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REVIEW

Challenges for patient selection with VEGF inhibitors

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Abstract As targeted therapies for cancer become increasingly integrated into standard practice, appropriate selection of the patients most likely to benefit from these therapies is now receiving critical scrutiny. Early experience with therapies directed at targets that are definitively overactive (e.g. the bcr-abl tyrosine kinase targeted by imatinib) or over-expressed [e.g. the human epidermal growth factor receptor 2 (HER2) targeted by trastuzumab] has generated the perception that pre-treatment target assessment is a pre-requisite for therapy with all targeted agents. However, emerging evidence suggests that this is not presently feasible for anti-angiogenic agents. Despite considerable evidence for the association of intratumoral and/or plasma vascular endothelial growth factor (VEGF) levels with tumor progression and/or poor prognosis, pre-treatment VEGF levels do not appear to be predictive of response to anti-angiogenic therapy. This may possibly be due to the complexity of the angiogenic pathways and the limitations associated with current methods of VEGF detection and quantification; e.g. low assay sensitivity and lack of standardized methods could prevent detection of very small increases in VEGF, which may be clinically important in patients with tumors that are highly dependent on this growth factor. In addition to a general lack of agreement as to the relative clinical relevance of circulating versus tumor VEGF levels, the absence of a 'gold standard' VEGF detection assay and the lack of a predefined, clinically relevant cut-off pose a significant hindrance to the clinical utility of VEGF measurements for therapy selection. Given the fundamental importance of angiogenesis for tumor growth and progression, and the key role of VEGF in these processes, presently it seems appropriate to view anti-VEGF agents such as bevacizumab (Avastin®) as having potential utility, independently of pre-treatment screening. Further research is needed to define the relationship between potential surrogate markers of VEGF pathway activity and clinical outcomes.

Keywords Angiogenesis · Vascular endothelial growth factor · Bevacizumab · Vatalanib · Epidermal growth factor receptor · Patient selection

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is necessary for tumor growth, progression, and metastasis [1, 2]. Since Judah Folkman first suggested that inhibiting angiogenesis might have therapeutic potential for treating cancer [3], researchers have identified many of the complex pathways involved in angiogenesis and proposed several selective targeted strategies. Certain anti-angiogenic agents are now in clinical practice and have shown encouraging results. Considering the recent clinical data for agents directed against molecular targets, in particular anti-angiogenic agents, is it necessary to identify which patients should be treated in order to increase efficacy and reduce toxicity of treatment?

The commonly held view of anticancer agents directed against molecular targets has been that they will only be effective in patients with tumors that express or overexpress the target compared with normal tissue. Although this may

be true for therapies directed at targets that are definitively overactive (e.g. the bcr-abl tyrosine kinase targeted by imatinib) or over-expressed (e.g. the human epidermal growth factor receptor 2 (HER2) targeted by trastuzumab) in subsets of cancers, emerging evidence suggests that other agents directed towards targets involved in angiogenesis or other growth factor systems may be effective regardless of the level of the target. For example, in agents targeting vascular endothelial growth factor (VEGF), a key regulator of tumor angiogenesis that is expressed in a wide variety of tumor types [4–6], there are a number of issues to consider. Although VEGF levels often correlate with the extent of angiogenesis and prognosis, this is not always the case, suggesting that factors other than the absolute level of VEGF may be important. Moreover, not only is there no consensus as to which form of VEGF is the most clinically relevant to test for (e.g. tumor vs. circulating, or bound vs. unbound VEGF), but there is also no 'gold standard' detection method. Recent data suggest that the efficacy of anti-VEGF agents does not relate directly to pre-treament VEGF levels.

Similarly, the use of agents targeting tumor necrosis factor-α in inflammation (etanercept, lenercept, infliximab, adalimumab) and T-cell activation in psoriasis (efalizumab, alefacept) does not require pre-treatment target assessment because the target has a well-established role in disease pathogenesis. In oncology, the use of imatinib to treat acute myelogenous leukemia does not require that target (c-Kit) levels are established prior to therapy because most of these tumors express the target. The clinical benefit of this agent is similar to or greater than that of agents, such as trastuzumab, that require patient selection based on target status being established prior to treatment.

This paper will review the rationale for VEGF inhibition as an anticancer strategy, and discuss the issues around VEGF detection in cancer and its utility for selecting patients for anti-VEGF therapy.

Tumor angiogenesis and VEGF

Angiogenesis is tightly regulated by a balance of pro- and anti-angiogenic factors [7–9]. Tumor cells and the tumor-associated stroma secrete a variety of pro-angiogenic factors that activate endothelial cells on nearby blood vessels, promoting new blood vessel formation via a complex series of events. Among the most prominent of these pro-angiogenic factors are VEGF, placental growth factor (PlGF), and angiopoeitin-1; with VEGF being considered the most important [8, 10, 11].

VEGF-A (referred to hereafter as VEGF) is a member of the VEGF/platelet-derived growth factor (PDGF) super gene family [12] that includes four other VEGF isoforms (VEGF-B, VEGF-C, VEGF-D, VEGF-E) and PlGF. Alternative exon splicing generates at least seven isoforms of VEGF, of which the four main isoforms are $VEGF_{121}$, $VEGF_{165}$ (the predominant species), $VEGF_{189}$, and $VEGF_{206}$ [13, 14]. Upon release into the extracellular matrix (ECM), the longer VEGF isoforms ($VEGF_{189}$ and $VEGF_{206}$) are almost entirely bound to heparin-containing proteoglycans, whereas $VEGF_{121}$ is freely soluble and $VEGF_{165}$ is partly soluble (approximately 50–70% of $VEGF_{165}$ is bound to the cell surfaces and the ECM) [15–17]. The protease plasmin, which plays a key role in the invasive process in angiogenesis [18] can release shorter forms of VEGF (e.g. $VEGF_{165}$) from bound VEGF, thus providing an additional source of soluble $VEGF_{165}$.

VEGF primarily binds to two transmembrane receptors with intracellular tyrosine kinase activity: VEGF receptor-1 (VEGFR-1, Flt-1) and VEGF receptor-2 (VEGFR-2, Flk-1/KDR) [13]. Binding of VEGF causes ligand-dependent receptor dimerization and tyrosine kinase autophosphorylation, resulting in activation of intracellular signaling pathways. It is now accepted that VEGFR-2 is principally responsible for mediating the mitogenic, angiogenic, and vascular permeability-enhancing effects of VEGF. Although VEGFR-1 has only weak tyrosine kinase activity compared with VEGFR-2, there is some evidence to suggest that VEGFR-1 is also able to mediate the growth and survival effects of VEGF, for instance via paracrine release of cell survival factors [19]. $VEGF_{165}$ also binds to neuropilin-1 (NRP1), which is proposed to enhance VEGFR-2-mediated signal transduction.

VEGF expression may be regulated by a variety of cellular factors and conditions. Hypoxia is the key regulator in malignancy, although the action of oncogenes and tumor suppressor genes, hormones, and cytokines and other growth factors may also be involved [6, 10, 19]. VEGF itself may contribute to tumor growth and progression through enhancement of microvascular permeability, promotion of angiogenesis (via activation of endothelial cells, alteration of endothelial cell proliferation, induction of enzymes/proteins important for endothelial cell migration and sprouting) and protection against apoptosis [6, 10]. Stimulation of angiogenesis and lymphangiogenesis by VEGF [20] also offers routes for metastatic spread.

