Am J Transl Res 2015;7(10):1675-1698
www.ajtr.org /ISSN:1943-8141/AJTR0013738

Review Article

Evading anti-angiogenic therapy: resistance to anti-angiogenic therapy in solid tumors

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Received July 31, 2015; Accepted September 27, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Vascular endothelial growth factor (VEGF) dependent tumor angiogenesis is an essential step for the initiation and promotion of tumor progression. The hypothesis that VEGF-driven tumor angiogenesis is necessary and sufficient for metastatic progression of the tumor, has been the major premise of the use of anti-VEGF therapy for decades. While the success of anti-VEGF therapy in solid tumors has led to the success of knowledge-based-therapies over the past several years, failures of this therapeutic approach due to the development of inherent/acquired resistance has led to the increased understanding of VEGF-independent angiogenesis. Today, tumor-angiogenesis is not a synonymous term to VEGF-dependent function. The extensive study of VEGF-independent angiogenesis has revealed several key factors responsible for this phenomenon including the role of myeloid cells, and the contribution of entirely new phenomenon like vascular mimicry. In this review, we will present the cellular and molecular factors related to the development of anti-angiogenic resistance following anti-VEGF therapy in different solid tumors.

Keywords: VEGF, vascular mimicry, biomarkers, myeloid cells

Introduction

Historically, the association between human diseases and vascular dysfunction has been envisaged as far back as the first century AD by Celsus (Aulus Cornelius Celsus, De medicina, c. A.D. 25). Celsus not only described four fundamental features of inflammations namely *tumor, rubor, calor,* and *dolor,* but also identified the phenotypic association between several inflammatory diseases and microvascular dilation, as well as hyperpermeability. Well over 100 years ago, Rudolph Virchow, a notable German scientist observed that growing tumors have a rich vascular network [1] and this observation was later substantiated by the seminal work of Ide [2] and Algire [3] who demonstrated the role of an abundant blood supply in the growth of tumors. In following years, a reciprocal causative role of tumor cells in the growth of tumor associated blood vessels was identified to support the hypothesis that tumors produce diffusible factors which can promote angiogenesis [4, 5]. This formed the foundation of Dr. Judah Folkman's landmark discovery in 1971 identifying key molecules of tumor angiogenesis and hence their importance in cancer therapy on the basis of "starve a tumor to death" hypothesis [6]. The discovery sprouted tremendous impetus to understand the biology of tumor associated blood vessels, both existing and growing, in the scientific community. In following years, many pro-angiogenic and anti-angiogenic targetable factors were identified, including VEGF which ultimately led to FDA approval for bevacizumab (Avastin) for the treatment of many solid tumors. Eventually, a new category of treatment modality in cancer therapy called anti-angiogenic therapy was opened [7-9] (**Table 1**). On the other hand, it was also revealed that tumor-associated or tumor-enticed blood vessels were far from normal blood vessels. For example, tumor directed new uncontrolled blood vessels were demonstrated to be disorganized and leaky [10, 11] indicating basic differences between normal endothelium and tumor associated endothelium. These important facts in the course of time formed the basis of (1) arguments in favor of anti-angiogenic therapy and (2) counter arguments for such therapy due to development of resistance to anti-angiogenic therapy in cancer.

Endothelial cells and angiogenesis

Studies have revealed a number of conceptual advantages for anti-angiogenic therapies including, (1) low tumor endothelial cell (TEC) to tumor cells ratios within the tumor, (2) least organ type differences among TECs and (3) genetically stable nature of TECs which makes them less prone to acquire drug resistance [12, 13]. The signal transduction events occurring within endothelial cells (EC) which together encodes for neovascularization/angiogenesis are poorly understood. Several studies have suggested the role of Rho family of small GTPases in angiogenesis and tumor progression [14-17]. A study by Fernandez-Zapico et al. [15] has revealed a novel role of hematopoietic specific GTPase exchange factor VAV1, in the tumorigenesis of pancreatic cancer demonstrating mechanistic effects of VAV1 which required its GEF activity and the activation of RAC1, PAK1, and NF-kappaB as well as involved cyclin D1 upregulation. We have also described an obligate role for the hematopoietic specific GTPase RAC2, in endothelial integrin signaling and in the postnatal neovascularization/pathological angiogenesis response *in vivo*. Using a RAC2 knockout mouse model, we discovered that despite the presence of both RAC1 and RAC2 proteins in endothelial cells, RAC2 was obligatory for the neovascular response and alphavbeta3/alpha4beta1/alpha5beta1 integrin-directed migration on vitronectin, H296, and CH271 fibronectin fragments, respectively. A genetic analysis of SYK-/+ or SYK-/+; RAC2-/+ mice revealed that SYK kinase is required for the integrin induced activation of RAC2. The analysis of endothelial cells from RAC2-/+ versus SYK-/+; RAC2-/+ mice provided genetic evidence that SYK-RAC2 signaling axis regulates integrin (alphavbeta3, alpha4beta1 and alpha5beta1) dependent endothelial cell migration, a key step for angiogenesis [18].

Cancer can be envisaged as a genetic alteration driven metabolic disease conjured up by the clonal selection pressure and intercellular cooperativeness between tumor cells, vascular cells, immune cells and tumor-associated fibroblasts (TAFs). The tumor induced angiogenesis effects on both the tumor and the endothelial cells. The relationship between the tumor and its surrounding endothelial cells is complex and multi-factorial. One of the crucial factors that ties tumor cells with the endothelial cells is the oxygen-dependent and oxygen-independent metabolism involving transcription factors HIF-1alpha, and NFκB as well as prolyl hydroxylase 2. The metabolic preference is for the high rates of glycolysis and glutaminolysis which provide most of the required biosynthetic intermediates and energy to support sprouting of endothelial cells (division and migration) without coupling to oxidative phosphorylation. The endothelial cells constituting neovasculature opts for the respiration-independent metabolism which allows them to resist the hostile environment of fluctuating oxygen tension. The accumulation of lactate largely contributes to the angiogenic phenotype through inhibition of prolyl hydroxylase 2 and the subsequent activation of HIF-1alpha and NFκB. Activation of NFκB in a hypoxia-independent manner leads to the increased production of IL-8/CXCL8 which drives the autocrine stimulation of endothelial cell proliferation and maturation of neovessels. Polet & Feron demonstrated that the addiction of proliferating endothelial cells for glucose and glutamine fuels the driving force of lactate to promote angiogenesis and provide novel potential treatment options without the disadvantages of conventional anti-angiogenic drugs [19]. Schoors et al., recently provided genetic evidence to demonstrate that the glycolytic activator phospho-fructokinase-2/fructose-2, 6-bisphosphatase 3 (PFKFB3) promotes vessel formation and blockade of PFKFB3 by the small molecule, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen1-one (3PO) reduced vessel sprouting in endothelial cell spheroids, zebrafish embryos, and the postnatal mouse retina by inhibiting endothelial cells proliferation and migration [20]. De Saedeleer et al., report that lactate inhibits prolylhydroxylase 2 activity and activates HIF-1alpha, a key contributor to glycolysis in normoxic oxidative tumor cells but not in Warburg-phenotype tumor cells and triggers tumor angiogenesis as well as tumor growth *in vivo* [21].

Anti-angiogenic therapy and resistance to anti-angiogenic therapy

There are several means of blocking the trophic support by which stromal cells normally provide to their malignant counterparts. Extensive study of the nature and function of tumor-associated growing blood vessels revealed funda-

mental differences between those vessels and normal blood vessels. The fact that there is a distinctive patho-physiological difference between tumor-associated blood vessels and normal blood vessels accounted for the normalization hypothesis suggested by Jain and his group to explain the effect of anti-angiogenics in the clinic. An alternative hypothesis to the widely held view that anti-angiogenic therapies starve tumor cells of oxygen and nutrients leading to cell death, was based on evidence put forward by Rakesh Jain that certain anti-angiogenic agents may transiently "normalize" the abnormal structure and function of tumor vasculature making it more efficient for oxygen and drug delivery [11]. The latter concept was based on the fact that tumor angiogenesis differs from normal angiogenesis in a way that the resulting vessels are tortuous, irregularly shaped, and hyper-permeable/leaky. These characteristics of tumor-associated blood vessels result in irregular blood flow and high interstitial fluid pressure within the tumor, which impair delivery of oxygen (radiation sensitizer) and cytotoxic drugs to the tumor. Normalization (recovery of normal state vasculature) required diminishing hyperpermeability, increasing pericyte coverage and restored the basement membrane to subsequently reduce hypoxia and interstitial fluid pressure [10, 22]. Hence anti-angiogenic therapy was believed to prune tumor vessels and normalize structure and function, thereby improving drug delivery and normalizing the tumor microenvironment. The normalization effect was suggested to account for the therapeutic benefit of combined anti-angiogenic and cytotoxic therapies. Thus a disorganized and leaky network of blood vessels irrigating solid tumors directly restrains the efficacy of conventional therapies by limiting intravenous drug delivery. In other words, tumor-driven vascular permeability compelled tumor-induced angiogenesis, blood flow disturbances, inflammatory cell infiltration, and tumor cell extravasations which accompanied tumor progression and, as collateral damage, impacted on efficiency of drug delivery.

