weak affinity for the IR, approximately 300 times less than that of insulin, and, conversely, insulin has an affinity for IGFR that is roughly 300,000 times less than that of IGF (91).

Upon ligation of IGF to an IGFR1-containing receptor, tyrosine autophosphorylation occurs and leads to attachment of a number of docking proteins, including insulin receptor substrates 1-4 (IRS1-4) and Src homology and collagen domain protein (Shc). The former activates the PI3k/Akt/mTOR pathway, which inhibits apoptosis and enhances cell proliferation. The latter activates the ras/raf/Mek/ERK/MAPK pathway, again facilitating cell proliferation. Additionally, IRS activation interferes with β-catenin: E-cadherin binding, with loosening of cellular moorings and translocation of β-catenin to the nucleus with subsequent transcription of target genes. Matrix metalloproteinases, hypoxia-inducible factor (HIF) 1α, and VEGF may also be induced by this receptor: ligand interaction.

3.3.2. IGF and insulin-like growth factor receptor (IGFR) in HCC

IGFII mRNA was found in 100% of HCC tissue, approximately 50% of peritumoral liver tissue, and in no background liver by Dong *et al* (93). Increase in IGFII mRNA relative to nonneoplastic liver was seen in 22% of human HCC samples in one study (94) and a separate study showed upregulation of IGFII at the protein level in HCC (95). Ubagai *et al* (96) demonstrated increased IGFII and IGF-1R expression in HCC cell lines exposed to aflatoxin B1, suggesting further investigation of this pathway in relation to this carcinogenic agent. In a global gene expression analysis of human HCC and hepatoblastoma, Luo *et al* (97) reported reduction of IGF-BP3, the main IGF-binding protein, in HCC relative to adjacent tissue, confirming reports of IGF-BP3 promoter hypermethylation and downregulation in HCC (98) with attribution of the observed downregulation to attachment of the poly(A)-binding protein TIA-1 to a downstream AT-rich region of the IGFBP3 gene (99). Separately, mutation or deletion of the IGFR2 gene may occur in some HCC, reducing its ability to sequester and degrade IGF (100, 101). Luo *et al* (97) also observed increased IGFII in human hepatoblastoma relative to HCC, confirming a known association in the former tumor (102) that may be related in part to uniparental disomy in this region (103).

Most analyses have focused on IGFII, which is the fetal form of IGF that appears associated with HCC. However, the study of Luo *et al* (97) also looked at IGFI and found that it was decreased in HCC samples as compared to normal liver.

Levels of IGFR1, among other proteins, may be related directly or indirectly to glypican-3 activity. Akutsu *et al* (104) showed that small interfering ribonucleic acid (siRNA) specific for downregulation of glypican3 led to reduction in IGFR1 as well as FGFR1 when tested in HCC cell lines. Separately, the micro-RNA Lin-28B was recently shown to be increased in HCC cell lines, and this also enhanced IGF-1R expression (105).

In an extensive study of 104 HCC, Tovar *et al* (106) found IGF pathway activation in 21%. This was associated with increased expression of IGFII, downregulation of IGFBP3 and allelic loss of IGFR2 in

varying combinations. An inverse association was seen with mIR-100/216. These investigators were able to inhibit many of the effects of IGF pathway activation by use of an antibody to IGFR1 (A12) suggesting this as a possible therapy.

3.3.3. IGFR pathway in HCC

Overexpression of IGFII in a transgenic mouse model resulted in a variety of tumors, with HCC as the most common type (107). Additionally, the IGF/IGFR pathway has been suggested as facilitating tumor aggressiveness/invasion and metastasis (108), as well as providing a mechanism of resistance against therapeutic agents.

Chen *et al*. (109) showed that inhibition of IGFII signaling reduced the migration and invasiveness of HCC in a murine model. They further showed that this was due to signaling through IGFR1 but not IR, and involved IRS2 but not IRS1 signal transduction, requiring induction of matrix metalloproteinase 2 (MMP2). Similar conclusions were reached earlier by Nussbaum *et al* (110), who showed proliferative and anti-apoptotic effects of IGFII to be mediated by IGFR1 and not IR. They also found a synergistic effect on HCC cell migration when IGFII and HGF overexpression were combined. A dependence of HGF effect on HCC cell migration was also shown to be related to prior exposure to IGFI by Price *et al* (111), in analysis of a rat HCC model. In some contrast to the above studies, de la Monte *et al* (112) analyzed IRS1 signaling in HCC cells and found aspartyl (asparaginyl)-beta hydroxylase (AAH) to be regulated by this pathway. AAH overexpression in Hep-G2 cells increased motility and invasiveness, suggesting this as a mediator for these features and identifying it as a potential therapeutic target in HCC.

Aleem *et al*. (113) suggested that the IGF pathway was active in the earliest phases of hepatic carcinogenesis. Using an N-nitrosomorpholine model of HCC, they demonstrated elevated IGFR1, IR1, IRS1 and 2, and ERK/MAPK activity in glycogen storage foci, which represent the earliest expression of altered hepatocytes in this model. They suggested that the insulin receptor contributes to glycogenosis, and the IGFR to increased cell proliferation in these foci.

The role of IGFB7 was examined in an HCC cell line in which it was shown that downregulation of this IGF binding protein was associated with increased proliferation, invasiveness, and resistance to apoptosis(114) as well as with resistance to alpha-interferon-induced injury(115).

IGFR activity also appears to be regulated, at least in part, by pathways that involve multiple pro-tumorigenic targets. MiR122, which is present in normal hepatocytes but often suppressed in HCC, was shown to suppress IGFR1 expression (as well as ADAM10 and serum response factor), and expression of this microRNA (miRNA) reduced a number of malignant phenotypic variables in several HCC cell lines(116, 117). Heat shock protein 90 (Hsp90) inhibition led to reduced viability of several HCC lines and was associated with reduced function of not only IGFR1, but also MET, raf1, protein kinase B, CDK4 and murine leukemia viral oncogene homolog 1(118).

The activities of the IGF: IGFR pathway appear to extend to crosstalk with other growth factor pathways involved in HCC. In one study of HCC cell lines, treatment with the anti-IGFR1 antibody AVE1642 led to upregulation and phosphorylation of ErbB3 (119). Indeed, combined exposure to AVE1642 and the EGFR TKI gefitinib was synergistic in reducing cell viability. Similar synergism was seen with the use of AVE1642 and the mTOR inhibitor rapamycin, suggesting that combining anti-IGF therapy with inhibitors of other growth factor pathways active in HCC is preferable to isolated target therapy (119, 120). Likewise, sorafenib treatment of human HCC in a xenograft model led to upregulation of IGFR-1, which was ameliorated by co-treatment with an antibody to IGFR-1(121).