Evidence suggests that expressions of VEGFR-1 and VEGFR-2 are up-regulated by hypoxic conditions [21, 22] as well as by a VEGF/VEGFR positive feedback loop [23, 24].

Experimental studies supporting the key role of VEGF and angiogenesis in tumor growth and progression gained from early studies showing that VEGF inhibition blocked tumor growth in xenograft models of human tumors [25, 26]. Other studies have demonstrated that VEGF is an important negative regulator of immune response through inhibition of dendritic cell maturation and natural killer cell

activity [7, 27]. These studies outlined the key role of VEGF in tumor growth and progression, and provided the rationale for the clinical development of agents targeting VEGF.

VEGF expression and detection in cancer

VEGF is expressed in approximately 30–60% of most solid tumors, and in up to 100% of renal cell carcinoma [28]. In general, tumor cells have been shown to express different VEGF ligands. Additionally, VEGFR-1 and VEGFR-2 are upregulated on intratumoral endothelial cells, and also expressed on circulating endothelial cells (CECs), endothelial progenitor cells (CEPs), and tumor cells [6, 29]

A number of different techniques have been used to evaluate VEGF expression in human cancers (Table 1), but to date there is no 'gold standard' test. VEGF mRNA and protein levels are usually evaluated in either tumor biopsy tissue or blood samples, but VEGF may also be measured in other body fluids, including malignant pleural effusions (ovarian, gastric, and colon cancers), malignant ovarian cysts, and urine (bladder cancer) [10].

VEGF mRNA can be detected and/or quantified using in situ hybridization (ISH), northern blot hybridization, reverse-transcription polymerase chain reaction (RT-PCR), microarray, and RNase protection assay techniques. Immunohistochemistry (IHC), enzyme-linked immunosorbent assays (ELISA), chemiluminescence immunosorbent assays (ICMA), and Western blotting techniques have been used to detect VEGF protein from tissue samples, whilst circulating levels of VEGF protein may be quantified using ELISA, ICMA (or other immunoassays), cell proliferation tests, and receptor binding assays.

Accurate and meaningful quantification of VEGF can be confounded by a number of factors (Table 1); e.g. increased VEGF mRNA expression is found in tumor cells adjacent to necrotic foci [30, 31]. In addition, VEGF mRNA expression

Table 1 Summary of common vascular endothelial growth factor (VEGF) detection methods

Method and description	Comments
Immunohistochemistry (IHC) detects VEGF protein expression in whole tissue sections (usually formalin-fixed, paraffin-embedded tissue)	Possible to differentiate between tumor and non-tumor VEGF expression
	Simple to perform
	Most common detection method
	No standardized methodology or scoring procedure
	Results variable and subjective
Enzyme-linked immunosorbent assay (ELISA) and chemiluminescence immunosorbent assay (ICMA) detect VEGF protein expression in tissue homogenate (fresh-frozen tissue), serum, or plasma	Serum and plasma measurements convenient vs. tissue samples
	Can be automated for high throughput
	Cannot distinguish between tumor and non-tumor sources of VEGF
	Circulating VEGF may be bound to serum proteins and unavailable to ELISA antibodies
	Serum measurements may be confounded by release of VEGF from platelets
Western blotting detects VEGF protein expression in tissue homogenate (fresh-frozen tissue)	Cannot distinguish between tumor and non-tumor sources of VEGF
	Less simple to perform than IHC
In situ hybridization (ISH) detects VEGF mRNA in whole tissue sections (ideally, fresh-frozen tissue)	Can distinguish between tumor and non-tumor VEGF expression
	May not relate directly to VEGF protein expression
	Less simple to perform than IHC
Northern blotting detects VEGF mRNA from tissue homogenates (fresh-frozen tissue)	Cannot distinguish between tumor and non-tumor VEGF expression
	May not relate directly to VEGF protein expression
	Less simple to perform than IHC
Reverse-transcription polymerase chain reaction (RT-PCR) detects VEGF mRNA in tissue homogenates (usually fresh-frozen)	Quantitative method that can be automated for high-throughput
	Cannot distinguish between tumor and non-tumor sources of VEGF
	Sensitive to contamination
	May not relate directly to VEGF protein expression
RNase protection assay detects VEGF mRNA in cellular extracts (tissue or circulating)	Cannot distinguish between tumor and non-tumor VEGF expression
	May not relate directly to VEGF protein expression
	Relatively complex to perform

has been found to correlate with vascular density in certain (e.g. carcinomas of the cervix or breast) but not all cancers [10]. IHC studies have shown that in addition to VEGF staining on tumor cells, antibodies to VEGF also stain tumor-associated blood vessels [10, 31] indicating that the vessels may provide a 'sink' for binding and retaining tumor-derived VEGF (since endothelial cells do not produce VEGF) [10]. Results from protein detection techniques involving tissue homogenates reflect a combination of tumor cell VEGF and associated blood vessel VEGF, and thus may not accurately reflect the degree of active tumor VEGF expression at a given time. Finally, primary tumors and metastases from the same patient may differ in their level of VEGF expression [32, 33], further complicating the interpretation of data.

Serum results are difficult to interpret because measurement may include VEGF released from non-tumor sources such as platelets and leukocytes [10], cells containing significant proportions of circulating VEGF [34, 35]. As serum VEGF concentrations have been shown to increase with longer clotting time, a standardized clotting time may help to reduce the variation in serum VEGF measurements between assays [34]. Cancer patients appear to have higher levels of VEGF per platelet than healthy volunteers [36, 37], which are presumed to result from platelet scavenging of tumor-derived VEGF. For this reason it has been proposed that measurement of VEGF in serum, rather than in plasma (which represents an equilibrium between free VEGF and VEGF sequestered in platelets, but also contain non-tumor-derived VEGF), may be more appropriate for providing an indirect estimate of tumor VEGF (and therefore better prognostic information) [38]. This is supported by a study in patients with primary colorectal carcinoma (CRC) in which increased serum, but not plasma, VEGF levels correlated with reduced survival in multivariate analyses [39]. Although the relationship between tumor production of VEGF and its blood concentration is not fully understood, it has been suggested that serum VEGF measurements should be corrected for platelet count to normalize data between patients and study groups [36], and allow for a more accurate estimate of tumor VEGF expression. However, it is not clear whether total or platelet-corrected serum measurements provide better prognostic information [38].

VEGF serum measurements may be further complicated by the binding of VEGF to serum proteins, rendering VEGF unavailable to certain assay antibodies [10]. Moreover, other non-tumor-related factors may contribute to increased VEGF in circulation of cancer patients (and/or controls), such as platelet activation, hypoxia, and inflammation, adding to the difficulty in relating circulating VEGF levels to tumor-related processes and their influence on prognosis [10, 40].

