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CLINICAL AND SYSTEMATIC REVIEWS

Targeting the vasculature in hepatocellular carcinoma treatment: Starving versus normalizing blood supply

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Traditional treatments for intermediate or advanced stage hepatocellular carcinoma (HCC) such as transarterial chemoembolization (TACE) and anti-angiogenesis therapies were developed to starve tumor blood supply. A new approach of normalizing structurally and functionally abnormal tumor vasculature is emerging. While TACE improves survival in selected patients, the resulting tumor hypoxia stimulates proliferation, angiogenesis, treatment resistance and metastasis, which limits its overall efficacy. Vessel normalization decreases hypoxia and improves anti-tumor immune infiltrate and drug delivery. Several pre-clinical agents aimed at normalizing tumor vasculature in HCC appear promising. Although anti-angiogenic agents with vessel normalizing potential have been trialed in advanced HCC with modest results, to date their primary intention had been to starve the tumor. Judicious use of anti-angiogenic therapies is required to achieve vessel normalization yet avoid excessive pruning of vessels. This balance, termed the normalization window, is yet uncharacterized in HCC. However, the optimal class, dose and schedule of vascular normalization agents, alone or in combination with other therapies needs to be explored further.
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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and ninth most common in women worldwide.[1] The disease carries a high mortality rate and represents the third most frequent cause of cancer death globally. The median survival following diagnosis is poor, ranging from four to 20 months.[2,3]

While potentially curative therapies such as surgical resection, liver transplantation or ablation can result in 5-year overall survival rates of $>70\%$,[4] they are applicable to less than 30% of patients with HCC.[5] Currently, treatment options for patients with intermediate and advanced HCC remain limited and are considered palliative.[6] Transarterial chemoembolization (TACE) which combines injection of chemotherapy and occlusion of the tumor blood supply, has been shown to improve survival in some randomized controlled trials of patients with unresectable HCC but not others.[7–9] After an initial objective tumoral response in approximately 25–40% of patients, treated tumors can revascularize and require retreatment until the capacity to keep the cancer under control is lost. For patients with advanced HCC, the only therapy with proven benefit is the multi-kinase inhibitor sorafenib which extends median overall survival by two to three months.[10]

Amongst its anti-tumor properties, sorafenib also exerts anti-angiogenic effects by inhibiting vascular endothelial growth factor (VEGF) receptor tyrosine kinases.[11] Hence, conventional treatments such as arterial embolization and sorafenib aim to starve the tumor of its blood supply (and therefore oxygen and nutrients). In contrast, an emerging concept in cancer treatment is the "normalization hypothesis" where tumor vessels, which are aberrant both in structure and function, are normalized to improve tumor perfusion and oxygenation. Such approaches have been associated with reduced metastasis and improved delivery of chemo-, radio- and immune therapies.[12]

In this review, we describe the structural and functional abnormalities in HCC blood vessels. We will then discuss the treatment of HCC by targeting the vasculature through two opposing approaches: the traditional method of starving the blood supply and the new paradigm of vasculature normalization.

CHANGES IN VASCULATURE IN HCC

Like other solid tumors, HCC cannot grow beyond a few millimeters in size without angiogenesis.[13] Through a process of angiogenic switch,[14] an HCC is able to evolve from a dysplastic nodule and grow in size by acquiring an increasing number and density of unpaired arteries (i.e., not accompanied by bile ducts) supplying it. This switch is the rate-limiting step in hepatocarcinogenesis and is stimulated by an imbalance of angiogenic factors in favor of those that are proangiogenic. Both tumor cells and adjacent cells secrete VEGF, basic fibroblast growth factors (bFGF), angiopoietins,