Since the acquisition of oncogenic mutations and promotion of angiogenesis are key hallmarks of cancer, it has been put forward that activated oncogenes and deregulated angiogenesis are tightly associated as mutations in cancer cells can lead to perturbation of pro- and anti-angiogenic balance, thereby causing aberrant angiogenesis [23]. Bottos & Bardelli proposed that normalization of the vascular network by targeting oncogenes in the tumor cell might lead to more efficient and sustained therapeutic effects compared to those therapies only targeting tumor vessels. They argued that pharmacological inhibition of oncoproteins in tumor cells restored a functional vasculature by bystander anti-angiogenic effects. As genetic alterations are tumor-specific, targeted therapy, which potentially blocks the angiogenic program activated by individual oncogenes, may lead to personalized anti-angiogenic therapy. A review by Abdollahi & Folkman describes current concepts in this field and proposes novel strategies to overcome tumor evasion of anti-angiogenic therapy. It was put forward that early detection of tumors, prediction of tumor evasive mechanisms, and rational design of anti-angiogenic combinations will direct anti-angiogenic therapy towards its ultimate goal, the conversion of cancer to a dormant, chronic, and manageable disease [24]. Normalization of tumor blood vessels as a treatment target has a "window effect". Arjaans et al., investigated the effect of blood vessel normalization in mouse xenograft models of human ovarian and esophageal cancer by bevacizumab on antibody (Zr-trastuzumab) uptake by tumors. In their study, bevacizumab treatment decreased tumor uptake and intra-tumoral accumulation compared with baseline in tumor models relative to controls. Bevacizumab treatment also reduced micro-vessel density (MVD) in tumors and increased vessel pericyte coverage. In explaining these findings, they opined that it is clinically important to have caution in designing combinatorial trials with therapeutic antibodies due to a possible reduction in tumoral accumulation that may be caused by bevacizumab co-treatment [25]. Recently, a new group of microRNAs (miRs) engaged in angiogenesis, called angiomiRs and hypoxamiRs, surfaced as new therapeutic targets in cancer. Some of those miRs were found to efficiently regulate cancer immunity and their dysregulation efficiently programs aberrant angiogenesis and cancer metastasis [26, 27]. The current progress of anti-angiogenic therapy for cancer has been recently reviewed by Vasudev and Reynolds [28].

It has been reported that plasmatic VEGF may constitute a predictive biomarker for bevacizumab efficacy among breast cancer patients

[29, 30]. The largest fraction of anti-angiogenic studies involved the VEGF-targeting mAb bevacizumab, which has been tested, most often in combination with conventional chemotherapy and/or targeted anti-cancer agents, in cohorts of patients affected by acute myeloid leukemia [31], multiple myeloma [32], head and neck squamous cell carcinoma (HNSCC) [33, 34], breast carcinoma [29, 35-40], melanoma [41], hepatocellular carcinoma [42-46], pancreatic cancer [47], ovarian carcinoma [48-52], prostate cancer [53], and several other advanced or metastatic solid tumors [54-57].

VEGF has extra-vasculogenic roles in tumor development. One of the primary extra-vasculogenic roles of VEGF is immune regulation. A primary mechanism of immune evasion is through inhibiting normal dendritic cells activation and maturation. Tumors secrete a number of factors that contribute to this role, and chief among them is VEGF-A [58]. Tumor-derived VEGF prevents dendritic cell maturation in patients and mice which impairs the generation of an antitumor response. Thus VEGF has been known to suppress dendritic cell maturation and modulates lymphocyte endothelial trafficking. Recently, Hodi et al., investigated the combination of CTLA4 blockade with ipilimumab and VEGF inhibition with bevacizumab in patients with metastatic melanoma. In their study bevacizumab has been shown to influence changes in tumor vasculature and immune responses with ipilimumab administration. Their findings has provided a basis for further investigating the dual roles of angiogenic factors in blood vessel formation and immune regulation, as well as future combinations of anti-angiogenetic agents and immune checkpoint blockade [59]. Long-term remission of a HER2 positive primary breast cancer under double monoclonal antibody therapy with trastuzumab and bevacizumab has been reported. Vascular endothelial growth factor (VEGF) over-expression is frequently observed in human epidermal growth factor receptor 2 (HER2) positive patients with breast cancer and over-expression of the proto-oncogene HER2 is associated with an up-regulation of VEGF. In this case report, a HER2 positive patient with breast cancer who refused cytotoxic chemotherapy with its potential side effects as well as mastectomy has been presented. This case report showed that (a) the combined double administration of bevacizumab and trastuzumab is safe, non-toxic and clinically effective, and (b) bevacizumab and trastuzumab can be used as a long-term application [60]. ^{89}Zr-conjugated bevacizumab has been investigated as a means to visualize neoplastic lesions by positron emission tomography (PET) in women with primary breast carcinomas which often secrete high levels of VEGF [61]. In the context of a randomized Phase III clinical trial, the addition of bevacizumab to docetaxel and trastuzumab has been reported to fail to improve the progression free survival (PFS) of HER2+ metastatic breast cancer patients [30]. Similarly, in patients with HER2- metastatic or locally recurrent breast carcinoma, the combination of bevacizumab with capecitabine failed to meet the non-inferiority criterion as compared with a therapeutic regimen involving bevacizumab and paclitaxel [37]. Earlier, the addition of bevacizumab had been suggested to improve the efficacy of multiple taxanes, including paclitaxel and docetaxel, against breast carcinoma [62, 63]. The US FDA revoked the authorization that was given to bevacizumab for use in metastatic breast cancer patients (in combination with paclitaxel) in February 2008 (which was originally granted under the FDA accelerated approval program) (source http://www.cancer.gov/cancertopics/druginfo).

Despite different demonstrated rationales in the support of anti-angiogenic therapy that led to the approval of the first anti-angiogenic drug, bevacizumab for treatment of metastatic cancer, there remain many unanswered questions in the field of tumor-angiogenesis and anti-angiogenic therapy. Even a decade after the approval of bevacizumab for treatment of metastatic colorectal cancer [9], the story of bevacizumab-based therapy in the context of managing cancer in the metastatic setting has been far from a full success.

Two major huddles of the anti-angiogenic therapy are (1) toxicity and (2) resistance. The toxicity to targeted therapies are either on-target or off-target toxicities. On-target toxicities are mechanism-associated effects, which can be stratified as to whether or not the targets are relevant to response. Off-target toxicities may be caused by the class of agent, e.g. antibody versus small molecule receptor tyrosine kinase inhibitor, or by immune reactions or toxic metabolites. The toxicities may be due to higher drug concentrations or altered end-organ