Furthermore, differences in the detection of VEGF isoforms contribute to the large variations in VEGF levels that have been reported in different studies. For example, reported levels of circulating VEGF in healthy subjects have ranged from 1 to 25 μg/l depending on the detection technique [41]. Even when the same technique is used, large variations are reported among studies: in healthy subjects, measurement of $VEGF_{121}$ and $VEGF_{165}$ using a commercial ELISA kit produced values ranging from <9–50 ng/l in plasma and 10–300 ng/l in serum in different studies [41].

Variations in tissue storage times and procedures may also alter the results. In one study in patients with renal cell carcinoma, VEGF expression in the membranes of tumor cells from paraffin-embedded tumor samples was found to significantly decrease with increased storage time [42]. Finally there is no definitive proof that circulating VEGF levels directly correlate with tumor angiogenesis or prognostic indicators [36], and it has therefore been suggested that quantification of tumor VEGF–VEGF receptor complexes might be a more useful indicator of VEGF activity [43].

Prognostic value of VEGF

Numerous retrospective studies have been conducted in the attempt to evaluate the relationship between VEGF levels with disease stage and prognosis in various human malignancies.

Increased VEGF expression, either in the circulation or in tumor tissue, was found to correlate with worse prognosis (decreased disease-free, relapse-free and/or survival) in several cancers including bladder [44], breast [45, 46], central nervous system [47], cervical [48], colorectal [49, 50], head and neck [51], hepatic [52], lung [53], pancreatic [41], ovarian [54] and renal cell cancer [55, 56], although a correlation was not seen in all studies [38] (Table 2) [57–107]. The differences in these study findings may be attributed, at least in part, to different testing methods and other contributing factors, as discussed previously. These differences make it difficult to draw definitive conclusions as to the prognostic value of either tumor or serum VEGF measurement [61, 108]. Several studies have found that increased VEGF expression is associated with tumor progression [45, 49, 50, 109, 110] and VEGF has also been shown to correlate with the extent of tumor vascularization in many studies [48, 49, 53, 110, 111].

Several studies have evaluated whether VEGF receptor expression correlates with prognosis. The results of studies in different cancer indications suggest that expressions of VEGFR-1 and VEGFR-2 may correlate with poor prognosis [112–114].

Table 2 Vascular endothelial growth factor (VEGF) expression in human cancer and correlation with prognosis

Cancer type	Tissue tested	Method	Correlation between VEGF levels and reduced patient survival (DFS, RFS, and/or OS)	Reference
Bladder	FFPE	ISH	Positive	[40]
	FFPE	IHC	No correlation	[53]
Brain	FFPE	IHC	Positive	[54]
	FFPE	IHC	Positive	[55]
	Frozen tumor tissue	RT-PCR	Positive	[56]
Breast	Tumor cytosol	ELISA	Positive	[57]
	Tumor cytosol	RNase	Positive	[58]
	Frozen tumor tissue	ICMA	Positive	[59]
	Tumor cytosol	ELISA	Positive	[60]
	Tumor cytosol	ELISA	Positive	[61]
	Tumor cytosol	ELISA	Positive	[62]
	Tumor cytosol	ELISA	Positive	[63]
	Tumor cytosol	ELISA	Positive	[64]
	Frozen tumor sample	IHC	Positive on UVA but not MVA	[65]
	Frozen tumor tissue	ELISA	No correlation on UVA (but trend towards reduced OS)	[66]
	Tumor cytosol	ELISA	No correlation (with DFS)	[67]
	Serum and plasma	ELISA	No correlation	[68]
Cervical	FFPE	IHC	Positive	[69]
Colorectal	FFPE	IHC	Positive	[45]
	Frozen tumor tissue	NBH	Positive	[70]
	FFPE	IHC	Positive	[71]
	FFPE	IHC	Positive	[72]
	Frozen tumor tissue	RT-PCR	Positive (in patients with $VEGF_{121}$ and $VEGF_{165}$ and$VEGF_{189}$ isoforms)	[73]
	Serum (preoperative)	ELISA	Positive	[35]
	Plasma (preoperative)	ELISA	Positive on UVA but not MVA	[35]
	FFPE	IHC	No correlation with OS	[74]
Endometrial	FFPE	IHC	Positive	[75]
Gastric	FFPE	IHC	Positive	[76]
	FFPE	IHC	Positive	[77]
	Serum (preoperative)	ELISA	Positive	[78]
	FFPE	IHC	No correlation	[79]
Head and neck	FFPE	ELISA	Positive	[47]
	Frozen tumor tissue	Western blot	Positive	[80]
	FFPE	IHC	Positive	[81]
	FFPE	IHC	No correlation	[82]
	Serum (pretreatment)	ELISA	No correlation	[83]
Lung	FFPE	IHC	Positive in NSCLC	[84]
	FFPE	IHC	Positive in NSCLC	[85]
	FFPE	IHC	Positive in NSCLC	[86]
	FFPE	RT-PCR	Positive in NSCLC	[87]
	Serum	ELISA	Positive in NSCLC	[88]
	FFPE	IHC	Positive in SCLC	[89]
	Serum (preoperative)	ELISA	Positive in NSCLC	[90]
	FFPE	IHC	Positive on UVA but not MVA in NSCLC	[49]
	Serum (preoperative)	ELISA	No correlation in NSCLC	[86]

Table 2 continued

Cancer type	Tissue tested	Method	Correlation between VEGF levels and reduced patient survival (DFS, RFS, and/or OS)	Reference
Melanoma	Serum	ELISA	Positive	[91]
	Serum (30 days after surgery)	ELISA	No correlation	[92]
Oesophageal	FFPE	IHC	Positive	[93]
	FFPE	IHC	Positive on UVA but not MVA	[94]
	FFPE	IHC	No correlation	[95]
Ovarian	FFPE	ISH	Positive	[96]
	FFPE	IHC	Positive	[97]
	FFPE	IHC	Positive	[98]
	FFPE	IHC	Positive	[99]
	FFPE	IHC	No correlation	[29]
Pancreatic	Frozen tumor tissue	RT-PCR	Positive	[100]
	Serum (preoperative)	ELISA	Positive on UVA but not MVA	[37]
Prostate	FFPE	IHC	Positive	[101]
	Plasma	ELISA	Positive	[102]
Renal cell	Serum	ELISA	Positive on UVA but not MVA	[52]
	FFPE	IHC	Positive on UVA but not MVA	[38]
Vulvar	FFPE	IHC	Positive	[103]

DFS disease-free survival, *ELISA* enzyme-linked immunosorbent assay, *FFPE* formalin-fixed, paraffin-embedded tumor tissue, *ICMA* chemiluminescence immunosorbent assay, *IHC* immunohistochemistry, *ISH* in-situ hybridization, *MVA* multivariate analysis, *NBH* northern blot hybridization, *NSCLC* non-small cell lung cancer, *OS* overall survival, *RFS* relapse-free survival, *RNase* RNase protection assay, *RT-PCR* reverse-transcription polymerase chain reaction, *SCLC* small cell lung cancer, *UVA* univariate analysis

VEGF inhibitors in the clinic: is VEGF testing necessary for patient selection?

The predictive value of VEGF levels for outcome of antiangiogenic therapy is not proven. As many of the numerous agents that inhibit several targets of the VEGF signaling pathway (monoclonal antibodies to VEGF or its receptors; small molecule VEGF receptor tyrosine kinase inhibitors (TKIs); ribozymes; soluble VEGF receptors [VEGF-Trap]) are reaching advanced stages of clinical testing or receiving regulatory approval, researchers and clinicians are beginning to question the utility of pre-treatment VEGF assessment for patient selection.