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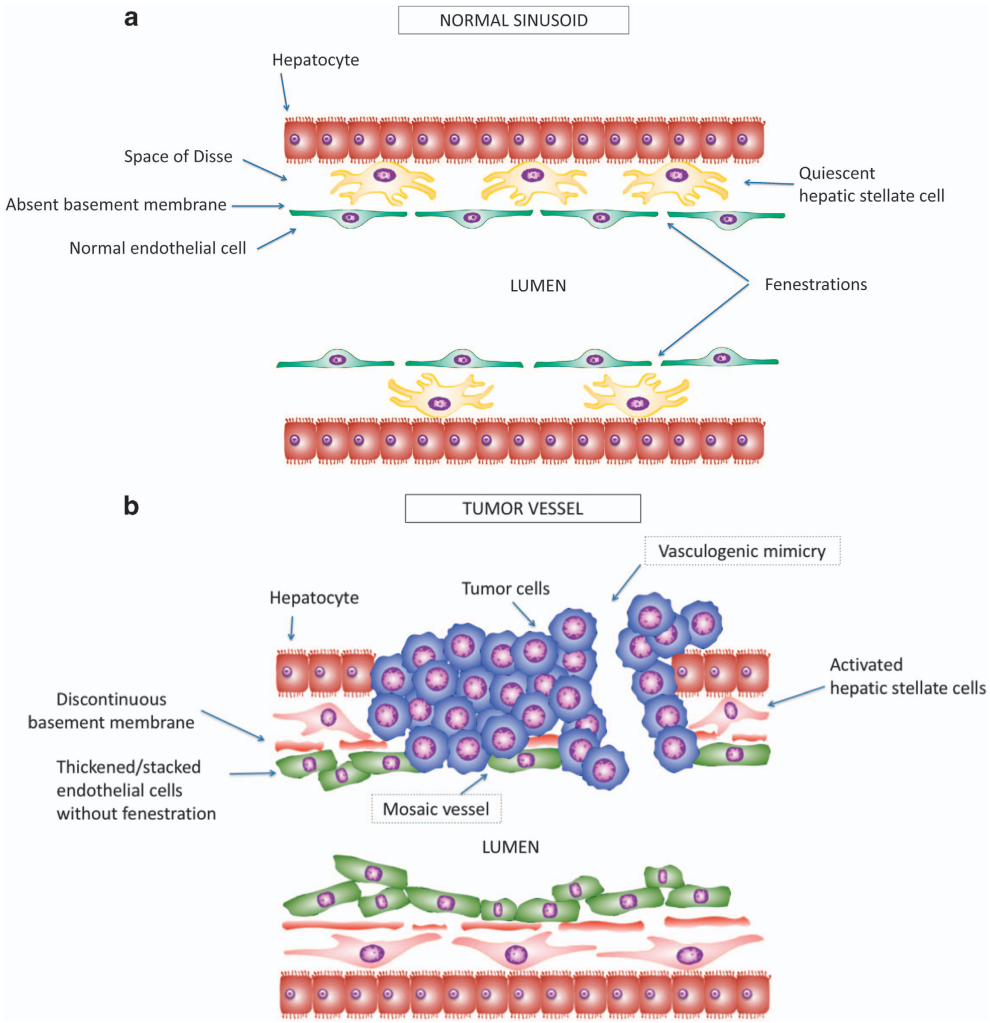

Figure 1 Structure of normal vessels vs. tumor vessels in the liver sinusoid. (**a**) In healthy liver sinusoids, the endothelium is regular, fenestrated and lacks a basement membrane. Hepatic stellate cells remain in a quiescent state. (**b**) In hepatocellular carcinoma, the endothelium is thickened and loses its fenestrations while a discontinuous basement membrane is formed through a process called capillarization. Tumor cells form the vessel wall in some areas. Hepatic stellate cells become activated and release vascular endothelial growth factor as well as other angiogenic factors. These vessels are structurally and functionally abnormal.

platelet derived growth factor (PDGF), placental growth factor (PlGF) and transforming growth factor among others. The tumor subsequently becomes hypervascular and draws blood from ectopic arteries to obtain nutrients for growth and to metastasize to distant organs.[15,16] However, these vessels are both structurally and functionally abnormal (Figure 1).

Tumor vessel structure. Macroscopically, tumor vessels are tortuous, with uneven diameters and irregular branching patterns. While normal liver sinusoidal endothelium is fenestrated and lacks a basement membrane (BM), HCC sinusoidal endothelium is thicker, has fewer fenestrations, shows BM formation and expresses the phenotype of capillary blood vessels. Hence this process is termed capillarization.[17] Endothelial cell proliferation is dramatically increased and circulating bone marrow-derived endothelial progenitor cells and hematopoietic stem cells are recruited to aid tumor angiogenesis.[18] Endothelial cells of HCC vessels can lose their polarity and detach from the BM causing them to stack upon each other (stratification) and protrude into the lumen.[19] Quantitation of these abnormal tumor vessels by immunostaining for endothelial cell markers such as CD34 (i.e., the tumor microvessel density), has been shown to be independent poor prognostic factors for disease-free and overall patient survival in HCC.[20] Furthermore, liver tumor cells themselves actively participate in the formation of new vessels either partially by occupying the vessel wall in mosaic vessels or completely in vasculogenic mimicry,[21,22] which is associated with high tumor grade, invasion and metastasis, and shortened survival.[23]

The BM of the capillarized sinusoids is affected to varying degrees ranging from relatively intact BM in differentiated HCCs to sharply defective BM in more anaplastic HCCs.[24] This degradation of BM is mediated by matrix metalloproteinases (MMP) which are highly expressed in HCC cells.[25] Moreover, degradation of the BM by MMP2 and MMP9

mobilizes proangiogenic factors such as VEGF sequestered in the BM, thus further potentiating tumor angiogenesis.[26] Both MMP2 and MMP9 expression have been demonstrated to be predictors of poor prognosis in HCC patients.[27,28]

Capillaries are enveloped by mural pericytes. In the liver, the hepatic stellate cell (HSC) expresses multiple smooth muscle cell markers and are considered the pericyte equivalent.[29] In HCC, HSCs become activated by secretion of cytokines such as PDGF and transforming growth factor-β by tumor cells. Tumor-activated HSCs in turn create a proangiogenic, prometastatic microenvironment by facilitating endothelial proliferation and survival through release of VEGF as well as other angiogenic factors. While pericytes are deficient in the abnormal vessels of non-HCC tumors,[30] activated HSCs proliferate and enhance their coverage of the sinusoids in cirrhosis and HCC.[29] Increased numbers of HSCs in the HCC microenvironment is associated with cell migration and invasion.[31]