sensitivity, which in turn can be a consequence of genetic polymorphisms controlling metabolism or tissue responsiveness. On-target toxicities are important to identify as some correlate with response and, hence, amelioration of these side effects is preferable to dose reduction or stopping drug. Toxicities secondary to relevant target's impact may be recognized when distinct types of agents, such as antibodies and small molecule kinase inhibitors, with the same target have a similar side effect. Both bevacizumab and vascular endothelial growth factor receptor (VEGFR) kinase inhibitors cause hypertension and these toxicities correlate with the response [64]. Bécouarn et al., in a study using FOLFIRI® and bevacizumab (*NCT00467142*) in first-line treatment for colorectal cancer patients reported grade 3/4 toxicities including neutropenia 16.1%; diarrhea 11.3%; nausea-vomiting 1.6% [65]. Toxicities to angiogenesis inhibitors (antibodies or small molecule inhibitors) belonging to the categories of either VEGF/VEGFR inhibitors (bevacizumab or ramucirumab) or multi-RTKs (sunitinib or sorafinib, pazopanib) encountered in the clinics are short-term toxicities and long-term toxicities. Knowledge regarding angiogenesis inhibitors in clinical trials indicates that short-term toxicities are mostly manageable [66]. However, long-term toxicities to these drugs are also important since anti-angiogenic agents are often used as long-term adjuvant or maintenance therapies. In a report related to the management of bevacizumab-related toxicities in patients with colorectal cancer (Phase III/IV) Dr. Saif identified hypertension (grade 3/4, 1%-18%), proteinuria (grade 3, 0%-2%), wound healing complications (1%), GI perforation (0%-2%), arterial thromboembolism (< 1%-2%), and bleeding (grade 3/4, < 1%-6%) as bevacizumab-associated adverse effects. Similar grades of toxicities related to bevacizumab-treatment in clinical trials and in community-based registry (BRiTE and first BEAT) studies have been reported and elegantly reviewed by Saif and Vasudev & Reynolds [28, 67]. Retrospective studies by Francesco Torino et al., indicate that TKi, sunitinib can induce hypothyroidism in 53-85% of patients, and in prospective studies this complication has been reported in 36-71% of patients. The other TKi, sorafenib has been also reported to be responsible for hypothyroidism in 18% of patients with metastatic renal-cell carcinoma [68]. Boehm et al. reported that the most frequent adverse events of sunitinib treatment in advanced renal cell cancer and malignant gastrointestinal stromal tumor in elderly patients included hand-foot syndrome, stomatitis, diarrhea, fatigue, hypothyroidism and hypertension. In their study, sorafenib as second-line treatment of advanced RCC and upfront treatment of advanced hepatocellular carcinoma caused dermatologic (hand-foot skin reaction, rash, desquamation), fatigue, diarrhea, nausea, hypothyroidism and hypertension. In addition, cardiovascular toxicity has increasingly been recognized as a potential adverse event associated with sunitinib and sorafenib treatment [69]. Similar categories of toxicities have also been reported following the treatment with pazopanib [70]. Knowledge regarding the molecular mechanisms involved in the toxicity of angiogenesis inhibition would facilitate more specific and more potent inhibitors to be developed in the coming years.

The initial hype of the effectiveness of Avastin (bevacizumab) in the clinic was soon followed by the identification of the development of a resistance phenomenon that lay inherent to the early success of the drug. The resistance to anti-angiogenic therapy was demonstrated at both the cellular and molecular levels. Interestingly, resistance was found to be *de novo* as well as acquired. Recently, proteomic characterization of breast cancer xenografts has been conducted to identify early and late bevacizumab-induced responses and to predict effective drug combinations [71]. To identify markers of response and/or resistance Reverse Phase Protein Array (RPPA) was utilized by this group to characterize treatment-induced changes in bevacizumab responsive and non-responsive human breast cancer xenografts. Data were combined with bio-informatics modeling to predict druggable targets for optimization of treatment. Integrating the bevacizumab-induced dynamic changes in protein levels with bio-informatics modeling in their study predicted inhibition of the PI3K-pathway to increase the efficacy of bevacizumab monotherapy which was tested in *in vivo* studies. In the light of recent reports of the failures of anti-angiogenic drugs, it is imperative to try to critically understand the strengths and limitations of anti-angiogenic therapies from different clinical trials in the context of genome wide changes. Accommodating the modest and

Table 1. Anti-VEGF drugs currently used/tested in the clinics

Drugs	Company	Mode of Action	Tumor Types Treated	Status
Bevacizumab	Genentech Inc.	Monoclonal anti-VEGF antibody	Metastatic Colorectal Cancer in Combination with Fluoropyrimidine-based Chemotherapy, Metastatic Renal Cell Carcinoma, Second-Line Treatment of Glioblastoma, First-Oine Treatment of Non-Small Cell Lung Cancer (NSCLC), Second-Line Treatment of Metastatic Colorectal Cancer, First-Line Treatment of Metastatic Colorectal Cancer,	FDA approved
Sorafenib	Bayer Healthcare and Onyx Pharmaceuticals	Small molecule multi-TKi including VEGFR	Liver cancer, Thyroid cancer and RCC,	FDA approved
Sunitinib	Pfizer Inc	Small molecule multi-TKi including VEGFR	Pancreatic neuroendocrine tumor, Gastrointestinal stromal tumor, Kidney cancer	FDA approved
Pazopanib	Glaxo SmithKline	Small molecule multi-TKi including VEGFR	Advanced soft tissue sarcoma, Advanced renal cell carcinoma	FDA approved
Vatalanib	Bayer Schering and Novartis	Small molecule multi-TKi including VEGFR	Colorectal cancer Non-Small Cell Lung Cancer (NSCLC)	(Phase I) (Phase II)
Axitinib	Pfizer Inc.	Small molecule Inhibitor of VEGFR1, VEGFR2, VEGFR3, c-KIT, PDGFT	Advanced renal cell carcinoma (after failure of one prior systemic therapy) Progressive, Recurrent/Metastatic Adenoid Cystic Carcinoma Advanced carcinoid tumor	FDA approved (Phase II) (Phase II)
Aflibercept	Regeneron Pharmaceuticals	Chimeric VEGF/PGF neutralizing receptor; vascular endothelial growth factor trap Functions as a soluble decoy receptor, binds to pro-angiogenic vascular endothelial growth factors (VEGFs)	Metastatic Colorectal Cancer K-RAS Mutant Patients With Resectable Liver Metastates (Phase II/III) Metastatic colorectal Cancer Myelodysplastic Syndromes Stage III-IV Melanoma Esophageal and Gastric Cancer Breast Cancer	FDA approved (Phase III) (Phase II) (Phase II) (Phase II) (Phase I)
Vandetanib	AstraZeneca	Small molecule dual TKi for VEGFR/EGFR	Medullary thyroid cancer Precancerous Head and Neck Lesions, Gastrointestinal Stroma Tumors	FDA Approved (Phase II)
Cediranib	AstraZeneca	Small molecule TKi for VEGFR	Alveolar Soft Part Sarcoma Recurrent Glioblastoma Ovarian cancer	(Phase II) (Phase III) (Phase III)
Nintedanib (BIBF 1120)	Boehringer Ingelheim	Small molecule inhibitor of angiokinase (inhibits VEGFR, FGFR, PDGFR)	Ovarian Neoplasms Peritoneal Neoplasms	(Phase III)
Ponatinib	ARIAD Pharmaceuticals	BCR-ABL inhibitor that also selectively inhibits certain other tyrosine kinases in preclinical studies, including FLT3, RET, KIT and the members of the FGFR PDGFR and VEGFR families of kinases.	Chronic myeloid leukemia or Philadelphia chromosome-positive acute lymphoblastic leukemia	FDA Approved
Brivanib		Vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor	Recurrent or Persistent Endometrial Cancer and Cervical Cancer	(Phase I)
Regorafenib	Bayer Healthcare	Inhibits vascular endothelial growth factor receptors (VEGFRs) 2 and 3, and Tie2, Ret, Kit, PDGFR and Raf kinases,	Colorectal cancer (metastasized) Gastrointestinal stromal tumor (locally advanced, cannot be removed by surgery, or has metastasized)	FDA Approved FDA Approved
Ramucirumab	Eli Lilly and Company	Binds to vascular endothelial growth factor receptor-2 (VEGFR-2)	Gastric or gastro-esophageal junction adenocarcinoma	FDA Approved

transient benefit of bevacizumab treatment as the tumors inevitably develop resistance, new therapies are being developed that attempt to inhibit angiogenesis through several different pathways. One of the promising new drugs, nintedanib, is an oral triple angiokinase inhibitor that acts by blocking not only VEGFR, but also FGFR and PDGFR, which are involved in the development of resistance to bevacizumab. An article by Durm and Hanna discusses the rationale for this approach and summarizes the clinical trial data on nintedanib, including the two most recent Phase III trials [72]. Similar multi-receptor tyrosine kinase inhibitors like sorafenib, sunitinib, pazopanib, ponatinib, axitinib and regorafenib have been either approved by FDA or are being currently tested in different phases of clinical trials as shown in **Table 1**. Recently, data from a phase 1 clinical trial conducted by Wagle and group showed that a patient with advanced bladder cancer experienced a complete response for 14 months to the drug combination of everolimus (mTORC1 inhibitor) and pazopanib (an anti-angiogenic inhibitor, a multi targeted receptor tyrosine kinases inhibitor including VEGFR, PDGFR etc). Genomic profiling of this tumor revealed two activating point mutations in mTOR (E2419K and E2014K) that may have caused this exceptional response [73]. This data clearly indicate that as a single agent pazopanib might not work in this patient and activating mutation of mTOR as well as activation of the PI3K-AKT-mTOR pathway (by different mechanisms) may play an important role for developing resistance to the anti-angiogenic therapy. This unexpected success may suggest that making a catalog of genomic alterations in the genes of the AKT-mTOR pathway may help to develop a combinatorial approach for future anti-angiogenic therapy. The study concluded that treatment with bevacizumab resulted in compensatory upregulation of several signaling pathways. As fundamental causes of resistance began to be revealed, three distinct features surfaced in the context of tumor-angiogenesis, (1) the distinctive patho-physiological nature of tumor-associated blood vessels, (2) VEGF-independent tumor-associated blood vessels, and (3) extra-endothelial trans-differentiation of tumor cells that serve as makeshift channels (vascular mimicry). These features form the structural basis for resistance to the anti-angiogenic therapies.