3.3.4. IGF/IGFR clinicopathologic correlations

A limited number of studies have addressed the clinicopathologic implications of IGF and its associated signaling molecules in HCC, with generally congruent results.

Polymorphisms in genes of the IGF signaling system were examined by Weng *et al*(122), who found a protective effect of the combined IGFII +3580 AA and IGF-2R +1619 GG genotype against development of HCC.

Dong *et al* (93) found that circulating IGFII mRNA correlated with metastatic HCC, but not with tumor size alone, and suggested that evaluation of this analyte might be helpful in diagnostic assessment. More recently, Qian *et al* (123) also found a correlation between circulating IGFII mRNA and metastatic HCC, again suggesting this as a potential assay for extrahepatic HCC.

In a separate study (124), examination of serum IGFI levels showed significantly lower levels in patients with HCC compared to healthy subjects. Reduction of IGFI was also seen in patients with HCV infection, and those with HCV and HCC had lower levels of circulating IGFI relative to patients with HCV infection alone. The results are compatible with a tissue-based study (97), noted above, in which HCC tissue samples showed reduced IGFI mRNA expression.

The *in vitro* effect of reduced IGFB7 was also mentioned above. In that study, Tomimaru *et al* (114) found downregulation of this IGF binding protein to correlate with poor clinical outcome and suggested its potential as a prognostic marker. They also reported absence of immunohistochemical IGFBP7 staining in HCC samples from patients who had poor response to therapy with alpha-interferon and 5fluoro-uracil (115).

Downregulation of miR-122, previously noted (above), was found by Zeng *et al* (117) to be a negative prognostic indicator in HCC patients.

3.3.5. IGF/IGFR therapeutic correlations

Agents targeting the IGF signaling axis are recognized as valid targets for the treatment of HCC, but have not yet reached the maturity of drugs targeting other growth factor pathways. A study of AVE1642 in HCC (NCT00791544) (ImmunoGen MA, Waltham, Sanofi-Aventis) was terminated due to a company decision to discontinue the drug development program, reportedly unrelated to safety or efficacy concerns. Cixutumumab (IMC-A12, Imclone) is a human IgG lambda monoclonal antibody directed against IGFR1 that is currently undergoing Phase I (NCT01008566) and II (NCT00906373) trials in combination with sorafenib to treat advanced HCC.

BIIB022 (Biogen IDEC, Weston MA) is listed in Phase I trial in combination with sorafenib for HCC (NCT00956436). Additional agents, including the IGF1R antibody dalotuzumab (MK0646, Merck) and other drugs that impinge upon the IGFR pathway by inhibition with growth hormone releasing hormone or growth hormone receptor also exist (125), but are not being evaluated in the context of HCC at this time.

3.4. The vascular endothelial growth factor (VEGF): vascular endothelial growth factor receptor (VEGFR) axis

3.4.1. Background

Vascular endothelial growth factors (VEGF) include several cysteine-knot-containing members of a platelet-derived growth factor (PDGF) subfamily, named VEGF-A (VEGF), VEGF-B, C, D and placenta growth factor (PlGF). Several isoforms exist, and the presence or absence of exons 6 and 7 in these variants dictates the presence or absence of heparan sulfate proteoglycan binding, affecting VEGF diffusion. VEGF is produced by a variety of mesenchymal and endothelial cells, and there is evidence that autocrine stimulation of endothelial cells by VEGF is required for survival. VEGF production can be upregulated by IGFI in hypoxic, and Sp1 in hypoxic and non-hypoxic conditions.

VEGF receptors (VEGF-R) are transmembrane proteins with extracellular immunoglobulin-like domains, a single transmembrane domain, and an intracytoplasmic tyrosine kinase domain. Three variants exist, namely VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4). VEGFR-2 appears to be the major receptor involved with signal transduction in the context of vasculogenesis, as its kinase activity is greater than that of VEGFR-1(126). VEGFR-3, which is primarily involved with lymphangiogenesis and neurogenesis (127), has recently been shown to actively participate in the leading edge of vessel growth.

Upon ligand binding to VEGFR domains 2 and 3, receptor dimerization occurs resulting in autophosphorylation of kinase domains. This can lead to activation of several signal transduction pathways including the ras/raf/MEK/ERK path via SOS activation, PI3k generation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) with activation of the Akt/PKB (protein kinase B) pathway, leading to nitric oxide production and protection against apoptosis, and the p38 pathway which, along with the FAK/paxillin pathway, contributes to actin reorganization and cell motility.

Recently, VEGF-associated changes have been examined in relation to the morphogenesis of vessel growth, leading to an appreciation of the complex cell-to-cell signal integration necessary to coordinate this process. Briefly, the leading edge of the new vessel sprout is comprised of one or a small number of “tip” cells, endothelial cells that proliferate in response to a VEGF gradient which typically guides them toward hypoxic areas. VEGF also induces delta like ligand 4 (DLL4), which is a Notch ligand and is thought to limit tip cell formation by suppressing filopodia formation, thus configuring endothelial cells to assume a supportive proliferative or “stalk” phenotype that serves to elongate the vascular tubes (128). This system in relation to normal and neoplastic environments has been recently reviewed in detail (129) (130).

3.4.2. VEGF and VEGFR members in HCC

Activation of the VEGF/VEGFR axis may occur early in HCC. Nakamura *et al*(131) reported increased VEGF in dysplastic nodules as well as in HCC, and suggested that an associated increase in fkl-1 in endothelial cells and HIF1α in hepatocytes might play a role in the sinusoidal capillarization that becomes more pronounced in fully developed HCC. Yamaguchi *et al* (132) used immunohistochemistry to demonstrate that VEGF expression was highest in well-differentiated as opposed to poorly differentiated HCC, and also decreased as tumor size increased.

Mas *et al* (133) found VEGF to be one of the proteins differentially expressed in plasma of HCC patients with underlying HCV, versus cirrhotic HCV patients without HCC.

3.4.3. VEGF/VEGFR pathway in HCC

Expression of the VEGF/VEGFR axis is typically associated with enhanced tumor growth in model systems, and, conversely, inhibition of this pathway leads to reduction in tumor growth. Dong *et al*. (134) inhibited the occurrence of HCC in a rat 2-fluorenylacetamide model by use of thalidomide, which downregulated both VEGF and NF-kappa B.

