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Clinical biomarkers of angiogenesis inhibition

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Abstract

Introduction—An expanding understanding of the importance of angiogenesis in oncology and the development of numerous angiogenesis inhibitors are driving the search for biomarkers of angiogenesis. We review currently available candidate biomarkers and surrogate markers of anti-angiogenic agent effect.

Discussion—A number of invasive, minimally invasive, and non-invasive tools are described with their potential benefits and limitations. Diverse markers can evaluate tumor tissue or biological fluids, or specialized imaging modalities.

Conclusions—The inclusion of these markers into clinical trials may provide insight into appropriate dosing for desired biological effects, appropriate timing of additional therapy, prediction of individual response to an agent, insight into the interaction of chemotherapy and radiation following exposure to these agents, and perhaps most importantly, a better understanding of the complex nature of angiogenesis in human tumors. While many markers have potential for clinical use, it is not yet clear which marker or combination of markers will prove most useful.

Keywords

Cancer; Angiogenesis; Biomarker; Imaging

1 Background

Angiogenesis, defined as the formation of new blood vessels, is a necessary process for tissue survival in physiologic and pathologic states. The study of angiogenesis has rapidly expanded since Judah Folkman first suggested in 1971 that angiogenic dysregulation could be required for tumor growth and metastasis [1]. Angiogenesis has since become an accepted target for anti-cancer therapy [2–5]. In 2003, bevacizumab became the first angiogenesis inhibitor to be approved by the FDA for use in the U.S. Currently there are several anti-angiogenic agents in clinical use or in testing for cancer therapy as well as many others that exhibit anti-angiogenic properties as part of their mechanism of action (Table 1). There are currently over 1,000 interventional clinical trials investigating over 40 anti-angiogenic agents in cancer treatment (Table 2, www. clinicaltrials.gov).

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Angiogenesis is a complex process with numerous potential therapeutic targets. In cancer, angiogenesis is initiated when a tumor cell produces a pro-angiogenic signal, or angiogenic factor, activating resting endothelial and stromal cells. The activated endothelial cells then acquire the ability to remodel adjacent extracellular matrix, proliferate, migrate, then differentiate and stabilize as new blood vessels [6,7]. Known angiogenic factors include vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMPs), transforming growth factor (TGF) -β, interleukin (IL) -8 and angiopoietins [4]. The most well-characterized and most commonly targeted pathway is VEGF and its tyrosine kinase receptors (VEGFR-1 and -2) [8,9].

The majority of the agents in use or in development target angiogenic factor pathways or the endothelial cell (Tables 1 and 2; reviewed in [6,10]). The most common target in anti-angiogenic therapy is disruption of the *angiogenic signal*. This can be done in many ways. First, the angiogenic factor itself can be targeted by either direct inhibition, such as with bevacizumab, a monoclonal antibody to VEGF-A, or by creating a sink for the factor, exemplified by VEGF-Trap (aflibercept), a soluble form of VEGFR. Inhibiting angiogenic factors is possible by decreasing production, (i.e. interferon (IFN) -α, COX-2 inhibitors) or activity (i.e. suramin). Toxins bound to angiogenic factors are another potential anti-angiogenic method currently in preclinical studies [11,12]. Targeting the receptor also disrupts the angiogenic signal, shown by cetuximab, a monoclonal antibody to the epidermal growth factor receptor (EGFR). Several drugs act on signaling pathways, specifically by targeting the tyrosine kinase receptors of angiogenic factors (i.e. sunitinib, sorafenib, erlotinib, gefitinib).

Agents that act on *endothelial cell functions* can be divided into those that (1) inhibit endothelial cell proliferation such as fumagillin analogs, squalamine and endoge- nous inhibitors endostatin and angiostatin; (2) inhibit endothelial cell invasion and motility like the matrix metalloproteinase (MMP) inhibitors (3) inhibit endothelial cell adhesion such as drugs targeting $\alpha_v\beta_3$ integrin. Other agents, often with multiple or unknown mechanisms are being investigated such as thalidomide and its analogs.