Resistance to anti-angiogenic therapy: intrinsic refractoriness or evasive escape

Targeting VEGF on one hand has produced some success in therapy against a number of human cancers. On the other hand, drug resistance related to anti-angiogenic therapies has been attributed to the compromised benefits of anti-angiogenic therapies in various clinical trials. In the metastatic setting, anti-VEGF approaches have yielded contrasting survival benefits in randomized Phase III trials [74]. The addition of bevacizumab, a VEGF specific antibody, to standard chemotherapy increased overall survival (OS) in colorectal and lung cancer patients and progression-free survival (PFS) in breast cancer patients. Multi-targeted tyrosine kinase inhibitors that block VEGF receptors and other receptor tyrosine kinases (RTKs) in both endothelial and cancer cells, had survival benefit in gastrointestinal stromal tumor and renal-cell-carcinoma patients. In contrast, the addition of bevacizumab to chemotherapy did not increase survival in patients with previously treated and refractory metastatic breast cancer and addition of vatalanib, a kinase inhibitor developed as a VEGF receptor-selective agent, to chemotherapy had no benefit in metastatic colorectal cancer patients [74]. The data from these and other advanced clinical trials have exposed several limitations of anti-angiogenic therapy and challenged the following views regarding the value of anti-angiogenic therapy in cancer: (1) promise of anti-VEGF monotherapy to increase survival in randomized Phase III trials and (2) possibility of clinical achievement of tumor-vessel regression following combined anti-VEGF treatment without compromising the delivery and efficacy of cytotoxic treatment in order to increase overall survival (OS) in previously treated chemotherapy-naïve colorectal cancer and lung cancer patients, as well as PFS in breast cancer patients. There may exist an escape route for tumors evading anti-angiogenic therapy. It is possible that this route may account for drug induced resistance triggered by therapy or it is also possible that even if synergistic effects are achieved by combining anti-VEGF agents and the cytotoxic agent(s), relapse can occur after combination therapy because they use alternative pathways for neovascularization from the very beginning of the event. It is known that by antagonizing VEGF receptor 2 (VEGFR2) signaling, ramucirumab (a human IgG1 also known as IMC-1121B) blocks the most prominent of

these interactions, i.e., neoangiogenesis [75]. Recently, ramucirumab has been tested as a standalone intervention in patients affected by advanced gastric or gastresophageal junction adenocarcinoma and hepatocellular carcinoma [76, 77]. The efficacy and safety of ramucirumab as first-line therapy in patients with advanced hepatocellular carcinoma was assessed and potential circulating biomarkers were explored. Forty-two adult patients with advanced hepatocellular carcinoma and no prior systemic treatment received ramucirumab 8 mg/kg every two weeks until disease progression or limiting toxicity. The primary endpoint was progression-free survival (PFS); secondary endpoints included objective response rate (ORR) and overall survival (OS). Median PFS was 4.0 months [95% confidence interval (CI), 2.6-5.7], ORR was 9.5% (95% CI, 2.7-22.6; 4/42 patients had a partial response), and median OS was 12.0 months (95% CI, 6.1-19.7). For patients with Barcelona Clinic Liver Cancer stage C disease, median OS was 4.4 months (95% CI, 0.5-9.0) for patients with Child-Pugh B cirrhosis versus 18.0 months (95% CI, 6.1-23.5) for patients with Child-Pugh A cirrhosis. The study concluded that ramucirumab monotherapy may confer anticancer activity in advanced hepatocellular carcinoma with an acceptable safety profile. Exploratory biomarker studies showed changes in circulating VEGF, PGF, and soluble VEGFR-2 that are consistent with those seen with other anti-VEGF agents [77]. Since vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR-2)-mediated signaling and angiogenesis contribute to the pathogenesis and progression of gastric cancer, another clinical trial (NCT00917384) has been recently launched to assess whether ramucirumab prolonged survival in patients with advanced gastric cancer or gastro-oesophageal junction adenocarcinoma. Ramucirumab is a recombinant monoclonal antibody (IgG1 class) that binds to VEGFR-2 and blocks receptor activation. Ramucirumab is the first biological treatment given as a single drug that has produced survival benefits in patients. An international, randomized, double-blind, placebo-controlled, Phase III trial (at 119 centers in 29 countries in North America, Central and South America, Europe, Asia, Australia, and Africa) was conducted in 355 patients with disease progression after first-line platinum-containing or fluoropyrimidine-containing chemotherapy who received either ramucirumab 8 mg/kg (n=238) or placebo (n=117) once every 2 weeks. The primary endpoint was overall survival. Median overall survival was 5.2 months (IQR 2.3-9.9) in patients in the ramucirumab group and 3.8 months (1.7-7.1) in those in the placebo group (HR 0.776, 95% CI 0.603-0.998; p=0.047). The survival benefit with ramucirumab remained unchanged after multivariable adjustment for other prognostic factors (multivariable HR 0.774, 0.605-0.991; p=0.042) [76]. These findings validated VEGFR-2 signaling as an important therapeutic target in advanced gastric cancer and advanced hepatocellular carcinoma. Ramucirumab has been also tested previously in combination with docetaxel for the treatment of stage IV NSCLC patients progressing upon one cycle of platinum-based therapy [78].

Resistance to anti-angiogenics (**Figure 1**) remains a text-book example of how failure to completely understand the basics of tumor biology led to the failure of a therapy in clinical practice. The finding that an "angiogenic switch" may be a critical step in carcinogenesis while leading to the overwhelming preclinical successes of bevacizumab and its combination with chemotherapy drugs, there has been less success in translating these preclinical findings into full-fledged clinical practice. The mechanism of development of resistance to anti-angiogenic drugs (drug induced or *de novo*), the role of resistance to anti-angiogenics in the failure of anti-angiogenic therapy in the clinic and the long term implications of resistance to anti-angiogenics in shaping the future of anti-angiogenic drug based cancer management was reviewed by Loges et al. [79]. Blood vessels, lymphatic vessels and the extracellular matrices of tumors constitute in large parts of the tumor microenvironment and it is established now that the microenvironments of tumors are different from their normal counterparts. Other stromal cells, including activated fibroblasts, macrophages, and immune cells are also part of the abnormal tumor microenvironment. These abnormalities create a microenvironment conducive for tumor growth which in turn shapes its stromal microenvironment in favor of the progression of the tumor, tumor metastasis, immune-suppression, and also induces stem-cell phenotypes [80]. As a corollary to the above, it is logical to argue that development of anti-angiogenic therapy resistance arise from the effects of anti-angiogenic drugs, (1) on the