Monoclonal antibodies to VEGF

Bevacizumab (Avastin®) is a humanized IgG_1 monoclonal antibody [115] that binds to and neutralizes all human VEGF-A isoforms (but not other members of the VEGF family) [19]. Preclinical studies demonstrated that bevacizumab inhibits the growth of human tumor cell lines in vitro and tumor growth in vivo [115]. In phase I studies in cancer patients, bevacizumab demonstrated direct antivascular effects on tumors, was generally well tolerated and not associated with dose-limiting toxicities, and did not exhibit overlapping toxicity with standard chemotherapeutic agents [116, 117]. Additional clinical trials have showed that a single infusion of bevacizumab induces a rapid and direct antivascular effect on tumors in patients with locally advanced rectal cancer [118].

Positive results from phase II and phase III studies [119–122] led to approval of bevacizumab for use in combination with 5-fluorouracil (5-FU)-based chemotherapy and carboplatin/paclitaxel for the first-line treatment for metastatic CRC and advanced non-small cell lung cancer (NSCLC). It should be noted that testing for pre-treatment VEGF status is not a requirement for the use of bevacizumab. A significant survival benefit has also been demonstrated with bevacizumab in combination with standard chemotherapies for metastatic breast cancer [123]. Although VEGF positivity (either circulating or tumor-associated) was not an eligibility requirement in any of the phase II or phase III bevacizumab trials discussed in this review, VEGF levels were often evaluated, allowing the relationship between outcome and VEGF expression to be explored and reported in several of these trials (Table 3) [124–128].

Pre-treatment serum VEGF levels were below the level of quantification in 8 of 15 patients with hormone-refractory metastatic prostate cancer treated with bevacizumab monotherapy; however, the potential relationship between VEGF levels and response was not reported for the patients who

Table 3 Summary of relationship between vascular endothelial growth factor (VEGF) levels and response in randomized bevacizumab trials

Trial description	Key efficacy findings	VEGF evaluation	Relationship of VEGF to key efficacy outcomes	Reference
Phase II randomized, placebo-controlled trial of single-agent bev in 116 patients with metastatic RCC.	Bev (10 mg/kg every 2 weeks) monotherapy significantly increased TTP vs. placebo (4.8 vs. 2.5 months; HR = 2.55, $P < 0.001$).	Plasma VEGF protein (free and bound) measured in 113 (97%) patients; 76 (67%) measurements below the lower limit of detection.	No significant associations with either clinical response or TTP.	[117]
Phase III randomized, controlled trial of bev/capecitabine vs. capecitabine alone in 462 previously-treated MBC.	Bev (15 mg/kg every 3 weeks) plus capecitabine significantly increased RR vs. capecitabine alone (19.8% vs. 9.1%; $P = 0.001$).	Tumor VEGF mRNA measured in FFPE tissue by ISH in 29% of patients, 32% of whom had no detectable VEGF expression. Results graded on a 0–3 scale	No association between VEGF overexpression (score of 3) and clinical response to bev/capecitabine therapy.	[118], [119]
Phase III randomized, controlled trial of bev/IFL vs. IFL in 813 patients with previously-untreated MCRC.	Bev (5 mg/kg every 2 weeks) significantly increased OS (20.3 vs. 15.6 months; HR = 0.66, $P < 0.001$), PFS (10.6 vs. 6.2 months; HR = 0.54, $P < 0.001$) and RR (44.8% vs. 34.8%, $P = 0.004$).	Tumor VEGF mRNA measured in FFPE tissue by ISH in 187 (23%) patients. Results graded on a 0–3 scale; approximately 30% had VEGF score of 3. Pre-treatment plasma VEGF protein measured by ELISA in 384 (47%) patients. Plasma VEGF was detected in 337 (88%) of samples.	No relationship between a VEGF mRNA score of 3 and survival benefit from bev. No association between plasma VEGF levels and survival benefit from bev.	[112], [120], [121]

MCRC metastatic coloretal cancer, *BC* breast cancer, *FFPE* formalin-fixed, paraffin-embedded, *HR* hazard ratio, *IFL* irinotecan/5-fluorouracil/leucovorin, *ISH* in-situ hybridization, *RCC* renal cell carcinoma, *TTP* time to disease progression, *OS* overall survival, *PFS* progression-free survival

had measurable VEGF [129]. Bevacizumab monotherapy was also evaluated in a phase II randomized, double-blind trial in 116 patients with metastatic renal cell carcinoma [124], a type of cancer in which VEGF is almost always over-produced due to mutations of the von Hippel-Lindau tumor suppressor gene [130–132]. Bevacizumab 10 mg/kg every 2 weeks significantly improved time to progression compared with placebo (4.8 vs. 2.5 months, $P < 0.001$) (Table 3). Measurements of plasma VEGF were available in 113 of the 116 enrolled patients; 76 patients had baseline levels below the lower limit of detection (40 pg/ml) suggesting poor assay sensitivity. There was no significant association between detectable pre-treatment VEGF and clinical response or time to progression, but the authors comment that limitations in assay sensitivity prevented definitive conclusions.

The relationship between VEGF and response to bevacizumab was explored in two phase III trials (Table 3). The addition of bevacizumab to capecitabine in patients with previously treated metastatic breast cancer significantly increased response rate (RR) versus capecitabine alone (19.8% vs. 9.1%, *P*:0.001) and demonstrated small non-significant increases in overall survival (OS) and progression-free survival (PFS) [126]. A retrospective analysis of VEGF mRNA levels by ISH (using a 0–3 grading scale, where 3 denotes the highest expression) was performed on pre-treatment tumor samples from 164 patients [125]. Approximately one-third of patients tested had no ISH expression; 37% of patients were graded as having 1+ expression, 20% were 2+, and 11% were 3+. The analysis failed to detect any relationship between VEGF mRNA levels and RR.

The pivotal phase III trial in 813 patients with previously untreated metastatic CRC demonstrated a significant survival advantage from the addition of bevacizumab to bolus irinotecan/5-FU/leucovorin (LV) (IFL) versus IFL plus placebo (20.3 vs. 15.6 months, $P < 0.001$) [119]. The addition of bevacizumab to IFL also significantly improved PFS (10.6 vs. 6.2 months; $P < 0.001$) and RR (44.8% vs. 34.8%; $P = 0.004$). VEGF levels were retrospectively evaluated in a subgroup of patients who had tumor samples collected [128]. VEGF mRNA expression quantified by ISH on tissue microarrays (using a 0–3 grading scale) in the tumor samples from 187 patients (83 from the IFL arm and 104 from the bevacizumab plus IFL arm) found that approximately 30% of patients had VEGF expression with a score of 3. Similar to the whole patient population, bevacizumab provided a survival benefit compared with placebo irrespective of VEGF mRNA levels, i.e. in patients with a VEGF score of 3 versus those with a score of 0–2 [128]. This retrospective subgroup analysis also showed that bevacizumab provides a survival benefit irrespective of the level of thrombospondin-2 (THBS-2; an endogenous inhibitor of angiogenesis) or microvessel density (MVD).