Several accessory molecules have been implicated in VEGF/VEGFR activity in HCC. The VEGFR co-receptor neuropilin-1 was found to be expressed in human HCC cells but not normal hepatocytes (135). Blocking this in a transgenic mouse model led to decreased vasculature and reduced tumor growth, suggesting a role of this protein in HCC. Chen *et al* (136) found that inhibition of the receptor tyrosine kinase EphA1 in HCC lines was associated with downregulation of VEGF as well as MMP-2 and 9 with subsequent decreases in tumor vasculature and growth upon inoculation into nude mice. Kaji *et al* (137) reported that aldosterone blockade led to downregulation of VEGF, leading to increased apoptosis in a murine HCC

model and supporting a role for the renin-angiotensin-aldosterone system in tumor angiogenesis. This agent has no effect on tumor cell proliferation *per se in vitro*.

VEGF is also a target gene of the androgen receptor, and Kanda *et al* (138) showed a relationship between HCV core protein and increased androgen receptor transcriptional activity, with enhanced VEGF expression *in vitro* in response to HCV infection.

Lian *et al* (139) found more frequent expression of VEGFR-3 short form splice variant in HBxAg-positive HCC and suggested that the viral antigen may interfere with signaling of this form of the receptor, since cell growth was accelerated by the viral protein in a ligand-independent fashion, but could be partially blocked by siRNA directed against the VEGFR. In a separate study (140), HBxAg increased VEGF, MMPs 2,9 and 14, and upregulated NF-kappa B signaling, suggesting a number of effects of this antigen.

3.4.4. VEGF/VEGFR clinicopathologic correlations

Alterations in the VEGF gene have been associated with HCC risk in some studies. Giacalone *et al* (141) found a +936 C->T polymorphism of the VEGF-A gene more frequently in their HCC patients vs. cirrhotic or normal control patients. Separately, He *et al* (142) were not able to find any association between an 18 bp insertion/deletion polymorphism (rs35569394) and HCC in their Chinese population.

Activity of the VEGF/VEGFR axis has been interpreted as a negative clinicopathologic indicator in a number of studies. Cui *et al* (143) retrospectively examined a series of patients with small HCC to uncover factors associated with tumor recurrence following treatment and found that a combination of increased VEGF, c-myc and Ki67 was seen more frequently in those patients who had tumor recurrence within one year after resection, compared to those without recurrence.

In a study of HCC tissue samples, Aucejo *et al*(144) found higher VEGFR2 levels in the vasculature of poorly differentiated, in contrast to well differentiated, HCC with higher levels also present in the arteries of background hepatic parenchyma in patients whose tumors were beyond the Milan criteria. They did not find any association with vascular invasion or posttransplant recurrence. Association of tumoral VEGFR2 and poor histologic differentiation was also reported by Huang *et al* (145), who did note a negative association with patient survival as well. Ho *et al* (146) looked at HCC mRNA and did not find any correlation between VEGF-A or C and recurrence free survival, but did report that patients with higher PlGF expression had earlier tumor recurrence. Jia *et al* (147) looked at HCC tissue by immunohistochemistry and were able to delineate a poor prognosis group by the expression of VEGF and VEGFR patterns in tumoral and peritumoral tissue.

Ferroni *et al* (148) looked at levels of VEGF in circulating platelets and found that the overall VEGF level corrected for platelet count was a predictor of decreased survival, although neither serum nor plasma VEGF levels alone were significant in this regard. In a separate study, no significant difference was found in serum VEGF levels between cirrhotic patients and those with HCC (149).

In some contrast to some of the above studies, Kaseb *et al*(150) found that baseline plasma VEGF level had prognostic significance in predicting survival in HCC patients, and incorporated this into the Cancer of the Liver Italian program score (CLIP score) to generate a V-CLIP score with greater predictive value. These authors suggest prospective validation of this scoring system. Nagaoka *et al* (151) found circulating soluble VEGFR1 level to correlate negatively with survival, and the ratio of PlGF to soluble VEGFR1 also had prognostic value.

3.4.5. VEGF/VEGFR therapeutic correlations

Sorafenib is a multikinase inhibitor that targets VEGFR2, 3, PDGFR-beta, Flt-3, c-kit and raf kinases. A global randomized clinical trial established this agent as effectively extending time to progression and patient survival, and it is currently accepted as the drug of choice for patients with inoperable or resistant HCC (152).

Although results with sorafenib are encouraging, objective tumor response *per se*, particularly complete remission, is only infrequently reported. Such results appear more common in Japan, suggesting an underlying genetic basis (153). Optimal use of this agent is still evolving. In a rat HCC model, sorafenib lowered the extent of vessel sprouting within tumors, but when combined with the mTOR inhibitor everolimus, vessel sprouting was absent (154). Clinical trials combining sorafenib with everolimus (NCT00828594, NCT01005199) or an alternative mTOR inhibitor (temsirolimus, NCT01008917, NCT01335074, NCT01013519, are underway.

In one such case, combination with the oncolytic poxvirus JX-594 (Jennerex Biotherapeutics, San Francisco CA) is undergoing clinical trial (NCT01171651). Phase I study showed this virus to effectively induce HCC necrosis; however, combination with sorafenib led to reduced viral replication due to drug effect on raf kinase, with subsequent reduced efficacy. In contrast, sequential administration was associated with objective tumor responses.

Sunitinib (Pfizer) is a tyrosine kinase inhibitor that targets VEGFR and PDGFR and demonstrated significant activity against HCC in preclinical studies. A trial comparing this agent vs. Sorafenib in HCC patients was halted due to a higher incidence of adverse effects in the sunitinib arm (NCT00699374). Several studies of sunitinib in HCC, either alone or in conjunction with other agents, are active at this time.

Brivanib (Bristol-Myers Squibb) is a dual TKI of VEGFR and FGF, and preclinical studies showed correlation with FGFR expression and sensitivity to the drug *in vitro* (155). A phase II study showed promising results in advanced HCC, with several partial and one complete remission (156).

ABT-869 (linifanib) (Abbott) inhibits VEGFR and PDGF and has been shown to have synergistic effect with the mTOR inhibitor rapamycin in a model HCC system (157). It is currently being evaluated against sorafenib in HCC (NCT01009593).

BIBF 1120 (Boehringer Ingelheim) is a multikinase inhibitor with activity against VEGFR 1,2,3, FGFR and PDGFR with activity against HCC in a murine model (158). Trials are underway comparing this drug to sorafenib for advanced HCC (NCT00987935, NCT01004003).