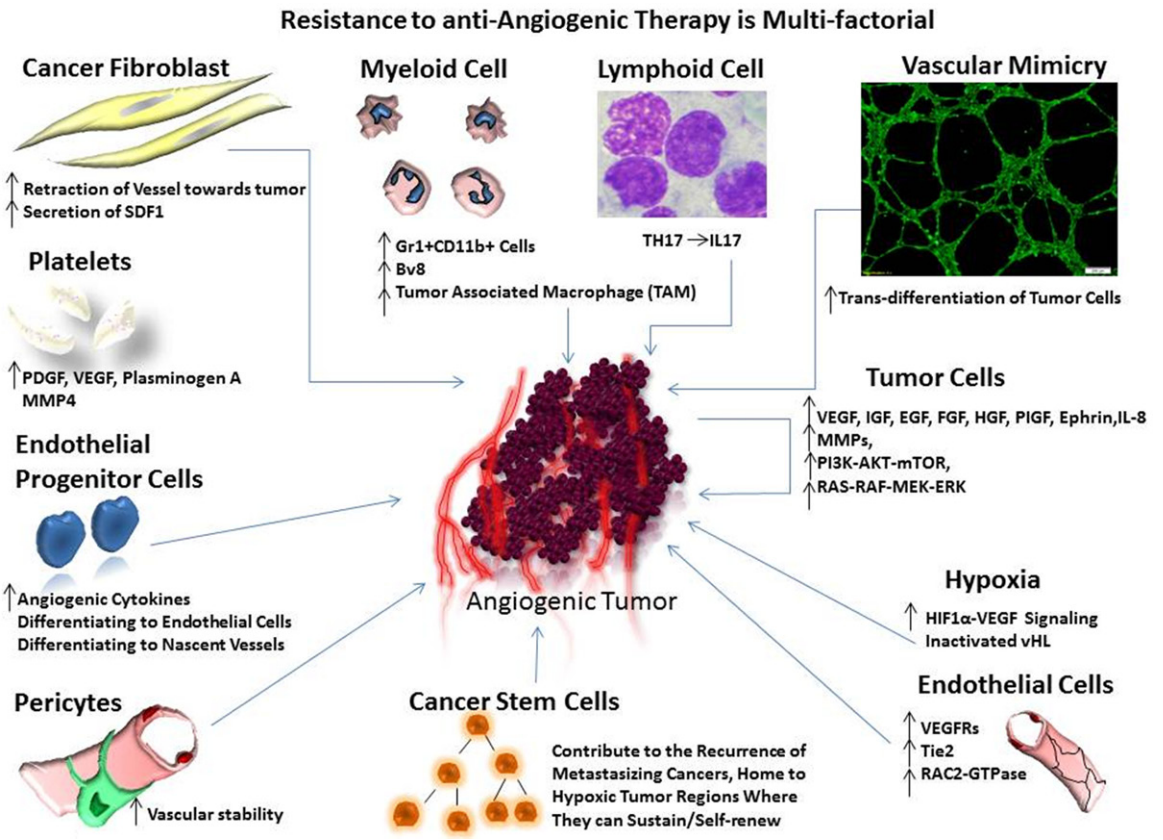

Figure 1. Resistance to anti-angiogenic therapy is multi-factorial.

abnormal tumor microenvironment and (2) on the manner in which this abnormal tumor microenvironment counter reacts to the drugs themselves. Preclinical and clinical studies have showed a limited efficacy of bevacizumab which have been contributed by upregulation of the compensatory signaling pathway(s) resulting in the development of the resistance to the anti-angiogenic therapies. Indeed Lindholm et al., demonstrated that since bevacizumab treatment caused compensatory upregulations of several signaling pathways, targeting such pathways proved efficacious for the anti-angiogenic therapy [71]. RPPA analysis was utilized to characterize treatment-induced changes in bevacizumab responsive and nonresponsive human breast cancer xenografts with the aim of identifying markers of response and/or resistance. The authors concluded that integrating bevacizumab-induced dynamic changes in protein levels with bio-informatics modeling predicted inhibition of PI3K-pathway to increase the efficacy of bevacizumab monotherapy. *In vivo* experiments combining bevacizumab and the PI3K/mTOR inhibitor BEZ235 confirmed their significant and additive growth inhibitory effect in a basal-like tumor model. Thus treatment with bevacizumab resulted in compensatory upregulation of several signaling pathways. Targeting such pathways increased the efficacy of anti-angiogenic therapy. Interestingly enough, there appeared a subtype specific response in this study between basal-like tumors and luminal-like breast tumors. Adding doxorubicin to bevacizumab showed significant and superior growth inhibition of basal-like tumors, whereas no additive effect was seen in the luminal-like model. The combination treatment corresponded to a continuous late attenuation of mTOR signaling in the basal-like model, while the inhibition was temporary in the luminal-like model [71].

Non-angiogenesis dependent pathways for tumor growth

The classical concept put forward by Folkman [81-83] that tumors are empowered with an inherent ability to stimulate the proliferation, maturation, and migration of stromal endothe-

lium (angiogenic capability) and that growth and metastatic progressions of a tumor are *only* angiogenesis-dependent have recently been challenged. The older concept was based on the hypothesis that tumor cells mediate/switch to their own pro-vascularization mode by producing "angiogenic molecules" [82, 84]. This switch was thought to depend on the balance of production of one or more of the positive regulators of angiogenesis (angiogenic cytokines and extracellular matrix degrading enzymes), including the vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), interleukin-8 (IL-8), placental growth factor (PlGF), transforming growth factor-b (TGF-b), platelet-derived endothelial growth factor (PDEGF), pleiotrophins [85]. However, studies by different groups have led to a newer hypothesis that neovascularization and angiogenesis are neither exactly interchangeable nor synonymous in many solid tumors. Histomorphological studies have indicated that some tumors can be vascularized (tumor-induced neovascularization) and/or metastasized with a significant involvement of endothelium-independent angiogenesis possibly (1) using existing vessels [86] (a process of vascular co-option) [87, 88], (2) forming vascular channels made of tumor cells themselves (a process called vascular mimicry) [89], (3) by bone marrow-derived stem cells serving as a precursor source of endothelial progenitor cells [90], or (4) by lympho-angiogenesis as a mechanism of *de novo* formation of lymphatics for the metastatic dissemination of tumor cells [91, 92].

VEGF-independent route to tumor angiogenesis

Vascular (vasculogenic) mimicry (VM) of solid tumor cells is an endothelium-independent trans-differentiation event characteristic to the highly plastic and aggressive tumor cells. This non-angiogenic phenomenon involves the formation of matrix-embedded capillary-like micro-circulatory structures whereby tumor cells mimic the pattern of embryonic vasculogenic networks. Studies from Yao et al., and others have provided evidence for participation of cancer stem cells (CSCs) in VM formation [93, 94]. Roles of CSCs in tumor vascularization, including production of pro-angiogenic factors, trans-differentiation into vascular mural cells such as endothelial and smooth muscle-like cells, and formation of non-endothelium-lined vasculogenic mimicry have also been reported [95]. Interestingly, CD133+ cells with cancer stem cell characteristics are found to be associated with VM in triple negative breast cancers [94]. Tubes lined by tumor cells have been reported histologically in a number of solid tumors including melanomas, ovarian carcinomas, inflammatory breast cancers [85] and other cancers [28]. Vascular mimicry of tumor cells refer to the characteristic plasticity of aggressive cancer cells forming *de novo* vascular networks which function (1) to rapidly perfuse growing tumors, transporting fluid from leaky vessels and/or (2) to connect with the constitutional endothelial-lined vasculature. This alternative mechanism of channel formation is derived from tumor cells [89] which contribute to establish tumor blood supply [96]. Vascular mimicry like EMT (epithelial-mesenchymal transition) and EndMT (endothelial-mesenchymal transition) demonstrate the multidirectional extent of phenotypic plasticity of cancer cells. Vascular mimicry has been reported in melanoma, head and neck carcinoma, hepatocellular carcinoma, choriocarcinoma, glioma, ovarian cancer, and breast cancers [89, 97-103]. Not only has vascular mimicry been reported in different solid tumors, but tumor cell-originated neovascularization including tumor-derived endothelial cell-induced angiogenesis along with vascular mimicry have been suggested to be involved in the development of resistance to anti-VEGF therapy as frequently observed in glioblastoma multiformae (GBM) [104]. Breast cancer cells are shown to trans-differentiate to drive vascular mimicry [105-109]. Studies of aggressive breast cancer have reported vascular mimicry in the absence of endothelial cells as well as the absence of central necrosis in tumor [110], which clearly indicated the presence of viable tissue without traditional intra-tumoral vasculature [111]. Vascular mimicry is also reported to represent a non-angiogenic pathway in breast-cancer metastasis [111]. Pezzella et al. reported that angiogenic primary breast carcinoma can relapse not only as angiogenic, but also as non-angiogenic lung metastases. They propose that this non-angiogenic pathway is a novel pathway of cancer progression and such tumors are likely to be resistant to anti-angiogenic treatment. Hence vascular mimicry may be regarded as one of the major causes of the develop-