The impact anti-angiogenic agents will have on treating cancer remains unclear. As monotherapy, anti-angiogenic agents have low objective response rates [13,14], potentially due to development of resistance by the induction of secondary pathways of angiogenesis [15]. Angiogenesis inhibitors in combination with other cytotoxic modalities may yield the best results for cancer patients [16]. Several clinical trials of angiogenesis inhibitors in conjunction with radiation therapy are underway (www.clinicaltrials.gov;[5])

Radiation and angiogenesis are connected at the molecular level [17,18]. Cells in a hypoxic environment are resistant to radiation ([19–21]. Tumors respond to radiation and stressors like hypoxia by producing hypoxia-inducible factor (HIF)-1, a strong survival mediator which inhibits apoptosis in endothelial cells [22,23]. Inhibition of HIF-1 activity results in long-term growth suppression in tumor xenografts [24]. It would be expected that inhibition of angiogenesis would cause an increase in HIF-1 expression and hypoxia as a result of impaired vascularization leading to radioresistant states in tumors. However, HIF-1 expression also leads to VEGF transcription and angiogenesis induction [13,25–28]. With this understanding, angiogenesis inhibition *in vivo* might either induce radiation resistance or sensitivity.

In fact, most studies favor increased radiosensitivity with inhibition of angiogenesis. One of the earliest observations of this phenomenon of sensitization to radiation with inhibition of angiogenesis also showed an improved oxygenation in tumors subjected to anti-angiogenic therapy [29]. Recent evidence shows some normalization of vasculature with angiogenesis inhibition and a subsequent period of increased oxygenation and susceptibility to radiation

[30–34]. Riesterer et al. demonstrated extended tumor growth delay and tumor-cell apoptosis with combination anti-angiogenic and radiation therapy as well as reducing the hypoxic response to radiation [35].

1.1 The pursuit of surrogate markers for angiogenesis

Traditional cytotoxic cancer therapies are typically titrated to achieve a maximum tolerated dose for a selected population. Unlike conventional cytotoxic chemotherapies, targeted therapies, such as angiogenesis inhibitors, may achieve therapeutic levels long before toxicities arise [36]. For this reason, it is necessary to identify biomarkers that accurately reflect the effect of a drug on its known targets and predict response to treatment [37–39]. Current angiogenesis inhibitors are typically cytostatic, and are thought to alter vessel structure instead of resulting in direct tumor kill. For this reason, investigators are aggressively pursuing suitable markers of anti-angiogenic modulation of tumor vasculature. This search has been challenging due to variations of tumor vasculature between tumor types, tumor histologies, tumor size, and degree of differentiation [40–44].

In general, markers of angiogenesis inhibitor effect can be divided into three major categories: invasive measures, minimally invasive measures, and non-invasive measures. Many technologies can be applied to more than one type of biospecimen such that they can be used as an invasive (biopsy) or minimally invasive measure (serum). Each of these markers can provide different information regarding the effect of the agent on tumor vasculature. Information can vary from anatomic to physiologic. It is not yet clear whether one marker may be most appropriate in certain clinical situations or whether a panel of markers may be required for an optimal assessment of angiogenic state of the tumor. We provide a general overview of each of these classes of markers and a brief discussion of the results and limitations of several markers that have been tested in this setting (Table 3).

2 Invasive measures: tissue biomarkers

The most intuitive method to measure the effect of any drug is to evaluate the target tissue, the tumor. Biopsies provide a way to thoroughly characterize tumor, histology and molecular processes with techniques such as immunohistochemistry, microarray, and proteomic analysis. These methods may be helpful in examining therapeutic effects of radiation, chemotherapy, targeted therapies, and their combinations. While evaluation of tissue provides an excellent mechanism to evaluate drug effect, the method raises practical and ethical concerns. The ethical implications of subjecting patients to serial biopsies in the context of a clinical trial have been debated [45,46]. At this time, there is no consensus in the research community regarding the appropriateness of repeated biopsies.