Vatalanib (PTK787/ZK222584, Novartis) is a VEGFR inhibitor that shows preclinical activity against HCC and enhances the effect of IFN/5FU in this setting (159). No clinical trials related to HCC are ongoing at present. Vandetanib (ZD6474, AstraZeneca) is a combined VEGFR and EGFR TKI that demonstrated activity against HCC in one study in which it was combined with an oral 5FU prodrug (160). Current status of clinical testing is unknown. AEE788 (Novartis), a dual VEGFR and EGFR TKI, has shown preclinical activity against HCC (161) but has not been clinically evaluated for this indication at this time.

The use of irradiation in conjunction with transcatheter arterial chemoembolization may have unintended consequences due to radiation-induced stimulation of VEGF production leading to tumor progression outside of the radiation field. Chung *et al*. (162) documented this in their patient series and suggest incorporating anti-VEGF therapy into these regimens. In this regard it is also noted that hepatic resection alone also caused an upregulation of VEGF in residual HCC cells in a rat HCC model (163).

3.5. The fibroblast growth factor (FGF): fibroblast growth factor receptor (FGFR) axis

3.5.1. Background

The FGF family contains 23 member glycoproteins including 4 that do not bind to the FGF receptor (FGFR). FGF are bound by heparan sulfate proteoglycans in extracellular locations and are cleaved from this interaction prior to binding cell surface FGF receptors (164).

FGF receptors are members of the immunoglobulin superfamily and consist of four transmembrane proteins with tyrosine kinase activity (FGFR1-4, CD331-334) that can give rise to nearly 50 different isoforms due to alternate splicing, leading to differences in ligand specificity and kinase activity. A fifth protein, FGFR5 (FGFR-like 1; FGFRL1), lacks tyrosine kinase activity and has been suggested as having a regulatory function (164).

The FGF: FGFR complex is stabilized by low affinity binding to a single chain cell-surface heparan sulfate proteoglycan. The specific FGF: FGFR interaction is dictated by the particular alternative splicing pattern of the extracellular IgIII domain of FGFR. This mechanism accounts for a degree of variation among ligand: receptor pairs among different cell types (e.g., epithelial vs. mesenchymal). Some FGF variants (FGF 19, 23) utilize members of the Klotho family as cofactors for binding (165). These isoforms appear to act in an endocrine fashion, in contrast to the usual autocrine/paracrine signaling modes of most FGF family members (165).

The FGF: FGFR complex undergoes dimerization and autophosphorylation with docking and phosphorylation of adaptor proteins including FGFR substrate 2 (FRS2). This in turn binds growth factor receptor bound-2 (GRB2) and son of sevenless (SOS) which activates the ras/raf/MEKK/ERK/MAPK pathway and phospholipase C gamma (PLC gamma) which feeds into the AKT and IP3 signaling pathways as well as leading to activation of NF-kappa B. Separately, the ligand: receptor complex activates PI3K which leads to JNK and p38 activation and nuclear translocation. Although many of these pathways are associated with cell proliferation and protection from apoptosis, FGF signaling may also be associated with growth arrest or apoptosis in some cases (166).

3.5.2. FGF and FGFR in HCC

Asada *et al* (167) examined a series of HCC cell lines and found mRNA for several FGFR in all lines except one. In some cases, receptors were expressed in both tumor lines and primary hepatocytes [FGFR1(IIIc), FGFR3(IIIc), FGFR4]. Other receptor subtypes [FGFR2(IIIb), FGFR2(IIIc), and FGFR3(IIIb)] were expressed in tumor lines but not in primary hepatocytes, suggesting that these may be associated with malignancy.

Similar but not identical results were obtained by Huang *et al*, (168) who reported that FGFR4 represents the only receptor subtype present in mature hepatocytes and that FGFR1 occurs in activated nonparenchymal cells but is also upregulated in HCC.

3.5.3. FGFR pathway in HCC

The initial interaction of FGF with FGFR utilizes concomitant binding with the heparan sulfate (HS) chain of proteoglycans and availability of FGF is related in part to sulfation status of the HS. One such HS proteoglycan is the membrane-associated glypican-3, which is one of the most highly transcriptionally upregulated genes in a majority of HCC (97). Several studies examined glypican-3 in relation to heparin desulfatases SULF1 and SULF2 in the setting of HCC. Lai *et al* (169) found that SULF2 was upregulated in the majority of HCC cell lines, was associated with glypican-3 upregulation, increased FGF2 binding, and increased ERK and AKT phosphorylation in HCC cells, leading to increased growth and migration. In contrast, SULF1 appeared to act as a tumor suppressor, and was downregulated in HCC lines in which it was inversely associated with FGF (and HGF) signaling (170). Since both isoforms possessed similar sulfatase activity, other mechanisms may account for this difference.

Given the complexity of the FGF receptor: ligand family, it is likely that subsets of family members may be

overrepresented in HCC or may demonstrate differential signaling effects. Gauglhofer *et al* (171) found that exposure of HCC cells to hypoxia or serum deprivation led to increase in FGFs 8, 17 and 18. The presence of these ligands was negatively correlated with apoptosis, and had additional effects on tumor stroma, with FGF 17 and 18 stimulating tumoral myofibroblast growth, and all three stimulating hepatic endothelial cell growth.

In a HCC cell line study, addition of FGF1 or FGF2 induced cell proliferation in several cases; however, addition of FGF7 showed no effect (167). Overexpression of FGF21 in liver led to delayed onset of HCC in a diethylnitrosamine rodent model (172), suggesting at least a weak suppressor activity.

FGF19 has a specific role in bile homeostasis (173) and demonstrates a reduced affinity for HS proteoglycans, which allows it to function as a hormone in addition to exerting short-distance effects. A transgenic mouse model of FGF19 expression in skeletal muscle is complicated by the development of dysplastic hepatocellular foci that evolve into hepatocellular carcinomas accompanied by elevated alpha fetoprotein (AFP) production and nuclear translocation of beta catenin (174). Wu *et al* (175) found that this is related specifically to its interaction with FGFR4, which is the main form of FGFR in the liver. Interestingly, they were unable to achieve similar results with exposure to FGF21, a separate member with hormonal activity that has in common with FGF19 the ability to interact with several FGFRs, but not with FGFR4. The situation may be complicated by other factors, and Luo *et al* (176) showed that the interaction of FGF19 and FGFR4 complexed with the beta Klotho protein led to apoptosis, and might be considered a tumor suppressor complex. However, they also observed downregulation of β-Klotho in both murine and human HCC, possibly explaining in part the opposing results of signal transduction. Huang *et al* (168) also posited a normally suppressive effect of FGFR4 on hepatocarcinogenesis. They found that although FGFR4-deficient mice did not spontaneously develop HCC, this deficiency was associated with accelerated tumor development following treatment with diethylnitrosamine. Co-expression of FGF19 and FGFR4 has been shown in primary liver tumors (177) and ectopic FGFR1 expression in these cells has also been associated with increased proliferation *in vitro* (168).