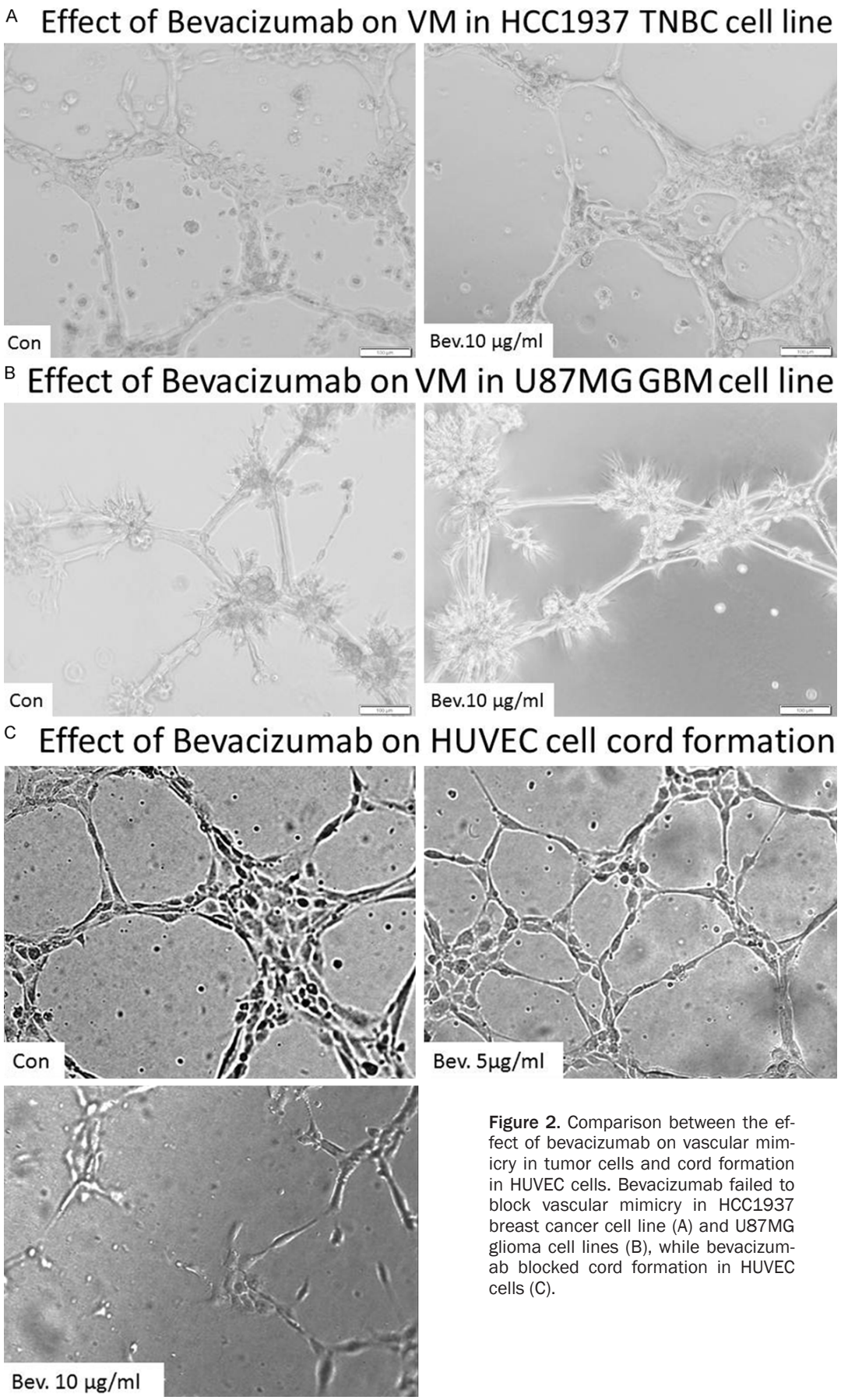

Figure 2. Comparison between the effect of bevacizumab on vascular mimicry in tumor cells and cord formation in HUVEC cells. Bevacizumab failed to block vascular mimicry in HCC1937 breast cancer cell line (A) and U87MG glioma cell lines (B), while bevacizumab blocked cord formation in HUVEC cells (C).

ment of resistance to anti-angiogenic therapy in solid tumors.

One of the confirmatory tests to determine the presence of endothelium-independent cell signaling in tumor cells which otherwise exhibit an endothelium-like phenotype of vascular mimicry is through the study of the effect of bevacizumab on this phenotype. In our laboratory, we have tested the effect of bevacizumab on the formation of vascular mimicry using both GBM and breast cancer cells and compared this with the effect of bevacizumab on cord formation in HUVEC cells. **Figure 2** shows that bevacizumab failed to decrease vascular mimicry in HCC1937 breast cancer (**Figure 2A**) and U87MG GBM cell lines (**Figure 2B**), while as expected, bevacizumab inhibited classical cord formation in HUVEC cells (**Figure 2C**). In fact, Shirakawa and colleagues [107] reported a haemodynamic connection between vascular mimicry in inflammatory breast cancer and angiogenesis. However, the underlying mechanism of vascular mimicry and its contributory association with the resistance to anti-angiogenic drugs in the context of the metastatic setting in different solid tumors are not yet clear. Recently, we have identified a mechanistic relationship between upregulation of the Wnt-beta-catenin pathway and vascular mimicry in the triple negative subset of breast cancer [112]. This study along with our previous studies [113, 114] on the association between the Wnt-beta-catenin pathway and metastasis in the same breast cancer subtype has helped to support the hypothesis that non-endothelial vessel formation may have a strong contributory role in driving metastasis in solid tumors.

Another distinct form of vascular pattern observed in solid tumors is where both endothelial and tumor cells contribute to the formation of the vascular tube [115]. Chang et al., observed the presence of "mosaic" vessels in which both endothelial cells and tumor cells form the luminal surface and this pattern has serious implications for metastasis, drug delivery, and anti-vascular therapy. Using CD31 and CD105 to identify endothelial cells and endogenous green fluorescent protein labeling of tumor cells, they showed that approximately 15% of perfused vessels of a colon carcinoma xenografted at two different sites in mice were mosaic vessels having focal regions where no CD31/CD105 immunoreactivity was detected and tumor cells appeared to contact the vessel lumen [115]. These regions occupied approximately 25% of the perimeter of the mosaic vessels or approximately 4% of the total vascular surface area in these colon carcinomas. Similar mosaic vessels were observed in human colon carcinoma biopsies. Their study provides a possible explanation for the anti-vascular effects of cytotoxic agents. From the above, it can be argued that if endothelium-independent vascularization is possible in tumors and this type of vascularization is demonstrated to have a significant contribution in the metastatic progression of the tumor then these factors can also contribute to the development of resistance to conventional anti-angiogenic therapy. The above indicates that there may exist a VEGF-independent route to tumor angiogenesis. The US FDA has approved several inhibitors of the VEGF pathway, which has enabled noteworthy advances in the therapy of cancer [116, 117]. The hypothesis that tumor progression is mediated by angiogenesis led to the birth of a therapeutic strategy based on the notion that tumor progression can be arrested by anti-angiogenic drugs. This was supported experimentally through a large body of evidence reported over three decades. The addition of bevacizumab to standard chemotherapies or to interferon therapy (in metastatic renal cell carcinoma), as well as the use of anti-VEGF receptor tyrosine kinase inhibitors (TKIs) with wide spectra of activities have been shown efficacious in multiple advanced cancers such as metastatic colorectal cancer, metastatic non-small-cell lung cancer, metastatic breast cancer, hepatocellular carcinoma and gastrointestinal stromal tumors (GISTs) as reviewed by Jain et al., [118]. However, despite these advances, enhanced survival of patients has yet to be achieved in Phase III clinical trials following anti-angiogenic agents that only target VEGF.