Abnormalities of tumor vasculature are also seen at the ultrastructural and molecular level. Schmitt *et al.* demonstrated VEGF-induced disruption of occludin-delineated tight junctions in HCCs and peritumoral normal liver parenchyma, thus facilitating a possible mechanism for tumor invasion.[32] The aberrant expression of claudins, which are integral structural and functional components of tight junctions, is observed in HCC and may have a causal role in tumor formation and progression by inducing epithelial–mesenchymal transition (EMT).[33] In addition, vascular endothelial (VE)-cadherin (a key protein in endothelial adherens junctions) is endocytosed and uncoupled from catenin-associated proteins in response to VEGF. As a consequence, endothelial cell-cell junctions are loosened and vascular permeability is increased in VEGF-induced tumor angiogenesis.[34]

Tumor vessel function. These abnormalities in endothelial cells, BM and cell junctions collectively contribute to tumor vessels which are excessively leaky in HCC with several consequences arising from this.[35,36] First, the extravasation of proteins and fluid into tumor interstitium leads to peritumor edema and interstitial hypertension via increases in oncotic pressure and hydrostatic pressure. The impaired diffusion of molecules and vascular collapse caused by capillarized sinusoids, peritumoral edema and interstitial hypertension reduces delivery of oxygen and therapeutic agents into the tumor.[37,38] This results in a tumor microenvironment of hypoxia, acidosis and potentially reduced efficacy of anti-cancer treatments. Prognostically, tumor pressure in HCC has been shown to correlate with differential grade, presence of vascular invasion and intrahepatic metastasis, as well as local and distant recurrence rates after treatment.[39,40]

Second, the poorly organized tumor vasculature with tortuous, irregularly shaped, and leaky vessels is less responsive to vasoactive signals and unable to support efficient blood flow.[41] There is considerable heterogeneity in tumor blood flow which is brisk in some areas and sluggish in others.[42] This variation is not only observed spatially, but also temporally as blood flow changes with continuous vessel remodeling. This patchy perfusion leads to non-uniform delivery of oxygen, nutrients and drugs to the tumor. The influx of immune effector cells into the tumor is also impaired due to alterations in leukocyte-endothelium adhesion molecule and chemokine expression.[43]

Third, the rapid proliferation of tumor and non-tumor cells, leaky vessels and regional hypoperfusion all result in hypoxia which is a potent stimulator of angiogenesis mediated by the expression of hypoxia-inducible factor 1 (HIF-1).[44] This leads to the formation of more non-productive HCC vessels which further aggravates hypoxia thereby establishing a vicious cycle (Table 1 and Figure 2). Tumor hypoxia has numerous other cancer-promoting effects in HCC which will be discussed in the following section.

TRADITIONAL TREATMENT APPROACH—STARVING HCC OF BLOOD SUPPLY

The recognition of the classical model of tumor angiogenesis as a therapeutic target was made in the 1970s by Folkman.[45] Simplistically, it was thought that limiting or obliterating the angiogenic response could improve outcomes, essentially starving the tumor to death. As HCCs typically have arterial hypervascularity, it would therefore seem logical to starve the tumor of its blood supply as a therapeutic approach. First described by Doyon *et al.* in 1974,[46] transarterial embolization (TAE) achieves angiographic occlusion of the HCC blood supply using embolizing agents. It capitalizes on the unique situation of HCC acquiring its blood supply from the hepatic artery while the surrounding liver (with dual blood supply) receives blood predominantly from the portal vein.[47] The selective arterial occlusion by embolic agents such as gel foam or polyvinyl alcohol results in tumor ischemia, hypoxia and ultimately necrosis while minimizing damage to liver tissue. The addition of regional chemotherapy (TACE) with lipiodol (theoretically) enhances anti-tumor effects as chemotherapeutic agents can be given at higher concentrations and remain localized in the tumor for longer periods.[48] As radioembolic agents do not work by creating ischemia from vessel occlusion,[49] they will not be discussed here.

Efficacy of arterial embolization. Over the past decade, TACE has become standard of care for patients with Barcelona clinic liver cancer (BCLC) stage B (intermediate) HCC.[50] Although early randomized controlled trials (RCTs) demonstrated strong anti-tumor effects in TAE or TACE compared with conservative or suboptimal treatments (e.g., tamoxifen or intravenous 5-fluorouracil), all failed to show a survival benefit. It was not until 2002, that two RCTs demonstrated improved survival.[7,9] Results from meta-analyses and systematic reviews are overall in favor of TACE over non-active treatment. Two meta-analyses which included almost identical studies (five out of six RCTs in common) both found TAE or TACE improved the two-year survival compared with non-active treatment in patients with unresectable HCC.[51,52] Overall, the improvement observed in survival with arterial embolization was 46–47%. On the other hand, a more recent Cochrane meta-analysis of nine RCTs revealed no difference in survival.[53] However, the inclusion of studies of patients with either early stage HCC (who benefit most from curative therapies) or advanced HCC (who have poor response and less tolerance to TACE) may have biased these results.[54] While studies of TACE may differ

procedurally in use of chemotherapy agents (or lack thereof), embolizing material and number of repeated treatments, it is clear that patient selection is important. The most ideal candidates for TACE are patients with liver confined tumors (unresectable, without vascular invasion), preserved liver function (Child-Pugh class A or B) and the absence of portal vein thrombosis.