Tsunematsu *et al* (178) showed that FGF2 expression in HCC cell lines increased in response to IL1-beta or IL6, and that this was associated with upregulation of MHC Class I-related chain A (MICA) and decreased HLA Class I on these cells. Both of these changes are associated with increased sensitivity to NK-mediated killing, leading these authors to suggest that FGF2 may play a protective role by decreasing the ability of these cells to escape innate immune-mediated killing mechanisms.

3.5.4. FGF/FGFR clinicopathologic correlations

Several polymorphisms of the FGFR4 gene were found in a series of HCC (179), and one (338R/R) was associated with AFP production, which is generally interpreted as a negative prognostic indicator.

Harimoto *et al* (180) examined FGFR2 in resected HCC specimens using immunohistochemistry and found expression to represent a negative prognostic indicator that associated with poor histologic differentiation, vascular invasion, high AFP production and decreased survival. However, Amann *et al* (181) found that reduced expression of an isoform of FGFR2, namely FGFR2-IIIb, was related to a 10q deletion and was associated with a more malignant phenotype of HCC. This isoform is normally expressed in hepatocytes and plays a functional role in liver regeneration.

Increased expression of FGFR3 in HCC samples relative to surrounding liver was reported by Qiu *et al* (182), who also noted an association with poor histologic differentiation and high expression of this ligand.

3.5.5. FGF/FGFR therapeutic correlations

The breadth of the FGF/FGFR signaling system raises the possibility of multiple areas of targeting at both the receptor and ligand levels. However, at present, most agents in trial are tyrosine kinase inhibitors with specificity for both FGFR and the closely related VEGFR and PDGFR ligands, as reviewed by Turner *et al* (164). Newer agents may be more selective and potent as regards the FGFR pathway, but a more comprehensive understanding of the biological complexity is needed to fend off untoward complications. For example, dystrophic calcification has been seen with blockade of FGF23 signaling (164), and targeting of FGFR1-IIIc has led to severe weight loss due to interference with hypothalamic signaling in preclinical models (183).

At present, most clinical trials using FGF pathway inhibitors are being undertaken in patient populations other than those with HCC. One active study of HCC patients (NCT00784290) utilizes an agent (TSU-68, Taiho Pharmaceutical Co. Ltd), with tyrosine kinase inhibitory activity against FGFR, VEGFR-2 and PDGFR. Other studies may include patients with advanced HCC as part of a more general population of patients with advanced tumors. These include studies of E-3810 (NCT01283945) (VEGFR/FGFR kinase inhibitor, Ethical Oncology Science,) and BGJ398 (NCT01004224), (pan-FGFR kinase inhibitor, Novartis).

Other agents may impinge upon the FGFR pathway. Cao *et al* (184) found that vitamin K2 significantly inhibited FGFR3 expression by suppression of promoter activity, and was associated with inhibition of HCC cell line proliferation *in vitro*. However, a clinical study did not find vitamin K2 to be an independent variable for prevention of HCC recurrence following treatment (185). However, this was based on a small patient sample and additional studies employing this agent appear to be desirable.

3.6. Platelet derived growth factors (PDGFs)

3.6.1. Background

The PDGF family is comprised of 4 polypeptides, PDGF-A, -B, -C and -D that form the dimers PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-

DD (186). PDGF-A and -B chains are secreted in a form that becomes active upon dimerization, whereas PDGF-C and -D require proteolytic cleavage to effect activation. PDGF-C is preferentially cleaved by plasmin and also by tissue plasminogen activator (tPA) (187) whereas PDGF-D is cleaved by urokinase plasminogen activator (uPA) (188) but not tPA (189). PDGF is synthesized by a number of mesenchymal cells, including fibroblasts and vascular smooth muscle cells. In the liver, myofibroblasts that have evolved from activated stellate (Ito) cells can produce PDGF. Interestingly, it has been demonstrated in the rat that this cellular transdifferentiation is first accompanied by that PDGF-B production, followed in later stages by PDGF-D and lastly by PDGF-C generation (190). The functional implications of this evolution are unknown.

The two PDGF receptors, PDGFR-alpha and PDGFR-beta, are transmembrane proteins with intracellular tyrosine kinase domains and form homo- or heterodimers that differentially bind their ligand types, which contribute a degree of heterogeneity to the system. Additionally, the various receptor dimers generate overlapping and non-overlapping signal components, indicative of further fine-tuning of signal transduction (186). Generically, however, ligand: receptor interaction leads to receptor phosphorylation with formation of docking sites for SH2-domain-containing adapter proteins such as GRB2, which leads to ras phosphorylation and raf/MEK/ERK/MAPK pathway similar to that described above for HGF: MET. Other SH2-containing proteins with direct signaling activity, such as PLC gamma, src, PI3K and GAP are also docked upon receptor phosphorylation, with activation of their subsequent pathways such as PIP2, rac, AKT, and ras, respectively. Additionally, the JAK/STAT pathway is directly activated, with at least involvement of JAK2 and STATs 1 and 3 in some cases (191). Among the negative regulators of this pathway are the SOCS (suppressors of cytokine signaling) proteins, which target JAK for proteasomal degradation.

In addition to positive effect on cell proliferation by, for example, the MAPK pathway, PDGF signaling may have a number of effects. These include stimulation of cell migration, protection from apoptosis, stimulation of angiogenesis by support of vascular mural cells, or pericytes, and increase or decrease in chemotaxis dependent upon cell type and receptor subtype (186).

In the liver, PDGFR are expressed by Ito cells, in addition to the more general expression on resident fibroblasts, vascular smooth muscle cells, pericytes, neurons and macrophages. Receptors are additionally expressed on platelets, which also represent the main storage site of PDGF.

3.6.2. PDGF and platelet derived growth factor receptor (PDGFR) in HCC

Only a few studies have specifically examined PDGF/PDGFR expression levels in HCC. Llovet *et al* (192) found PDGFR-alpha to be among the genes upregulated in early HCC as contrasted with dysplastic nodules. Stock *et al* (193) found PDGFR-alpha levels to be increased in HCC compared to background liver in 64% of cases (14/22). Of these, 3 also had increased PDGFAA and 4 had higher PDGF-CC relative to adjacent liver.