Biomarkers of anti-angiogenic therapy

The identification of biomarkers assist in the (1) validation of mechanistic hypotheses, (2) identification of responsive patients, (3) optimization of treatment dosing and scheduling of therapeutic drugs to predict efficacy of regimens and (4) detection and prevention of tumor escape. Disease progression occurring in patients abetted by inherent/acquired resis-

tance to anti-angiogenic drugs, has lead to the identification of pathways mediating VEGF-independent tumor angiogenesis as well as different predictive biomarkers for identifying those patients who are most likely to respond to such treatments. Recent evidence suggests that both tumor and non-tumor (stromal) cell types are involved in the reduced responsiveness to the anti-VEGF treatments. The review by Napoleone Ferrara examined the role of tumor- as well as stromal cell-derived pathways involved in the tumor growth and in the refractoriness to anti-VEGF therapies [119]. Pohl et al., have examined circulating endothelial progenitor cells (EPC), serum-VEGF levels and tumor tissue VEGF expression of patients with mCRC (metastatic colorectal cancer) under a bevacizumab containing chemotherapy. Five-member VEGF family (A-E) is a vasculature specific (vascular development, angiogenesis and lymphangiogenesis) growth factor (s) which initiates the proliferative and migratory signals in cells via its cognate cell surface receptor tyrosine kinase (RTKs) called VEGFR. VEGF receptors have an extracellular domain consisting of 7 immunoglobulin-like domains, a single transmembrane domain and an intracellular split tyrosine-kinase domain. Among three-member family (1-3) of VEGF receptors, VEGFR2, also known as KDR is the predominant receptor to mediate endothelial cell responses to VEGF and its therapeutic inhibition has been shown to impact in the clinic for the treatment of a number of diseases including cancers [120]. Recently, ramucirumab, an antibody that binds to VEGFR2/KDR has been approved by FDA for gastric or gastro-esophageal junction adenocarcinomas. Circulating endothelial progenitor cells (EPCs) were originally identified by Asahara as CD34+ VEGFR2+ mononuclear cells which differentiated into an endothelial phenotype, expressed endothelial markers [121]. EPCs are clinically useful prognostic and predictive tools in cancers associated with pathological angiogenesis and targeting EPCs can be a key to successfully manage cancer patients [122]. Patients with a partial remission after six months of immuno-chemotherapy showed a reduction of CD34 negative KDR (Kinase insert domain receptor; a type III receptor tyrosine kinase) positive cells as early as 3 weeks after start of therapy. Neither serum nor tissue markers were of significant predictive value in their pilot study [123]. As reviewed by Jain et al., most biomarkers are disease and/or agent specific and they are required to be validated prospectively. In their review, (1) the challenges in establishing biomarkers of anti-angiogenic therapy, (2) the definition of systemic, circulating, tissue, and imaging biomarkers, (3) the identification of their advantages and disadvantages and (4) the likely future opportunities for validating biomarkers of antiangiogenic therapy has been elegantly and elaborately discussed [118].

There remains a paucity of fully validated biological markers that predict responsiveness or development of evasion to anti-angiogenic therapy of cancers [74, 79]. The identification of such biomarkers remains vital to move these anti-angiogenic therapies forward, as failure to respond to anti-angiogenic therapy is associated with rapid tumor progression that may have been counteracted or prevented had the intrinsic or acquired evasion to anti-angiogenic therapy been identified [9, 124]. The situation is challenging, as there are multiple contributing factors associated with the resistance to anti-angiogenic therapy. Using several experimental models, it has been demonstrated that both tumor and non-tumor (stromal) cell types may be involved in the reduced responsiveness to anti-angiogenic treatments [125, 126]. The importance of stroma has been recently reported in the progression of GBM by Ricard et al. [127]. In their study, they focused on an untreatable highly vascularized GBM whose progression was dependent on oxygen and metabolites supplied by blood vessels. They searched whether correlations existed between blood vessel density, tumor cell density, and proliferation in control tumors. Extensive vascular remodeling and the formation of new vessels accompanied the growth of U87 tumor cells, but no strong correlation was found between local cell density and the extent of local blood vessel density irrespective of the tumor area or time points. Bevacizumab treatment massively reduced tumoral vessel densities but only transiently reduced U87 tumor growth rate (in a xenograft model) while AMD3100 achieved a potent inhibition of tumor growth without significant reduction in blood vessel density. Together, these observations indicated that in brain, tumor growth can be sustained without an increase in blood vessel density and GBM growth is governed by stromal properties that in this case are not synonymous to endothelial properties.

With the increasing availability of anti-angiogenic agents for the treatment of cancers, the identification and establishment of biomarkers of response as well as biomarkers of resistance have become imperative for scientific and clinical professionals. This urgency is due to the conviction that "selected patients" are most likely to benefit from these high-cost therapies. It is also necessary to identify new targets to prevent the obvious escape from these therapies. As stated earlier, although there are candidate biomarkers of anti-angiogenic therapy, validated biomarkers remain elusive [128]. According to Duda et al., one of the reasons for this is the unclear mechanism(s) of action of these drugs. For example, blockade of VEGF produced both anti-vascular and normalizing effects on tumor vasculature that had limited translational effect as evaluated by current criteria based on tumor size measurements such as the Response Evaluation Criteria in Solid Tumors (RECIST). It is also commented that excessive anti-vascular effects (when using high doses) might induce a transient response with severe toxicities. Additionally, vascular normalization alone (with no cytotoxic treatment) may not have sufficient capability to shrink tumors/halt their growth and anti-angiogenic agents could have systemic effects. Based on aforesaid arguments Duda et al., concluded that it is most likely that for each cancer and each agent, one might need a specific set of biomarkers for good prediction and these biomarkers will be mechanism specific. The work of Jain's group suggests that patients who have elevated pretreatment levels of plasma sVEGFR1 are not likely to benefit from anti-VEGF therapies and increased levels of SDF1 were correlated with escape from anti-VEGF therapies. Other evasive pathways emerging from preclinical and clinical studies include Ang2 and cMET as reviewed in a recent article by Jain [80]. Recently biomarkers of reactive resistance associated to an early disease progression have been studied by Hayashi et al., during chemotherapy plus bevacizumab treatment for colorectal carcinoma. In their study, Hayashi et al. demonstrated that an early increase in the serum VEGF-A concentration after the initial decrease is a potential predictive marker of a poor response and reactive resistance to bevacizumab plus chemotherapy [129]. From the perspective of a reactive resistance, it has been shown that sunitinib (pan receptor tyrosine kinase) by way of its main effect on endothelial tumor cells, increases the number of renal cancer stem-cells and thus contribute to its own resistance [130].

Myeloid cells in VEGF-independent tumor angiogenesis

To date, anti-angiogenic therapy predominantly targets VEGF, either the ligand or its receptor. Like all other therapies, inherent/acquired resistance to anti-VEGF drugs occurs in cancer patients, which culminates into disease recurrence and faster progression. It is understood that both tumor and non-tumor (stromal) cell types are involved in the reduced responsiveness to current anti-angiogenic treatments. When VEGF is blocked, there occurs a paradigm shift in the angiogenic milieu of a tumor. The most prominent among them are increase of other pro-angiogenic factors, such as fibroblast growth factor (FGF), ephrin, and members of the angiopoietin family that together increases the chance of circumventing an anti-angiogenic therapy over a course of time. An additional mechanism involves the recruitment of bone marrow-derived pro-angiogenic cells. VEGF inhibitors have been known to induce the expression of stromal derived factor-1 (SDF1), placental growth factor, stem cell factor, interleukin-6, and other cytokines in non-tumor tissues and can be argued to stimulate metastasis and angiogenesis in a VEGF-independent manner [131]. These cytokines may recruit bone marrow-derived ECs and myeloid progenitors that then might initiate and promote the formation of a pre-metastatic environment [132]. These autocrine/paracrine events remain less clear due to the inherent difficulty in establishing a suitable preclinical experimental model. The complexity of the event is furthermore heightened by the simultaneous occurrence of events involving the immune system, tumor cells, endothelial cells and bone-marrow derived progenitor cells demanding a simultaneous modeling of tumor and endothelial compartments, immune system and bone-marrow compartment in an *in vivo* setting. Even the most recent advancement of patient derived xenograft (PDX) preclinical tumor model has limited application to model these events collectively [133].