Pre-treatment plasma VEGF protein levels were also quantified using ELISA in 384 patients (191 in the IFL arm and 193 in the bevacizumab plus IFL arm) from this study [127]. Plasma VEGF was detected in 333 (88%) samples; 4 samples were above the upper limit of quantification (889 pg/ml) and 47 samples were below the lower limit of detection (12.5 pg/ml). There was no difference in OS between patients with the lowest and highest VEGF levels. Thus, the level of VEGF expression is not a predictive factor of survival benefit from bevacizumab treatment; however, the analysis suggested that the baseline level of plasma VEGF is prognostic for OS.

Finally, a recent phase II study in 52 patients with metastatic pancreatic cancer treated with gemcitabine and bevacizumab showed that pre-treatment plasma VEGF levels did not correlate with outcome [133].

Taken together, the results of these retrospective analyses suggest that response to bevacizumab therapy may not be related to pre-treatment tumor VEGF expression or circulating VEGF levels. Thus, pre-treatment VEGF measurement using actual methodologies has little predictive value for patients receiving bevacizumab.

VEGF receptor tyrosine kinase inhibitors

Several VEGFR TKIs have entered clinical development. The most advanced are sunitinib malate (SU11248, Sutent®; inhibits VEGFR-2, PDGF receptor (PDGFR), c-Kit, and Fms-related tyrosine kinase/Flk2/Stk-2 [Flt-3] [134, 135]) approved for the treatment of advanced renal cell carcinoma (RCC) and gastrointestinal stromal tumor, and sorafenib (Nexavar®; inhibits Raf kinase and VEGFR-2 and VEGFR -3, PDGFR-β, Flt-3, and c-Kit receptor tyrosine kinases [136]) approved for the treatment advanced RCC. Other VEGFR TKIs that have entered phase II/III testing include vatalanib (PTK-787/ZK 222584; inhibits VEGFR-1, VEGFR -2 and VEGFR -3, PDGFR, c-Kit and c-Fms [137], and ZD6474 (which has activity against VEGFR-2 and VEGFR -3, and epidermal growth factor receptor (EGFR) [138, 139].

Sunitinib malate has reported results of VEGF or VEGFR testing in clinical studies. A phase I trial of patients with advanced cancer found that VEGF levels were significantly increased within 4 weeks of sunitinib malate treatment [140], and plasma VEGF levels increased by ≥3-fold in 70% of patients [89, 141]. Another phase I/II trial of 28 patients with advanced solid cancers, sunitinib malate increased plasma VEGF concentrations during the first month of treatment, whereas plasma sVEGFR-2 levels decreased [142]. Additionally, in a multicenter phase II metastatic RCC trial by Motzer et al., sunitinib malate increased the levels of VEGF-A and placental growth factor (PlGF), and decreased soluble VEGFR-2 (sVEGFR-2); changes in all the biomarkers were highly significant in all cycles through cycle 8 ($P \leq 0.002$) [143]. During the 2-week treatment break of the 6-week cycle, the levels of all three biomarkers returned to near baseline levels. No correlations were reported between clinical response and plasma changes of these factors [143]. There are several hypotheses that could explain these changes in biomarkers, including the dislodgement of VEGF bound to the external domain of VEGFR-2, the rapid release of stored VEGF from known sources (e.g. platelets, α2-macroglobulin, Thrombospondin-1), the compensatory increase of VEGF in various tissues perhaps secondary to an induced state of local hypoxia, the block of the VEGF-A clearance by the kidney due to the inhibition of VEGFR-2 and, finally, the lack of VEGF clearing by VEGFR-2 after anti-VEGFR-2 therapy. In addition, the mechanism behind the consistent decrease in sVEGFR-2 levels observed with sunitinib malate studies is not entirely understood at present, as biochemical characterization of the naturally occurring sVEGFR-2 protein has only recently begun. Probably, these data could reflect a feedback regulatory loop [143].

Vatalanib has been evaluated as a single agent and in combination with chemotherapy [144–152], but only one trial reported data on pre-treatment VEGF status [153]. In this phase I trial, plasma VEGF levels increased following vatalanib administration, but there was no correlation between plasma VEGF levels and disease progression [153]. A pooled analysis of data from two phase I trials of vatalanib monotherapy in patients with advanced CRC showed that plasma VEGF levels increased during the first cycle of therapy, and then declined during the second cycle of therapy [146]. There was a positive correlation between changes in VEGF levels during the first cycle of therapy and clinical outcome (non-progressive disease; $P = 0.027$), but not between pre-treatment VEGF levels and outcome. The authors noted that pre-treatment VEGF levels varied among patients, which may have been due to differences in disease stage and tumor size. The phase III trial of vatalanib plus 5-FU/LV/oxaliplatin (FOLFOX4 regimen) (CONFIRM-I study), failed to meet its primary endpoint of improved PFS, and did not report any relationship between pre-treatment VEGF status and outcome [154]. However, a subset of patients with high levels of lactate dehydrogenase, a marker possibly related to hypoxia, had a significantly better outcome with the addition of vatalanib versus chemotherapy alone.

Additionally, a recent preclinical study found that the antitumor efficacy of the VEGFR-2 TKI GW654652 correlated with high VEGF and low VEGFR-2 expression in tumor xenografts [155]. The authors concluded that VEGF receptor tyrosine kinase inhibitors might be more effective in patients with this pattern of tumor VEGF/VEGFR-2

expression. However, this hypothesis has not yet been tested in the clinic.

What level of VEGF is clinically meaningful, and how should it be measured?

Tumor VEGF expression does not always translate to increased tumor angiogenesis [69, 111], and the expression level gives no indication as to the degree of dependence of a tumor on VEGF signaling. There is evidence that tumors are more dependent on VEGF in earlier stages of disease, with the role of VEGF (vs. other angiogenic mediators) decreasing during disease progression [62].

Results of preclinical [156–159] and clinical [119, 160] studies have demonstrated that agents targeting VEGF improve the anticancer activity of conventional cytotoxic agents. It has been suggested that in addition to causing vascular regression and preventing the formation of new vasculature, both of which have direct effects on tumor growth, anti-VEGF agents 'normalize' the tumor vasculature, (which is typically abnormally structured and hyperpermeable), reduce intratumoral pressure, and cause the abnormal vasculature to regress. These vascular effects are also proposed to allow improved penetration and effectiveness of cytotoxic agents [6, 161, 162]. In patients with rectal cancer, bevacizumab reduced tumor perfusion, vascular volume, microvascular density, and interstitial fluid pressure, and increased the fraction of vessels with pericyte coverage [118]. In addition, there is accumulating evidence for the existence of VEGF receptors on tumor cells, suggesting a direct antitumor effect of anti-VEGF agents [6]. Data from the Eastern Cooperative Oncology Group (ECOG) E3200 trial support this theory [160]. The addition of bevacizumab to FOLFOX4 resulted in significantly better OS (12.9 vs.10.8 months, *P*:0.0018) and PFS (7.2 vs. 4.8 months, $P < 0.0001$) compared with FOLFOX4 alone in patients with previously treated advanced CRC.