3.6.3. PDGF pathway in HCC

PDGF can activate STAT3, and Bagnyukova *et al* (194) examined this in HCC. Their results support a model in which this pathway is inhibited in early tumors by expression of the negative regulator Socs1. However, in later tumors Socs1 downregulation via promoter methylation occurs and results in activation of the JAK/STAT pathway with subsequent tumor progression. In addition to PDGF, this pathway can also be activated by TGF-beta, again underscoring the interconnections and redundancies in growth factor communication networks.

An interaction between TGF-beta, ras and PDGF was also found by Fischer *et al* (195), who observed that upon EMT, p19(ARF) null hepatocytes showed TGF-beta - induced upregulation of PDGFA and PDGFR with autocrine signaling, resulting in nuclear beta-catenin accumulation. Interference with PDGFR signaling reduced TGF-beta associated migration and suppressed tumor growth *in vivo* (196). Further studies identified interleukin-like EMT inducer protein in collaboration with ras serving as a stimulant to PDGF signaling in this circumstance (197).

PDGF-C overexpression in mice leads to hepatic fibrosis, steatosis, and hepatocellular dysplasia that proceeds to HCC (198). Campbell *et al* (199) also found angiogenesis in association with this, and were able to decrease this with the tyrosine kinase inhibitor imatinib, suggesting a possible role for this or similar agents. More recently, a model of HCC in PDGFB transgenic mice has been developed (200). In this model, liver fibrosis spontaneously develops related to upregulation of TGFBR by PDGF and treatment with diethylnitrosamine and phenobarbital leads to dysplasia and HCC.

The role of Hsp90 inhibition in reducing viability of HCC lines and downregulating expression of IGFR1 and several other molecules was discussed above. Lang *et al* (201) also found that Hsp90 inhibition led to reduction of PDGFR-beta on vascular smooth muscle cells, raising the possibility that this also may contribute to the increased anti-tumor effect of these two agents together compared to mTOR inhibitor alone. The increase is suggested to be due to blockade of the paradoxical Akt activation with mTOR inhibition that is due at least in part to IGF1R signaling (201). A hypoxia-induced AKT/HIF1-alpha/PDGF-BB autocrine loop has also been identified in HCC cells (202) and might conceivably also contribute to this bypass.

3.6.4. PDGF/PDGFR clinicopathologic correlations

Mas *et al* (133) examined a number of circulating growth factors in HCV patients with and without HCC, and found PDGF, among several other factors, to be differentially expressed in the HCC group. A separate study of an Egyptian population with HCV infection also showed increasing levels of circulating PDGF to correlate with increasing disease stage, with highest levels seen in patients developing superimposed HCC (203).

3.6.5. PDGF/PDGFR therapeutic correlations

Sorafenib is a multikinase inhibitor that targets PDGFR-beta in addition to other receptor tyrosine kinases. Trials employing this drug are discussed under the heading of VEGF. Sunitinib is a dual VEGFR and PDGFR TKI and was also discussed previously.

3.7. The transforming growth factor-beta (TGF-beta)/TGF-beta receptor axis

3.7.1. Background

Transforming growth factor-beta (TGF-beta) protein exists as three isoforms, TGF-beta 1 (TGF-beta), TGF-beta 2 and -beta 3. These are secreted as precursor molecules that are cleaved into active forms by substances including but not limited to MMP-9, plasmin, thrombospondin-I and reactive oxygen species (204). The resulting peptide forms a characteristic "cysteine knot" comprised of disulfide bonds from 8 cysteine residues that aids in resisting denaturation, whereas a ninth cysteine enables dimerization to a second TGF-beta molecule.

Three different forms of TGF-beta receptors exist on the cell surface. Types I and II are transmembrane serine/threonine kinases with a higher affinity for TGF-beta 1 than for TGF-beta 2. In contrast, TGF-beta receptor type III, which includes both beta-glycan and endoglin, represents cell surface proteoglycans without signaling capabilities and with high affinities for both TGF-beta 1 and 2.

Initial binding of TGF-beta isoform dimers to Type II receptor induces multimeric aggregation that leads to phosphorylation of the type I receptor, inducing phosphorylation of the receptor-regulated Smad-2 and Smad-3 transcription factors, followed by their separate dimerization with Smad-4 and subsequent translocation to the nucleus. This pathway can be negatively regulated by the inhibitory Smad-7, and, in the case of Smad-2, also by Smad-6. A regulatory role for peroxisome proliferator-activated receptor gamma (PPAR-gamma) induced by TGF-beta activation of cytosolic phospholipase A92) alpha has also been recently proposed (205) and the transcription factor serum response factor (SRF) has also been found to negatively regulate this system. (206) Activation of the Akt pathway may also occur, and, in conjunction with signals from other growth factors or cytokines, activation of ras, p38, and JNK pathways.

The type III TGF-beta receptors may play a regulatory role by affecting the availability of the ligand to interact with the type I and II receptors.

TGF-beta is produced by most immune cells, and is associated with downregulation of immune functions such as proliferation and activation, i.e., it is immunosuppressive. It is also mitoinhibitory for a number of other cells types and has a number of other effects including facilitation of epithelial-mesenchymal transition (EMT).

3.7.2. TGF-beta and TGF-beta receptor in HCC

TGF-beta may also be produced by novel sources within HCC. Alisa *et al* (207) al showed that intratumoral regulatory CD4+ T cells that are specific for a peptide within alpha-fetoprotein exist and elaborate TGF-beta.

Ogata *et al* (208) found that SOCS3deficient mice developed STAT3 hyeractivation, and this in turn was associated with increased TGF-beta, suggesting this as a target gene of STAT3. They also observed reduced SOCS3 expression in the livers of HCC patients, and suggested that loss of this negative regulator may contribute to upregulation of TGF-beta in this tumor. TGF-beta may also be upregulated directly by HCV core protein, which binds to the TGF-beta 1 promoter (209).

Lu *et al* (210) found TGF-beta 1 in approximately 70% and TGF-beta RII in approximately 60% of their clinical HCC specimens, increased relative to surrounding liver.

Kiss *et al* (8) showed TGF-beta RII to be downregulated in HCC relative to surrounding liver in 50% of cases, equally expressed in 42% and overexpressed in 8%. TGF-beta RIII is downregulated in HCC, and a study of 10 tumors showed LOH in two (211).