Myeloid cells have been demonstrated to play a role in VEGF-independent tumor angiogenesis as elegantly reviewed by Napoleone Ferrara

[116]. In earlier studies Shojaei et al., [134] screened a number of murine cell lines in order to establish experimental tumor models that were responsive/refractory to treatment. Their data showed that refractory tumors like Lewis lung carcinoma and EL-4 were associated with a significant increase in the frequency of tumor infiltrating CD11b+Gr1+ cells compared to sensitive ones (B16-F1, TIB-6). CD11b+Gr1+ cells isolated from refractory tumors, but not from sensitive tumors, were able to mediate refractoriness to anti-VEGF treatment. The combination of an anti-Gr1 antibody with anti-VEGF delayed the onset of refractoriness. They also demonstrated that anti-VEGF therapy refractoriness was observed in immunocompetent C57BL/6 or in immunocompromised x-linked immunodeficiency mutation (XID) mice, suggesting that accumulation of CD11b+Gr1+ cells and their role in mediating refractoriness were not dependent on the adaptive immune system. Studies conducted to identify mediators of VEGF-independent angiogenesis found that the Bv8 protein (also known as prokineticin-2) [116, 135] was upregulated in CD11b+Gr1+ cells associated with resistant tumors. Granulocyte colony-stimulating factor (G-CSF) strongly induced the expression of Bv8 in CD11b+Gr1+ cells [136]. Bv8 blockade by neutralizing antibodies resulted in suppression of tumor angiogenesis and growth as well as exhibited additive effects with anti-VEGF antibodies in slowing growth of human and murine tumor cell lines [136]. Also, production of G-CSF by tumor or stromal cells was found to be strongly correlated with refractoriness to anti-VEGF therapy in mouse models [137]. This study showed for the first times that G-CSF neutralization reduced tumor angiogenesis and suppressed mobilization of CD11b+Gr1+ cells. In addition to the production of G-CSF by tumor or stromal cells, neutrophils infiltrating human tumors strongly expressed Bv8, indicating that this protein played a pathogenic role in human malignancies [138]. It has been also demonstrated that subsets of patients among most solid tumor classes exhibited 'leukemoid reactions' [139], wherein leukocytosis was associated with a paraneoplastic syndrome linked to production by the tumor of colony-stimulating factors, most frequently G-CSF [139, 140]. The above findings suggest that G-CSF or Bv8 may contribute significantly to this phenomenon and thus can serve as therapeutic targets. Recently, the oncogenic RAS pathway activation has been identified in promoting resistance to anti-VEGF therapy in mouse models including a genetically engineered model of pancreatic adenocarcinoma through G-CSF-induced neutrophil recruitment [141]. This data showed that activation of the RAS/MEK/ERK pathway regulated G-CSF expression through the Ets transcription factor. G-CSF release was markedly reduced with a MEK inhibitor *in vitro* and synergized with anti-VEGF antibodies to (1) reduce CD11b(+)Ly6G(+) neutrophil mobilization, (2) decrease tumor growth, and (3) increase survival.

Resistance to cancer therapies which specifically target VEGF signaling is an eminent clinical problem and a successful therapy should a pharmacological way to circumvent this problem. The first step of resolving this problem is to understand the mechanisms of the development of the resistance following the drug treatment, which are poorly understood. Increasing evidence indicate that stromal micro-environment containing immune cells in conjunction with the tumor cells is critical in mediating anti-angiogenic drug resistance. Chung et al., demonstrated that IL-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. IL-17 released in the tumor micro-environment in response to anti VEGF drugs have been shown to trigger stromal derived inflammatory and VEGF-independent angiogenic programs which leads to the drug refractoriness. Tumor-infiltrating T helper type 17 (T(H)17) cells and IL-17 have been shown to induced the expression of granulocyte colony-stimulating factor (G-CSF) through NF-κB and ERK signaling. This mobilizes immature myeloid-cell and their subsequent recruitment into the tumor microenvironment. The occurrence of T(H)17 cells and Bv8-positive granulocytes has been identified within clinical tumor specimens. Tumors resistant to treatment with antibodies to VEGF were rendered sensitive in IL-17 receptor (IL-17R)-knockout hosts deficient in T(H)17 effector function and pharmacological blockade of T(H)17 cell function sensitized resistant tumors to therapy with antibodies to VEGF. The study highlights the importance of immuno-modulatory strategies in improving the efficacy of anti-angiogenic therapy [142].

Tumor associated fibroblasts in anti-angiogenic therapy

In addition to the role of myeloid cells in this process, additional tumor-infiltrating cell types,

like tumor-associated fibroblasts (TAFs), are also known to have specific roles in the reduced responsiveness to current anti-angiogenic treatments. Tumor-associated fibroblasts have been identified as "Trojan Horse" mediators of resistance to anti-VEGF therapy [143]. Tumor-associated fibroblasts (TAFs or cancer-associated fibroblasts CAFs, hereafter referred as TAFs) from sensitive and resistant tumors exhibit distinct angiogenic and tumorigenic properties. In contrast to normal skin fibroblasts or TAFs from TIB6 tumors, those are sensitive to anti-VEGF treatment (TAF-TIB6), TAFs from resistant EL4 tumors (TAF-EL4) stimulate TIB6 tumor growth under VEGF inhibited conditions. Crawford et al. report that tumors resistant to anti-VEGF therapy stimulate tumor-associated fibroblasts to express pro-angiogenic PDGF-C. They demonstrated that PDGF-C mediated the angiogenic and tumorigenic properties of fibroblasts associated with tumor refractoriness to anti-VEGF treatment [144]. They reported that TAFs could mediate tumor refractoriness to anti-VEGF therapy and platelet-derived growth factor (PDGF)-C was identified as a key mediator in the process. This result showed that platelet-derived growth factor C is upregulated in TAFs from resistant tumors. PDGF-C-neutralizing antibodies blocked the angiogenesis induced by such TAFs *in vivo*, slowed the growth of EL4 and admixture (TAF-EL4 + TIB6) tumors and exhibited additive effects with anti-VEGF-A antibodies. These data provide evidence for an additional mechanism for TAF-mediated tumorigenesis and suggested that upregulation of PDGF-C might play a role in overcoming the inhibition of VEGF-mediated angiogenesis in certain resistant/refractory tumors.

Conclusion

All successful anti-angiogenic drug-mediated cancer therapies are limited by the inevitable development of drug resistance. Angiogenesis inhibitors targeting the vascular endothelial growth factor (VEGF-VEGFR) signaling pathways are found to have demonstrable therapeutic efficacy in mouse models of cancer and in an increasing number of human cancers. However, in both preclinical and clinical settings, the benefits are at best transitory and are mostly followed by a restoration of tumor growth and progression. Nearly a decade after approval of the first anti-angiogenic drug, the primary or the acquired resistance still remains as the major challenge in the clinic. Further clinical investigations are needed to optimize anti-angiogenic treatments in solid tumors management, as well as the identification of reliable markers that predict the relapse and the response to these therapies. In addition to several areas of clinical research of high priority, including the optimization of drug regimes, the use of predictive biomarkers to identify putative responders versus non-responders, the development of anti-angiogenic treatment, the development of vessel normalization drugs, and the development of VEGF-independent anti-angiogenic drugs, the development and management of anti-angiogenic drug resistance deserves serious attention for the future improvement of anti-angiogenic therapies. An anti-angiogenic drug may not work across all the tumors since angiogenic mechanisms vary from one tumor type to another. Therefore, these mechanisms should be targeted in addition to anti-angiogenic therapies to achieve better results for patients with solid tumors. The only way to better combat the anti-angiogenic drug mediated resistance in cancer is to understand its mechanism of development following the anti-angiogenic therapy. On-treatment biopsies (obtaining patient tumor samples during therapy) are of utmost importance to validate preclinical data in parallel to clinical observation. The importance of on-treatment biopsies may provide us a unique scope for (1) testing drugs at the translation levels (genomics and proteomics) and (2) informing treatment strategies such as discontinuous therapy to delay the emergence resistance. Increased knowledge regarding the mechanisms of development of the drug resistance will aid in the improvement of effective therapies for patients with cancer. The future of anti-angiogenic drug based therapy in various cancers rests on the shoulder of basic scientists who will reveal the biology of tumor cells' response to the drug and on the shoulder of the clinicians who will translate the knowledge to the benefit of the patients. In the new era of genomic knowledge driven precision medicine, the introduction of anti-angiogenic drug into the treatment regime in different settings needs to be delicately weighed against the (1) the toxicities of drug, (2) drug induced resistance and (3) other options of targeted therapies.

Acknowledgements

Authors acknowledge Avera Research Institute, Sioux Falls, SD and Dept. of Internal Medicine, University of South Dakota, SSOM, Sioux Falls, SD. Authors acknowledge Ms. Hannah Koble

for her prompt and efficient secretarial assistance with table preparation.

Disclosure of conflict of interest

None.

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