Although plasma VEGF levels correlated with tumor VEGF levels, and tumor VEGF levels correlated with microvessel density (a marker of angiogenesis), plasma VEGF levels did not correlate with microvessel density in patients with advanced CRC [163]. These results suggest that while plasma VEGF levels may relate to the level of tumor VEGF expression, they may not be a good indicator of tumor VEGF activity. Moreover, in patients with NSCLC, tumor VEGF expression, but not preoperative serum VEGF, was found to correlate with survival [90]. This study also found that there was no correlation between tumor and serum VEGF levels, a finding that was attributed in part to the different antibodies used in each assay (anti-$VEGF_{121}$ for IHC and anti-$VEGF_{165}$ for ELISA).

Measurement of tumor VEGF levels is even more problematic, with issues surrounding tissue availability, tissue source (primary vs. distant metastases), tissue preparation, and storage techniques, all of which can affect results. Depending on the level of dependence of the tumor on VEGF signaling, even small changes in tumor VEGF expression may be clinically important [28]. However, the level of sensitivity of commonly used assays may be too low to detect these clinically meaningful changes in VEGF expression, while difficulties remain in defining a relevant 'cut-off', particularly with so many studies using different detection methods.

It has been suggested that quantification of tumor VEGF–VEGFR complexes might be a more useful indicator of VEGF activity. In a study in patients with NSCLC, VEGF–VEGFR-2 complex levels correlated with poor survival even in patients with low tumor VEGF expression [43]. Similarly, a recent retrospective study evaluating tumor specimens from 202 patients with primary breast cancer found that levels of intratumoral soluble VEGFR-1 and VEGF, and the ratio of sVEGFR-1 to total VEGF are potent and independent prognostic factors [68]. The authors suggested that the determination of VEGF and sVEGFR-1 is useful to distinguish anti-VEGF therapy-sensitive tumors from less sensitive tumors. However, these findings are complicated by subgroup analyses which found that the prognostic value of total VEGF and sVEGFR-1 is specific for estrogen receptor-positive and negative tumors, respectively. In addition, total VEGF, sVEGFR-1, and the ratio of sVEGFR-1 to total VEGF were significant prognostic indicators for low but not high HER2-expressing tumors.

Potential of using CECs and CEPs as biomarkers of anti-angiogenic activity

At least two distinct populations of CECs have been identified: bone marrow-derived CEPs and mature CECs, which are thought to be derived from mature vasculature [164]. VEGF stimulates the mobilization of CEPs from the bone marrow compartment, where they enter the circulation, move to sites of ongoing angiogenesis, incorporate into growing blood vessels, and differentiate into endothelial cells [164, 165]. Thus, although their role in tumor vascularization remains to be determined, it is thought that CECs and CEPs contribute to angiogenesis.

Preclinical and clinical studies support a role of CEPs in angiogenesis and as a measure of antiangiogenic therapy. The effect of angiogenesis inhibitors on CECs/CEPs was recently evaluated in mice bearing Lewis lung-carcinoma [166]. In control mice, exogenous administration of VEGF increased levels of both CEC and CEPs. Co-administration of ZD6747 inhibited this increase in CEC and CEP levels,

but had no significant effect in the absence of exogenous VEGF. In contrast, in mice-bearing Lewis lung-carcinoma, ZD6474 had differential effects, causing a dose-dependent increase in mature CECs but not CEPs, accompanied by a decrease in tumor microvessel density and tumor volume after 3 days of treatment [166]. The apoptotic fraction of mobilized CEC was not significantly increased by treatment. In the same study, ZD6126, a vascular-targeting agent, was evaluated. After treatment with this agent, a fivefold induction in mature CECs was observed ($P = 0.04$). These CECs were predominantly (95%) mature CECs, although a small increase in CEPs was also observed [166]. Additional preclinical data showed a striking correlation between genetically heterogeneous bFGF- or VEGF-induced angiogenesis and intrinsic CEC or CEP levels in eight different inbred mouse strains [165]. Furthermore, in different strains of genetically altered mice, regulation of intrinsic CECs or CEPs was affected by the regulators of angiogenesis VEGF, Tie-2 and thrombospondin-1 [165]. Treatment with a targeted VEGFR-2 antibody (DC101) caused a dose-dependent reduction in viable CEPs that correlated with antitumor activity in tumor-bearing mice [167].

There is evidence suggesting that measurement of CECs could be used to evaluate response to antiangiogenic therapy. A phase II study of the thrombospondin-1 mimetic peptide, ABT-510, in patients with soft tissue sarcomas showed that patients with high baseline CEC levels exhibited reduced time to progression [168]. Similarly, changes in the levels of viable CECs from baseline to week 3 inversely correlated with PFS ($P = 0.015$) in patients with previously treated metastatic breast cancer receiving letrozole plus bevacizumab [169].

In locally advanced rectal carcinoma, a single infusion of bevacizumab (5 or 10 mg/kg) significantly reduced the percentage of viable CECs at day 3 and 12 compared with baseline ($P < 0.01$) [170]. The kinetics of CEPs showed similar trends (data not reported), although CEPs were detected at concentrations that were two orders of magnitude lower than those of viable CECs. Interestingly, the decrease in CECs occurred despite the significant increase in the levels of plasma VEGF and PlGF. Based on these results, the authors propose that the kinetics of CECs in the circulation, in conjunction with the levels of VEGF family proteins in the plasma should be further investigated as a potential surrogate marker of anti-VEGF agents [170].

Metronomic (continuous, low dose) chemotherapy is proposed to have anti-angiogenic properties. A recent study investigated the correlation between CEC kinetics and clinical outcome in patients with advanced breast cancer receiving a metronomic schedule of chemotherapy [171]. CECs decreased in patients with no clinical benefit (defined as a clinical response or a stable disease) as compared to those who had a clinical benefit ($P = 0.015$). This difference was due to an increased fraction of apoptotic CECs. After a median follow-up of 17.4 months, univariate and multivariate analyses indicated that CEC values greater than 11/μl after 2 months of therapy were associated with a longer PFS ($P = 0.001$) and an improved OS ($P = 0.005$). CEPs were always less than 5% of the CEC population, and no correlation was found with clinical outcome. In the same study, clinical benefit did not correlate with CEC or CEP count and viability in patients receiving thalidomide in addition to chemotherapy (data not shown) [171].

Other studies that support CECs as potential biomarkers include a phase I study of ZD6126 in patients with advanced solid tumors, which showed that CEC levels increased either after the first (week 1) or second (week 2) dose, and maximum levels were achieved a median of 4 h after infusion [172].This study also showed that CEC levels did not correlate with the magnitude of CEC increase, and CEC increase did not correlate with ZD6126 dose, peak plasma concentrations or drug exposure. Additionally, in patients with metastatic imatinib-resistant GISTs, sunitinib malate significantly increased the levels of mature CECs (it is not clear whether these were viable, non-viable or total CECs) after 6–20 days of therapy in responders versus non-responders [173]. In another recent study, objective response or stable disease with a combination of bevacizumab and erlotinib in breast cancer has been associated with a post-treatment increase in non-viable CECs at 3 weeks after treatment versus the baseline [174].