TAE vs. TACE. Although TACE is the more widely accepted treatment approach, it remains doubtful whether it is superior to TAE alone. Several RCTs and two meta-analyses have indicated no survival difference between the two treatments.[51,55–59] These results suggest TACE derives its anti-tumor effects predominantly from the ischemic effect due to embolization rather than the addition of chemotherapy.

Table 1 Detrimental effects of hypoxia in carcinogenesis

Detrimental effects of hypoxia in carcinogenesis
Induction of a more aggressive tumor phenotype
Upregulation of growth factors
Induction of apoptosis resistance[60]
DNA hypermethylation of tumor suppressor genes[68]
Induction of EMT[70]
Impaired anti-tumor immune response
Impaired entry of anti-tumor immune cells, e.g., CD8+ T cells[36]
Recruitment of immunosuppressive Treg cells and MDSCs[80,83]
Increased expression of immune checkpoints[85]
Stimulation of angiogenesis[60]
Induction of chemoresistance and radioresistance[60]

EMT, epithelial-mesenchymal transition; MDSC, myeloid-derived suppressor cell.

Limitations of TACE and the effect of tumor hypoxia. Aside from the aforementioned restrictions on patient selection, TACE holds other drawbacks in HCC treatment. Although the intention of TACE is to starve the HCC of its blood supply, the resulting hypoxia in the tumor has subsequently been shown to stimulate dedifferentiation, proliferation, angiogenesis and metastasis of the cancer itself.[60] Recently, Lai *et al.*[61] demonstrated a significant association between hypoxia in TACE treated HCCs and the induction of CK19, a marker for an aggressive tumor phenotype. A histological study of 24 HCCs treated with TAE in patients undergoing surgical resection found the proliferative activity of tumor cells and intratumoral endothelial cells was increased after TAE compared to untreated tumors.[62] Accordingly, the rate of local recurrence after initial TACE is upwards of 80% and recurrent tumors have significantly shorter doubling times compared to primary HCCs.[63]

Central to these processes is the role of HIF-1α, a heterodimer transcription factor, which induces the expression of genes involved in cell survival, proliferation and angiogenesis.[44] During normoxia HIF-1α is hydroxylated, ubiquinated and rapidly degraded by proteosomes. In response to hypoxia, HIF-1α binds to the promoter region of VEGF and induces its transcription. Protein levels of both activated HIF-1α and VEGF are significantly increased following TACE.[64,65] During hypoxia, HIF-1α and VEGF through the modulation of other proteins (such as myeloid cell factor 1 and Bcl-2) create an environment of apoptosis-blocking and tumor cell survival.[66,67] Specifically, arterial embolization has been shown to upregulate the anti-apoptotic protein Bcl-2 which causes HCC cells to escape apoptosis induced by anoxic injury, rendering them resistant to further embolization treatments.[65] In addition, hypoxia can contribute to carcinogenesis via HIF-1α

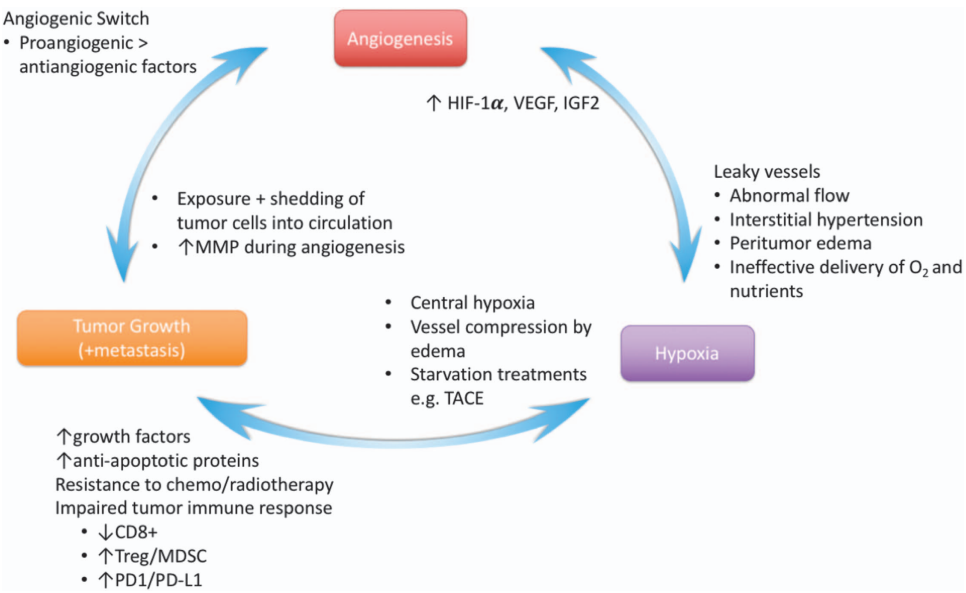

Figure 2 Vicious cycle of hypoxia, non-productive angiogenesis and tumor growth. Angiogenesis is required for a tumor to grow beyond a few millimeters. However, this neovascularisation produces abnormal leaky vessels which give rise to interstitial hypertension, edema and tumor hypoxia. Although some treatments (e.g., TACE) aim to achieve hypoxia in order to kill the tumor by starvation, hypoxia has been demonstrated to stimulate further angiogenesis and tumor growth through a variety of mechanisms (see text: Limitations of TACE and the effect of tumor hypoxia). HIF-1α, hypoxia-inducible factor 1α; IGF-2, insulin-like growth factor-2; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinases; TACE, transarterial chemoembolization; VEGF, vascular endothelial growth factor.