3.7.3. TGF-beta pathway in HCC

Upregulation of the Type III TGF-beta co-receptor endoglin was demonstrated in endothelial cells from clinical HCC tissues relative to endothelial cells from nearby non-neoplastic tissue using immunohistochemistry (212). Expression was found strongly at the edge of tumors, and such cells had a greater migratory capacity *in vitro*. The authors hypothesized that TGF-beta, elaborated by tumor cells, acted on endoglin-positive vessels to facilitate angiogenesis.

A number of studies have examined the effects of mediators of the TGF-beta pathway on hepatocarcinogenesis. Embryonic liver fodrin (elf), a beta-spectrin that acts as a scaffolding protein for Smad3/4, appears necessary for at least some of TGF-beta effects (213). Although homozygous loss of elf is lethal, elf+/- mice develop HCC in a high proportion of cases. This is associated with abnormal vasculogenesis, suggesting that this molecule normally inhibits this process. *In vitro*, siRNA blockade of elf resulted in raised levels of retinoblastoma protein in HepG2 cells, indicating a role in cell cycle progression as well. These results led the investigators to suggest that agents enhancing the effect of elf may be useful in prevention or treatment of HCC (213). In a separate study, ELF was found to be reduced in 8 of 9 HCC specimens (214).

The TGF-beta pathway appears to act in concert with other signaling pathways as regards HCC. An enhanced predisposition to tumor development in mice that overexpress TGF-alpha and have inactivation of the TGF-beta receptor, relative to those overexpressing TGF-alpha alone, was mentioned above (58).

The dichotomous effect of TGF-beta on HCC has long been recognized. In this regard Herzer *et al* (215) recently showed TGF-beta upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as one mechanism of TGF-beta -mediated apoptosis of HCC cells. Kim *et al* (216) showed the transcription factor FoxO3a to

also be an important mediator in this process. In contrast, a group of microRNA induced by TGF-beta in Huh-7 HCC cells was shown to counteract the apoptotic effect in a manner dependent upon Smad (217)

A primary challenge for the neoplastic cell lies in exploiting the useful functions of TGF-beta such as induction of the epithelial-mesenchymal transition while escaping its deleterious effects such as apoptosis and autophagy (218). Several mechanisms, not mutually exclusive, have been suggested. Caja *et al* (65) showed that TGF-beta upregulated TGF-alpha and HB-EGF in a rat HCC cell line, leading to activation of EGFR with subsequent AKT activation and inhibition of apoptosis. Although these ligands were also upregulated in normal hepatocytes by TGF-beta, they did not undergo significant proteolytic activation and the EGFR pathway was not activated. Knockdown of EGFR enhanced TGF-beta -mediated and NADPH oxidase 4 (NOX4)-dependent apoptosis (219-221) in tumor cells, but this effect was not seen if there were additional alterations affecting this pathway (222). A separate process may involve the microRNA miR-183, which was found to be increased in a majority of HCC in one study (223). This transcript was found to inhibit programmed cell death 4 (PCD4), which is involved in TGF-beta -associated apoptosis of HCC cells (223, 224). Another modification may involve methylation of a single TGF-beta -responsive promoter site in tristetrapolin, a negative regulator of c-myc, which was found in a majority of HCC cell lines and clinical tumors, and may result in resistance to the cytostatic effect of TGF-beta (225).

A potential protumorigenic effect of HBV X protein on TGF-beta signaling was shown by Murata *et al* (226), who demonstrated increased expression of linker-phosphorylated Smad3, associated with c-myc expression, in contrast to C-terminally phosphorylated Smad3, associated with p21(WAF1) expression. Both of these pathways are activated by TGF-beta signaling, and can lead to proliferation or cell cycle arrest, respectively (227). In their series, patients with HBV and linker-phosphorylated Smad3 were more likely to develop HCC.

Choi *et al* (228) found the HCV NS5A protein to interact directly with TGF-beta RI and to inhibit Smad 2 phosphorylation as well as the dimerization of Smads 3 and 4. They hypothesized that this provided a means for the virus to inhibit TGF-beta signaling.

Other effects of TGF-beta may also modulate tumor development. Yang *et al* (229) found that TGF-beta 1 could downregulate the tumor suppressor PTEN primarily by accelerating its turnover via the ubiquitin-proteasome pathway, and upregulation of carbonic anhydrase IX, which is associated with metastatic potential, has also been shown to be related to TGF-beta in Hep3B HCC cells (230).

An interesting association has been found between activation induced cytidine deaminase, an enzyme associated with somatic hypermutation and other alterations of immunoglobulin genes, and HCC. Transgenic mice overexpressing AICD develop both lung adenocarcinoma and HCC. With this backdrop, Kou *et al* (231) found increased AICD in HCC and cirrhotic liver and further found that TGF-beta led to upregulation of this enzyme. Since this was often associated with p53 mutation, this process may be of particular interest in those HCC with this genetic change.

In the p19 (ARF) null hepatocyte model, TGF-beta signaling induces PDGF autocrine signaling by PDGF-A production and expression of PDGF receptors (196). Fischer *et al* (195) demonstrated that this subsequently led to beta-catenin activation and nuclear translocation in conjunction with PDGF and accompanied by EMT. TGF-beta also phosphorylates beta1 integrin via Smad 2 and 3, and this can mediate vascular invasion (232). Blockade of TGF-beta signaling by the TGF-beta R kinase inhibitor LY2109761 upregulated E-Cadherin production and decreased *in vitro* migratory capabilities of HCC cells (233). In a xenograft HCC model this treatment was associated with decreases in tumor proliferation rate, vascular invasion, and metastasis (234). The authors suggested that this agent might find clinical use to inhibit HCC spread.

LY2109761 was also found to inhibit the TGF-beta -dependent upregulation of connective tissue growth factor, which is associated with an increase in tumor stroma and evidence of invasive tumor behavior (235).

Overexpression of Smad7 is also frequently present in HCC tissues and this negative regulator of TGF-beta signaling has been suggested as a means whereby tumor cells can escape the growth inhibitory effect of TGF-beta (236).

3.7.4. TGF-beta clinicopathologic correlations

The polymorphism -509C->T is associated with several types of cancer, and Qi *et al* (237) examined the distribution of this in their population of Chinese patients with HBV infection. In this group, those with the CC genotype had higher median plasma TGF-beta 1 levels and this genotype was found more frequently in HCC patients. Assay of circulating TGF-beta 1 was found to have a high sensitivity and specificity for the diagnosis of HCC at levels over 1.2 mcg/L in the series of Dong *et al*. (238).