Thus, data suggest that a decrease in viable CECs, or an increase in non-viable CECs (resulting in an overall increase in total CECs compared to the baseline), might function as early biomarkers of efficacy for anti-angiogenic therapy. Current understanding of the mechanisms that regulate CECs and their role in angiogenesis is at very early stage. One proposed mechanism for the observed changes is that anti-angiogenic treatments damage and/or remove the survival signals for endothelial cells, either in circulation or in tumor-associated blood vessels, with subsequent release into the circulation [171].

Although early data are promising, further work is needed to better characterize CECs and CEPs, and their value as biomarkers of angiogenesis and/or angiogenesis inhibition [175]. Some questions need to be resolved, including: (1) the sensitivity and reproducibility of the methods employed; (2) accounting for the great variance of CEPs levels among animals with different genetic constitution; (3) do tumors mobilize sufficient CEPs to be detected in clinical practice; (4) the best antigen panel to characterize these cells; and, (5) what is the role of viable and non-viable cells.

Potential of using phosphorylated VEGFR-2 as a biomarker of anti-angiogenic activity

A pilot study of bevacizumab alone or in combination with doxorubicin and docetaxel in patients with inflammatory breast cancer assessed tissue VEGF, activated VEGFR-2 status (phosphorylated VEGFR2 [p-VEGFR-2]), total VEGFR2, tumor microvessel density (MVD), tumor cell apoptosis and proliferation, and vascular permeability (via dynamic contrast-enhanced magnetic resonance imaging [DCE-MRI)] [176]. The phosphorylation of VEGFR-2, at tyrosine sites 951 and 996, was evaluated by IHC and compared to baseline in tumor cells. p-VEGFR-2 was significantly reduced with bevacizumab monotherapy, and this change persisted during combination chemotherapy. These changes in p-VEFGR-2 were only observed in patients with partial response and stable disease, whereas levels of p-VEGR-2 were high in patients with progressive disease. These results indicate that bevacizumab has direct effects on tumours, by reducing the activity of VEGFRs expressed on their surface, and thereby suggest the potential of using p-VEGFR-2 to measure efficacy of anti-VEGF agents.

Potential of using vascular imaging for monitoring anti-angiogenic activity

Imaging modalities are relatively non-invasive assays useful to evaluate tumor vascularity. The combination of AIs and cytotoxic drugs make it advantageous to simultaneously assess tumor volume and tumor vascularity (a marker of anti-angiogenesis efficacy). A few techniques have been evaluated in conjunction with anti-angiogenic therapies in the clinic, and there is good evidence to support a potential role of four techniques: dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), PET scan, dynamic CT scan, and contrast-enhanced ultrasound [177, 178].

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)

DCE-MRI is a technique that yields parameters associated with tissue perfusion and vascular permeability. The paramagnetic contrast agent gadopentetate dimeglumine (Gd-DTPA) is injected as a rapid iv bolus and diffuses out of the blood vessels into the extravascular space. The changes in signal intensity of Gd-DTPA are recorded by serial images acquired before, during, and after the injection. Relative changes in semiquantitative parameters, such as the maximum gradient of the signal–intensity–time curve, the maximum increase in contrast enhancement, and the AUC are indirectly related to tissue perfusion, vascular permeability, and vessel surface area [177, 178].

The potential of DCE-MRI was evaluated in patients with advanced solid tumors receiving vatalanib (50 to 2,000 mg once daily) [179]. A substantial reduction in contrast enhancement (Ki) was evident for all dose groups on day 2 and at the end of cycle 1 (EC1). This reduction was more pronounced in the higher dose group. A significant inverse relationship was found between increasing PTK dose, AUC and reducing Ki on both day 2 and EC1. Patients with non-progressive disease had a significantly greater reduction in enhancement on day 2 and EC1, and there was a relationship between reduction in Ki and disease progression. The authors identified a dose in which the lower limit of exposure was associated with at least 40% reduction in contrast enhancement (60% baseline Ki), a level that was associated with non-progressive disease. These results suggested that DCE-MRI could be a useful biomarker for defining the pharmacological response and dose of PTK.

Another phase I study evaluated the role of DCE-MRI as a pharmacodynamic measure of response after acute dosing of AG-013736, an oral available angiogenesis inhibitor, in 31 patients with advanced solid tumors [180]. AG-013736 caused significant decreases in DCE-MRI vascular parameters by day 2 compared with baseline, and this decrease appeared to be dose-dependent. However, despite these promising results, because of the small number of patients evaluated ($n = 17$), there was no established association between vascular and clinical response [180].

The study by Wedam et al. [176], also evaluated DCE-MRI at baseline, and after cycles 1, 4, and 7 of therapy. A decrease in K transf, representative of vascular permeability and flow measured, was observed after the first infusion of bevacizumab and continued during combination therapy. A greater change in K transf was observed from cycle 1 to cycle 4 than from cycle 4 to cycle 7, suggesting that the overall tumor rate of change in treatment effect occurred in the earlier courses of therapy. However, no significant difference in any of these parameters was found between clinical responders and non-responders.

Despite these interesting results, there are also several issues to be taken into account. In order to obtain valid estimation of tumor response it is important that the same region of tumor is imaged on each patient visit. For this reason, 3-dimensional (3D) protocols which provide data over the whole tumor, are preferable to single-slice protocols. However, current MRI technology does not permit “fast” protocols in 3D, so investigators must choose between complete assessment of perfusion from “slow” 3D protocols in which follow-up scans are truly comparable.

Whichever parametric analysis is used, it is fundamental to achieve standardization among participating institutions,

and the use of retrospectively applied threshold values should be avoided. There is not one optimal approach, and a technique that offers excellent statistical power, and cross-center agreement for one type of tumor may be of little value in other tumors (due to tumor heterogeneity). Until now, all the studies showed that, at higher doses, eventually a dose is found at which the majority of patients manifest the desired change in the DCE-MRI end-point. Below that dose, the majority of MRI studies do not show the anticipated change. Thus, to date, developed methodologies appear to identify the minimum effective dose rather than the optimum biological dose. It is also important to establish the reproducibility of the measurement. For example, if an agent caused a 20% decline in a vascular parameter measured by MRI but the day-to-day variation in that parameter was 25%, then it would not be possible to say whether that drug was active. Studies in the upper abdomen and thorax can be compromised by respiratory motion artefacts. Finally, the results must be validated in larger prospective studies [177, 178].

PET scan

PET is a sensitive and quantitative technique that can be used to monitor the pharmacokinetics and pharmacodynamics of drugs radiolabeled with positron-emitting radioisotopes. It has been used in some studies to assess tumor blood flow with oxygen-labeled water and tumor metabolism with fluoro-labeled fluorodeoxyglucose as biologic end-points of response to antiangigenic agents.