independent processes such as DNA hypermethylation of tumor suppressor genes.[68] The upregulation of HIF-1α and VEGF along with other angiogenic factors such as insulin-like growth factor-2 (IGF-2) induced by hypoxia play a major role in the stimulation of neovascularisation[69] and EMT[70] which facilitate tumor progression and metastasis. Moreover, reciprocal positive regulation exists between these factors as IGF-2 increases HIF-1α stability which in turn induces VEGF expression.[71] Prognostically, increased plasma levels of HIF-1α, VEGF and IGF-2 are all associated with the development of metastasis and poor outcomes in post-TACE patients and HCC patients in general.[72–75]

Tumor hypoxia is also a known driver of chemoresistance in HCC. Through both HIF-1α-mediated and HIF-1α-independent pathways, hypoxia can protect tumor cells from chemotherapy induced apoptosis.[76,77] In addition, hypoxia elicits the expression of multidrug resistance-related genes such as multidrug resistance protein 1 and lung resistance protein in HCC cell lines.[77] An autocrine signaling loop involving PDGF-BB, Akt and HIF-1α which confers cisplatin resistance in HCC cell lines under hypoxic conditions has also been discovered.[78] Conversely, HIF-1α downregulation by antisense gene therapy enhances the therapeutic efficacy of doxorubicin against HCC.[79] These mechanisms could explain the lack of an additive effect seen in chemoembolization over bland emolization alone, as discussed previously, while the use of systemic chemotherapy in HCC has similarly been ineffective.

The tumor microenvironment is altered by hypoxia in its immune status. Although not yet extensively studied in HCC, the effect of hypoxia on the intrahepatic immune infiltrate may be crucial.[75] Hypoxia has been shown, in other cancers, to promote chemokine-mediated recruitment of immunosuppressive Treg cells[80,81] and myeloid-derived suppressor cells.[82] Furthermore, recruited monocytes and resident macrophages in the hypoxic tumor microenvironment differentiate into tumor associated macrophages (TAMs). The polarization of these TAMs favor a tumor-promoting M2-like phenotype over a tumor-suppressive M1-like phenotype.[81] This concept that hypoxia converts the tumor microenvironment from immunosupportive to immunosuppressive appears to also apply in HCC.[83] Indeed, both the presence and balance (CD8+ effector cells vs. Treg) of within tumor infiltrating lymphocytes have proved to be independent prognostic factors in HCC.[84] Furthermore, programmed death-ligand 1 (PD-L1) expression is increased by HIF-1α in hypoxia and facilitates the evasion of anti-tumor immunity by HCCs.[85,86]

Thus, through the exacerbation of hypoxia (in an already hypoxic microenvironment), the anti-tumor effects of TAE or TACE are modest and self-limiting. Arterial embolization may, in fact, paradoxically promote the HCC to become more aggressive and evasive leading to progression and metastasis. The limitation of starving tumor blood supply to treat HCC is further highlighted by the lack of efficacy seen when combining TACE with anti-angiogenic therapies such as sorafenib[87] and bevacizumab.[88] Clearly other approaches need to be explored.

Mechanisms of action of current anti-angiogenic therapies. Although TACE is the *par excellence* example of targeting HCC via vascular starvation the current small molecule tyrosine kinases also do this to a certain extent. Sorafenib, regorafenib, lenvatinib, sunitinib, cediranib and axitinib are multi-targeted tyrosine kinase inhibitors trialed in HCC with activity against receptor tyrosine kinases involved in neovascularization and tumor progression including VEGF receptors (VEGFR) 1–3 and/or PDGF receptors (PDGFR)-α and -β. Both VEGF and PDGF pathways are key mediators of angiogenesis which are overexpressed and play significant roles during hepatocarcinogenesis.[89] When VEGF interacts with VEGFRs on the endothelial cell surface, it causes autophosphorylation of its intracellular tyrosine kinase and activation of downstream proteins resulting in a mitogenic effect on endothelial cells.[90] The binding of PDGF to its receptors, after dimerization and activation of the intracellular tyrosine kinase, stimulates endothelial cell migration (rather than proliferation) as well as survival and migratory signals to pericytes that provide support to vascular endothelial cells.[91] Inhibition of these intracellular tyrosine kinase receptors has been shown to reduce endothelial cell proliferation, tubule formation, microvascular area and density in tumors (i.e., starving the tumor by inhibiting angiogenesis).[11,92] Multi-kinase agents which target both VEGFRs and PDGFRs have additive effects on limiting angiogenesis as inhibiting PDGFRs has been shown to cause pericyte detachment from the endothelium, leaving endothelial cells more susceptible to VEGF inhibition.[91] Furthermore, some multi-targeted have direct anti-cancer effects. For example, sorafenib inhibits Raf kinase which is part of the Raf/MEK/ERK signaling cascade involved in cell growth and survival and is overactivated in HCC.[11] Hence multi-kinase inhibition may enable these small molecules to be used as monotherapies, something that has not proved effective with the pure VEGF antagonist bevacizumab. The efficacy of these anti-angiogenic therapies in clinical trials is discussed below.