Expression microarray analysis suggests that TGF-beta -related gene signatures are of use in predicting behavior and survival of HCC. Coulouarn *et al* (239) defined a model TGF-beta signature by differential analysis of hepatocytes from wild type and TGF-beta RII knockout mice exposed to TGF-beta, and further identified an early signature with genes associated with apoptotic and cell cycle arrest pathways, and a late signature that highlighted genes associated with cell adhesion, cytoskeletal organization and matrix remodeling. Applying this to clinical HCC, they found that the late signature was associated with decreased mean survival time, more frequent tumor recurrence, and metastatic disease. Similar outcomes were found when this was applied to lung

cancers, suggesting this as reflecting a mechanism common to multiple cancer types.

Immunohistologic studies have yielded inconstant results. Lu *et al* (210) found that HCC that expressed TGF-beta 1, TGF-beta RII and Smad4 were more likely to show vascular invasion and worse histologic differentiation than HCC without this phenotype. In contrast, Mamiya *et al* (240) found a reduction of TGF-beta RII to be associated with these features.

3.7.5. TGF-beta therapeutic correlations

The TGF-beta RI kinase inhibitor LY2109761, mentioned above has been associated with an inhibitory effect on HCC in preclinical models (241). No clinical trials involving this agent are ongoing at present.

Several antibodies have been developed against TGF-beta 2 (Lerdelimumab, Cambridge Antibody Technology, Cambridge UK), TGF-beta 1 (Metelimumab, Cambridge Antibody Technology), or TGB-beta 1-3 (Fresolimumab) (GC1008), (Genzyme, Cambridge MA). Lerdelimumab and Metelimumab are undergoing clinical trials for nonneoplastic conditions associated with fibrosis, and Fresolimumab is currently being assessed in several forms of cancer, with a focus on metastasis suppression (242). No trials include HCC at present.

Several agents target other components of the TGF-beta signaling pathway. Trabedersen (AP12009) (Antisense Pharma, Regensburg, Germany) is a phosphorothioate antisense oligodeoxynucleotide directed against TGF-beta 2 mRNA that has shown some effect against glioblastoma multiforme/anaplastic astrocytoma(243) and is currently being examined in several tumors (such as pancreatic adenocarcinoma(244)) known to overexpress TGF-beta (NCT00844064). No studies of this agent in hepatocellular carcinoma are underway at present.

Given the importance of the TGF-beta pathway in hepatocarcinogenesis, further clinical development of these agents and incorporation into therapeutic protocols for HCC are eagerly awaited.

A number of agents in preclinical development have been recently reviewed by Giannelli *et al* (245).

4. PERSPECTIVE

This overview has highlighted only a small subset of investigations related to the intersection of growth factors and HCC, but two main themes emerge, namely the tremendous recent progress in this area and, secondly, the profound plasticity and adaptability of HCC in orchestrating and exploiting these molecules for continued growth and progression. Examples such as tumoral upregulation of the EGFR pathway in response to therapeutic blockade of MET activity, or modest response to most single targeted agents, should serve as case studies to avoid a myopic approach. Analysis of crosstalk among different growth factor pathways, as well as studies of interaction of growth factors with other tumor cell, stromal cell, and interstitial functions and factors is needed to vet out these potential roadblocks to therapy. The concept of oncogene addiction is applicable to some tumors and allows for specific therapy, but the interlinked multi-network approach employed by HCC suggests that creative design of molecular targeted agents, in combination with existing therapies, is needed to effectively reverse the course of most of these tumors. Studies defining the biology of growth factors and their subversion by HCC have provided cause for much optimism for control of this deadly tumor in the near future.

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Dr. Nalesnik and Dr. Michalopoulos contributed equally to this article.

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Abbreviations: HGF: hepatocyte growth factor; HCC: hepatocellular carcinoma; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; IGF: insulin-like growth factor; IGFR: insulin-like growth factor receptor; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; TGF-beta: transforming growth factor-beta; TGF-alpha: transforming growth factor alpha; HBV: hepatitis B virus; HCV: hepatitis C virus; HGFA: HGF activator; TNF-alpha: tumor necrosis factor-alpha; GAB1: GRB-associated binding protein; GRB: growth factor receptor; CrkL: v-crk sarcoma virus CT10 oncogene homolog-like protein; p120-Ras-GAP: p120 ras-GTPase activating protein; PLC gamma 1: phospholipase C gamma 1; PI3K: phosphoinositide 3-kinase; STAT3: signal transducer and activator of transcription 3; SHP2: SH2-containing protein-tyrosine phosphatase; src: v-src sarcoma viral oncogene homolog; SHC: SH2 domain-containing transforming protein adaptors; IL1: interleukin 1; IL6: interleukin 6; ras: rat sarcoma; SOS: son of sevenless; raf: v-raf-1 murine leukemia viral oncogene homolog 1; MEK: mitogen-activated protein kinase kinase ; ERK/MAPK: mitogen-activated protein kinase; JNK: c-JUN N-terminal kinase; Cox-2: cyclooxygenase-2; mTOR: mammalian target of rapamycin; IKK: I kappa B kinase; NF kappa B:

nuclear factor kappa light chain enhancer of activated B cells; GSK3-beta: glycogen synthase kinase 3 beta; BAD: bcl-2-associated death promoter; PAK1: p21 protein-activated kinase 1; FAK: focal adhesion kinase; HAI: HGF activator inhibitor; HBX protein: hepatitis B virus X protein; SHARP trial: sorafenib HCC assessment randomized protocol trial; PET: positron emission tomography; TKI: tyrosine kinase inhibitor; c-kit: v-kit feline sarcoma viral oncogene homolog protein; FLT3: fms-related tyrosine kinase 3; Tie-2: Tie family angiopoietin 1 receptor tyrosine kinase; HB-EGF: heparin binding EGF-like growth factor; ADAM: a disintegrin and metalloprotease; IFN gamma: interferon gamma; JAK: Janus kinase; EMT: epithelial to mesenchymal transition; SFK: src-family kinase; PTEN: phosphatase and tensin homolog; IGF-BP: insulin-like growth factor binding protein; IRS: insulin receptor substrate; HIF: hypoxia-inducible factor; siRNA: small interfering ribonucleic acid; MMP: matrix metalloproteinase; AAH: aspartyl (aspariginyl)-beta hydroxylase; miRNA: microRNA; PIP3: phosphatidylinositol (3,4,5)-triphosphate; PKB: protein kinase B; DLL4: delta like ligand 4; CLIP: Cancer of the Liver Italian Program; 5FU: 5 fluorouracil; AFP: alpha fetoprotein; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator; SOCS: suppressors of cytokine signaling; Hsp: heat shock protein; ELF: embryonic liver fodrin;

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