The use of oxygen-labeled water offers several properties for measurement of blood flow. It is freely diffusible, has a short half-life of 2 min, and has favorable dosimetric properties. However, there are potential limitations. In small tumors, partial volume effects may be significant if the tumor size is less than twice the resolution of the scanner. Second, there is a phenomenon called "*spill over*" or "*spill in*" from surrounding structures with high blood flow, such as the heart, aorta, and liver, thereby limiting the use of PET scan in the lung, liver, and mediastinum, respectively. Further issues are that many imaging modalities, such as CT and PET, use ionizing radiation, limiting the number of studies that can be performed. Furthermore, many PET isotopes are short-lived, requiring synthesis of the relevant compound either at the patient's bedside, as in the case of oxygen-labeled water, or within a day of administration. In addition, tumors may not have a uniform exchange of water between blood and tissue. Necrotic areas may have a poor exchange between blood and tissue and a lower volume of distribution of tracer. The heterogeneity of delivery of drugs to solid tumors may lead to variability in the results obtained from PET scan and other imaging modalities [177, 178].

Dynamic or functional CT scan

Using dynamic or functional CT scan, it is possible to determine the absolute values of tissue perfusion, relative blood volume, capillary permeability, and leakage. All these parameters provide physiological correlates with microscopic changes correlated to tumor angiogenesis. Tumor microvessels are too small to image directly, but their increased density translates in vivo to increased tumor perfusion and blood volume. Dynamic CT is simple, widely available, and reproducible and has been validated against oxygen-labeled water PET scan. Quantification is simpler than for MRI, as the relationship between signal and contrast concentration is much more linear than that seen with MRI, although the sensitivity is less. The problem is that this technique uses ionizing radiation, and there is a limit to the number of studies that can be performed in any one patient. As yet, reduction in tumor perfusion by anti-angiogenic compounds has not been demonstrated by dynamic CT in clinical studies. The main reason for this has probably been the lack of commercially available software to perform the more precise quantitative analyses involved in calculating perfusion, blood volume, and capillary permeability. This situation is likely to improve with the rapid development of new CT software, such as 3D assessment of spiral CT. Finally, it may be possible to label monoclonal antibodies to VEGF and image in that way. This technique is currently under evaluation at several institutions [177, 178].

Functional utrasound

Ultrasound is one of the most widely used imaging modalities and also one of the most rapidly evolving technologies. New quantitative approaches, such as 3D scanning methods, and the increasing availability of microbubble contrast agents, open exciting new avenues for functional ultrasound imaging. Conventional Doppler imaging is able to direct image flow in vessels down to approximately the millimeter level. It is, thus, best seen as a tool for imaging the macrocirculation, rather than the microcirculation. Current Doppler methods often perform relatively poorly when directly correlated with measures of tumor angiogenesis, such as MVD. This is potentially attributable to sampling errors in heterogeneous tumors. A much more promising clinical application at the moment appears to be imaging the response of a tumor blood supply to cancer therapy. Although ultrasound media have been developed for the assessment of vascularity, these studies have been hampered by the operator dependency and difficulties in obtaining images when flow rates are very low. This makes measurements of reproducibility difficult, which obscures determination of drug effects. Also, depth of penetration is

poor; therefore, organs such as lungs and brain are inaccessible [177, 178].

Conclusions: open questions and future directions

VEGF is an effective target for anticancer therapy, and clinicians are now faced with the challenge of how best to integrate anti-VEGF agents into clinical practice. Experience with other targeted cancer agents, such as imatinib and trastuzumab, has generated the perception that targeted cancer therapies are most suitable for patients having tumors with overexpression of the target. However, accumulating data suggest that this may not necessarily be true for agents targeting the VEGF signaling pathway. There is no consistent evidence to definitively link VEGF levels with tumor-associated VEGF activity or any predictive or prognostic indicators, although many of the discrepancies between studies may be attributed to numerous drawbacks associated with current methods of VEGF detection and quantification. The absence of a 'gold standard' VEGF detection test, and lack of a predefined, clinically relevant cut-off is a significant hindrance to the clinical utility of VEGF measurements for therapy selection. In addition, there is no consensus regarding the most relevant form (e.g. tumor or circulating) of VEGF to measure.

Furthermore, the VEGF signaling pathway is very complex. The binding of VEGF to its receptors involves a complex series of molecular events within the cell, including direct activation of the intracellular signaling pathways and co-activation of other receptors and downstream components. There are also different VEGF isoforms, and their role in cancer needs to be further characterized, but redundancy, cross-talk, and autocrine circuits appear to exist within the VEGF signaling pathways in many tumors. In addition, different location of VEGF receptors have been identified and VEGFR-2 is over-expressed by some tumor cells, raising the possibility that VEGF inhibitors might have direct effects on tumor cell growth [181].

Given the diversity of the VEGF signaling network, it is important to consider VEGF expression in the context of other determinants of molecular activity, such as specific isoforms, other ligands, receptors and co-receptors, downstream components, and the cross-talk with other molecular pathways. Recent data suggest that VEGF bioavailability, not total expression, determines the response to VEGF inhibition [182].

Furthermore, the pattern of specific VEGF-A isoform expression may influence the response to therapy without substantial changes in total VEGF mRNA expression [183]. Taking into account tumor cell heterogeneity, the subjectivity associated to scoring VEGF expression, and the complexity of the VEGF signaling pathway, it is unrealistic to expect that the level of VEGF expression (regardless of the method of detection) would predict response to bevacizumab or other anti-angiogenesis compounds [184].

A better knowledge of the precise mechanisms of action of anti-VEGF compound is the key to develop a new generation of predictive tools. Regarding bevacizumab, several potential mechanisms have been postulated, including inhibiting the survival signals for VEGF-dependent immature vessels, normalizing the vasculature so as to improve delivery of chemotherapy, inhibiting the growth of new vessels and/or the recruitment of CEPs, direct activity on VEGFR-2-expressing tumor cells, and enhancing the immune response [162].

Regardless of the choice of marker and method of detection, there are also relevant disparities between the characteristics of primary tumor and metastatic sites. It has been documented that hepatic metastases have a significantly higher apoptotic index, decreased MVD, lower proliferation index, and decreased VEGFR-2 as compared to primary colon tumors [185, 186]. The level of VEGF expression may be site-specific in patients with metastatic disease, with decreased expression reported in liver metastases relative to primary tumors and abdominal metastases [32].

Finally, there are important differences between preclinical/animal models and human disease that could explain in part the lack of reproducibility of the results of experimental models [187].

Presently, regarding anti-angiogenic therapy, the identification and validation of prognostic and predictive markers still remains a challenge. Perhaps, there is a need for different biomarkers for different agents. Furthermore, a combination of markers or a "signature" might prove to be of greater value than only a single factor.

Recently published data have suggested potential biomarkers of antiangiogenic activity including sVEGFR-2 and CEC/CEP evaluation in the circulation, and new imaging strategies such as DCE-MRI, PET and dynamic CT scan, but these preliminary results were only from small phase I/II studies. These promising results need to be confirmed in large prospective phase II and III studies of anti-VEGF agents that include measurement of biomarkers as secondary endpoints. A relevant challenge in biomarker validation, particularly in the evaluation of new genomic or proteomic technology, is to perform rigorous evaluations of analytical, statistical methodology as well the clinical performance of the assay. In fact, the test should be safe and effective for the specific treatment choices [188].

In conclusion, although a number of surrogate markers for anti-VEGF activity are currently being investigated, none has been clinically validated. In addition, multiparametric analyses suggest [119] that anti-VEGF agents, such

as bevacizumab, improve outcomes in all subgroups of patients.

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