NOVEL TREATMENT APPROACH—NORMALIZATION OF VASCULATURE IN HCC

The normalization hypothesis. Realizing the limitations of starving tumor vasculature due to the adverse consequences of hypoxia, a growing number of pre-clinical and clinical studies have explored the emerging (yet counterintuitive) paradigm of normalizing vasculature to treat cancer. Normalization of the vasculature adopts approaches that reverse the classical phenotype of tumor blood vessels at the cellular and molecular level as opposed to simply obliterating the vasculature. This essentially means a change to the tumor microenviroment with less hypoxia, less vascular leak, increased pericyte numbers together with an increase in the infiltration of CD8+ T cells and a decrease in the neutrophil to lymphocyte ratio (i.e., the opposite to changes seen in new HCC tumor vessels as described above).[36] Such changes have the potential increase delivery of other cancer directed therapies and may be synergic with immune directed therapies such as checkpoint inhibition.

Pre-clinical studies. Examples of effective vessel normalization have been now been demonstrated at the experimental level. Hamzah *et al.*[93] observed that vessel normalization in

Table 2 Pre-clinical agents that have potential normalization activity

Pre-clinical agents that have potential normalization activity
PlGF inhibitors[96,97]
Some traditional Chinese medicine compounds[102–104]
HIF-1α inhibitors[99]
VE-Cadherin modulator (e.g., Blockmir to inhibit miR-27a)[106]

HIF-1α, hypoxia-inducible factor 1α; PlGF, placental growth factor; VE-Cadherin, vascular endothelial cadherin.

Table 3 Features of vessel normalization

Features of vessel normalization[36]
Reduced vessel diameter and tortuosity
Decrease in vascular permeability
Decrease in tissue hypoxia
Decrease in interstitial pressure and edema
Increase in pericyte coverage around blood vessels
Improvement in number and function of intratumoral immune cells

tumor-bearing mice deficient in Rgs5 (a protein overexpressed by pericytes in aberrant tumor vasculature) was associated with significant increases in infiltrating tumor-specific CD4+ and CD8+ T cells and prolonged survival. In a separate study of mice treated with a designer angiostatic peptide, anginex, increased leukocyte infiltration was also seen through improved leukocyte-vessel wall interactions in tumor vessels.[94]

Several *in vitro* and *in vivo* studies of vessel normalizing agents have now also documented anti-tumor activity against HCC (Table 2). Placental growth factor is a member of the VEGF subfamily. After binding to its receptor VEGFR-1, it induces pro-cancer responses in endothelial, malignant, and immune cells. Increased expression of PlGF is associated with poor prognosis in HCC.[95] Importantly, PlGF inhibition reduces tumor growth and induces vessel normalization in experimental HCC models.[96,97] Histidine-rich glycoprotein (HRG) is a host-produced protein deposited in the tumor stroma which can induce a change in polarization TAMs in favor of the M1-like phenotype. This effect of HRG on TAM polarization also indirectly resulted in tumor vessel normalization. Both these processes are PlGF-mediated. Furthermore, Vandewynckel *et al.*[98] recently demonstrated that PlGF inhibition possibly exerts its anti-tumor effects by improving intratumor hypoxia which is a potent activator of the pro-survival, PKR-like endoplasmic reticulum kinase (PERK) pathway in HCC cells.

A number of pharmacological agents that target the driver of abnormal vasculature, HIF-1α, have also been linked to vasculature normalization. EZN-2968 is a RNA antagonist that specifically binds HIF-1α mRNA and reduces its expression by 80% and the expression of VEGF mRNA by 50% in mice livers.[99] A phase I study presented results demonstrating its anti-tumor activity in patients with advanced malignancy including HCC.[100] Another suppresser of HIF-1α is NVP-BEZ235, a dual PI3K/mTOR inhibitor which induces apoptosis of hypoxic cells. This novel agent has exhibited promising activity against HCC.[101]

Several vasoactive traditional Chinese medicine compounds have exhibited anti-tumor efficacy against HCC in mouse xenograft models. Sinomenine hydrochloride, a known inducer of vascular normalization,[102] has also been shown to inhibit HCC growth by promoting cell cycle arrest and caspase-dependent apoptosis.[103] Tanshinone IIA, a herbal extract from Chinese sage (*Salvia miltiorrhiza*), inhibited HCC metastasis and improved survival after palliative resection through the promotion of VEGFR-1/PDGFR-related vascular normalization.[104] This anti-metastatic potential was also seen in a study of Buyang Huanwu decoction.[105]

As the benefits of vascular normalization extend beyond cancer, novel targets also arise from non-oncological studies of vascular biology. We have developed a novel Blockmir to inhibit miR-27a, a microRNA which targets VE-cadherin resulting in its downregulation. This led to vascular normalization and potently enhanced recovery from ischemic limb injury in mice.[106] Early experience with the same Blockmir in a subcutaneous isograft HCC model demonstrated an inhibition of tumor growth (Zhao *et al.* Accepted Cancer Res. 2017). In the B16F10 melanoma model, the Blockmir decreased vascular leak and tissue hypoxia, increased pericyte numbers, induced greater infiltration of T cells into the interior of the tumor and showed a reduction in tumor growth by 60% (Zhao *et al.* Accepted Cancer Res. 2017). Interestingly, such vascular normalization effects plus the effect of an anti PD-1 monoclonal antibody showed a synergistic anti-tumor effect.

CURRENT CLINICAL ANTI-ANGIOGENIC THERAPIES: DO THEY HAVE VESSEL NORMALIZING POTENTIAL?

Non-HCC directed therapies. The normalization hypothesis was initially proposed in an attempt to explain the observation that some anti-angiogenic therapies potentiate the effects of chemotherapy and radiotherapy.[12] Multiple clinical trials have shown that combination therapy with bevacizumab (humanized anti-VEGF monoclonal antibody) and conventional chemotherapy improved patient survival and response rates over either therapy alone. This effect was observed across multiple advanced stage (metastatic) cancers: colorectal cancer, non-small cell lung cancer, breast cancer and renal cancer.[107–110] Given that anti-VEGF therapy aims to starve tumor blood supply and is associated with vessel pruning while chemotherapy relies on this same blood supply for drug delivery, these findings were unexpected. It was hypothesized by Jain *et al.* that anti-VEGF therapy augmented chemotherapy delivery and efficacy by transiently reversing vessel abnormalities (and hence the tumor microenvironment) without destroying them. In a landmark study, the same group went on to demonstrate that within 12 days of infusion, a single dose of bevacizumab in rectal carcinoma patients reduced microvascular density and improved vessel permeability, pericyte coverage and interstitial hypertension.[111] These are all markers of vascular normalization (Table 3). Conversely, Yang *et al.* recently showed that discontinuation of anti-VEGF treatment in mice created a period of profound structural abnormality in liver sinusoidal capillaries with enlargement of pore sizes, loss of VE-cadherin and hyper-permeability.[112] These changes led

Table 4 Potential approaches using vessel normalization in hepatocellular carcinoma

Potential approaches using vessel normalization in hepatocellular carcinoma
Normalization alone
Normalization and established chemotherapy drugs (increase drug delivery)
Normalization and multi-kinase inhibitors (increase drug delivery)
Normalization and immunotherapy e.g., checkpoint inhibitors (increase entry and activity of anti-tumor immune cells)

to tumor extravasation and marked promoted liver metastases in a mouse colorectal cancer model.

It is important to recognize that beneficial effects of anti-VEGF therapy may be dose dependent. According to Jain and colleagues, a paradox exists with anti-angiogenic therapy where judicious application leads to selective pruning of immature tumor vessels leaving a relatively normalized network of vessels.[12] While on the other hand, sustained or high doses may result in excessive regression of vasculature leading to the same adverse effects of hypoxia seen in treatment attempts to starve the tumor.[113] This delicate balance has been termed the "normalization window".[12] This refers to a transient pharmacologically induced time period after the commencement of anti-angiogenic therapy during which tumor vessels exhibit features of normalization and improved functionality (reduced hypoxia) resulting in increased vulnerability of cancer cells to cytotoxic therapies.[114] The commencement and duration of this window has been studied across different cancers using different vessel normalizing agents and can vary widely. For example, the time period of increased oxygenation in mice melanoma, breast carcinoma and ovarian carcinoma models treated with bevacizumab was demonstrated to be between day two and day four after starting treatment.[115] In comparison, mice with human glioma xenografts treated with anti-angiogenic agent suramin showed improved oxygenation compared to controls for up to 5 weeks afterwards.[116] The normalization window in human HCC is currently not known.

HCC directed therapies. In contrast to the aforementioned studies in other cancers, the synergistic effects of anti-angiogenic therapies have not been reproduced in HCC. Phase II trials of bevacizumab combined with systemic chemotherapy[117,118] did not show numerically superior response rates (RR), progression-free survival (PFS), or overall survival (OS) compared to bevacizumab alone or chemotherapy alone.[119,120]

Thalidomide exerts anti-angiogenic effects via inhibition of VEGF, bFGF and HIF-1α to improve tumor hypoxia and interstitial hypertension—changes associated with vessel normalization.[121–123] However, clinical trials of thalidomide either alone[124,125] or in combination with chemotherapy[126] or radiotherapy[127] in unresectable HCC have been met with disappointing results ($<$5% RR).

Although sorafenib and sunitinib can destroy tumor vasculature, they also both demonstrate the ability to normalize tumor vasculature.[128] Currently, sorafenib is licensed for use as monotherapy but it might prove effective when combined with chemotherapy on the basis of its vascular normalizing properties. An exploratory phase II RCT of sorafenib plus doxorubicin vs. doxorubicin alone in advanced HCC found encouraging results with greater median PFS and OS seen in combination therapy.[129] However, the same authors could not confirm the superiority of sorafenib plus doxorubicin compared with sorafenib in a phase III trial of 346 patients.[130] No survival benefit was seen at the cost of higher toxicity. Trials of sunitinib with reduced dosing showed modest activity with 2.9% RR, 3.9 months PFS, and 9.8 months OS.[131] Interestingly, a greater decrease in tumor vascular permeability (K^{trans}, measured by dynamic contrast-enhanced magnetic resonance imaging) at day 14 after treatment was associated with partial response or stable disease. This suggests that degree of vascular normalization may be a determinant of HCC response to sunitinib. Nevertheless, a phase III trial of sunitinib vs. sorafenib in untreated patients with advanced HCC was terminated early due to significantly worse survival (7.9 vs. 10.2 months median OS) and higher toxicity.[132]

In recent developments, another multi-kinase inhibitor lenvatinib has been reported to have non-inferior overall survival compared to sorafenib for first-line treatment in patients with unresectable HCC.[133] In the second-line setting, regorafenib was also found to significantly improve OS in patients with HCC who progressed on sorafenib.[134] However, the effects of these treatments on vessel normalization are not known.

Targeting the epidermal growth factor signaling pathway via erlotinib can reduce vascular permeability, tumor hypoxia and enhance responses to chemotherapy and radiation.[135] Phase II studies in advanced HCC have demonstrated 0–9% RR, 3–4 months PFS and 10–13 months OS.[136,137] However, the combination of erlotinib with other anti-angiogenesis agents such as sorafenib and bevacizumab failed to show any additional benefit.[138,139]

Other agents directed against VEGFR such as cediranib, axitinib and ramucirumab have all demonstrated normalization properties in other cancers but have not been shown to benefit HCC patients beyond the effect seen with sorafenib.[140–145] Thus, although normalization effects are seen with some of the above agents, synergistic effects in human HCC either with chemotherapy or anti-angiogenic therapies have not so far been seen.

FUTURE PERSPECTIVES

Vascular normalization with anti-angiogenic agents is now an emerging approach to treat many cancers. In human HCC clinical trials, current anti-angiogenic therapies (beyond sorafenib) have not found the "normalization window" in order

to improve outcomes. It is likely that the optimal class, dose and schedule of these agents required to achieve normalization and yet avoid excessive pruning is not known. Moreover, the exact timing of combination chemotherapy or radiotherapy in order to capitalize on the normalization window when their anti-tumor effects are enhanced is similarly unclear. These unknowns may partially explain why benefits seen in pre-clinical studies have not translated into clinically significant improvements. Another unanswered question is whether and how this transient normalization window can be prolonged. Clearly further characterization of the normalization window is needed. Current approaches to treat HCC using anti-VEGF therapies and multi-targeted tyrosine kinase inhibitors are unlikely to be sufficient even if used appropriately. Therefore, a multipronged approach involving several anti-angiogenic pathways is likely to be required. Other relevant mediators of vessel normalization and their drug targets also need to be explored. Novel strategies such as targeting VE-cadherin particularly in combination with checkpoint inhibitors appear to be promising (Table 4).

Contributing to this challenge is a lack of validated surrogate biomarkers to signify when, or indeed if, vascular normalization has occurred. In addition to guiding appropriate dosing and scheduling of therapy, biomarkers can be used to identify patients who may benefit most from vascular normalization, while avoiding futile treatment and toxicity in others. Traditional methods to assess response based on tumor shrinkage[146] may not accurately reflect reduction in viable tumor burden (without necessarily reducing size) due to necrosis caused by anti-angiogenic therapies. Functional imaging of HCC vasculature such as perfusion computed tomography, dynamic contrast-enhanced magnetic resonance imaging or ultrasound have proved to be useful biomarkers in HCC but are limited by their complexity, cost and need for specialized technologies and expertise.[131,147] Blood-based biomarkers for antiangiogenic therapy have similarly shown promise in predicting outcomes[131] but require validation in large prospective RCTs.

Since the cancer-promoting effects of hypoxia impacts on numerous different mechanisms, emergence of new anticancer therapies will continually provide opportunities for combination therapy. For instance in the advent of immune checkpoint inhibitors used to treat other advanced cancers, combination therapy between sorafenib, anti-programmed death receptor-1 (PD-1) antibody and other immunotherapies to concomitantly target the hypoxic and immunosuppressive microenvironment has shown promise.[146]

CONCLUSION

Hepatocellular carcinoma is a hypervascular tumor with a poor prognosis and heavy global burden. There has been much interest in targeting its vasculature as a therapeutic approach.

While TACE improves survival in carefully selected patients it may eventually become a victim of its own success due to the detrimental effects of tumor hypoxia and thus limit its overall efficacy. To date the clinical efficacy of anti-angiogenic agents in advanced HCC, either alone or in combination with other therapies, has been modest at best. Although there is evidence pointing to benefits of vasculature normalization, the results have failed to demonstrate comparable efficacy with the current standard of care, sorafenib. A better understanding of the normalization window is required to guide dosing of anti-angiogenic therapy in relation to concomitant therapies. The development of biomarkers may help in selecting patients who benefit from these targeted therapies.

CONFLICT OF INTEREST

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