In addition to ethical concerns, the logistical and monetary costs of multiple biopsies are significant, making this option impractical for larger studies. Smaller series have successfully used this technique and gained a wealth of information. An excellent example of the successful use of tissue biopsies to evaluate tissue effects of an anti-angiogenic agent is a study of combined chemoradiotherapy and bevacizumab in rectal cancer patients [47,48]. Finally, the use of tissue markers requires biopsy of a portion of tumor. This technique may lead to sampling error, disruption of normal tumor biology after each biopsy, and the potential for wound healing problems when performed concurrently with the delivery of an anti-angiogenic agent, radiation, or cytotoxic chemotherapy.

2.1 Microvessel density and structure

Evaluation of microvessel density (MVD) is performed by immunostaining endothelial cells in tissue, identifying “hot spots” of angiogenesis and counting the number of vessels per high

power field. Within tumors, MVD has been identified as a potential prognostic indicator of progression, overall survival, and disease-free survival in multiple histologies [49–52]. MVD has also been explored as a method to predict and evaluate the efficacy of anti-angiogenic therapy [53,54]. Regardless of the prognostic value of MVD, its utility in evaluating response to anti-angiogenic therapy has been disappointing.

Pre-clinical data [53,55] and numerous clinical trials show that microvessel density does not predict response to anti-angiogenic therapy, nor does it predict the dose required to elicit an anti-angiogenic response [56–61]. In addition, MVD does not appear to be associated with other non-invasive serum markers and imaging techniques used as markers of angiogenic state [62]. However, two recent trials in gastric and head and neck cancer patients found statistically significant decreases in MVD before and after COX-2 inhibitor therapy, which is thought to act partially as an anti-angiogenic agent [63,64].

There are several potential reasons for the conflicting reports of the utility of MVD in the setting of evaluation of efficacy of anti-angiogenic therapy [65,66]. For one, the choice of immunostained antigen may affect MVD prognostic and predictive value. Several studies have measured MVD with antibodies to CD105, a protein expressed in higher quantities in proliferating tumor endothelial cells compared to normal microvasculature [67–70]. Because other antigens such as CD31 and CD34 are present on the surface of tumor endothelial cells regardless of proliferation status, they may not reflect the presence of targeted proliferating neo-vessels as effectively as CD105 [71].

One major concern with any biopsy marker, such as MVD, involves sampling error. As mentioned, measurement relies on selection of “hot spots” within tissue that could vary substantially. Also, the MVD of the biopsy may not be representative of the remainder of the tumor. Additionally, as MVD is a measure of vessels per area of tumor, the measurement reflects the balance of tumor cells and vessels. If a proportional number of tumor cells and vessels are eliminated with a therapy, the vessel density measurement may remain stable, even though both tumor and endothelial cells have been killed [53]. Questions also arise concerning whether MVD reflects the functionality of vasculature present as well as thedegreeofthe tumor’s dependence upon the vasculature identified [65].

Yet another concern is the affect additional treatments, besides anti-angiogenic therapy, will have on MVD. In 1945 it was discovered that radiation has an independent effect on MVD as well as vessel length and diameter. Both single dose [72,73] and fractionated radiation [74] result in altered tumor vascularization, noted as early as 12 h post-irradiation. These changes reflect a decrease in the intercapillary distance in irradiated tumors, possibly leading to reoxygenation following radiation [74].

The concerns about MVD sampling challenges, modulation by other therapies and the observed lack of correlation with anti-angiogenic response indicate that MVD cannot be supported as a direct measure of angiogenesis in clinical trials. More promising results might be found with greater characterization of neoangiogenesis as well as more qualitative structural analysis of tumor vasculature [71]. While anti-angiogenic therapy may not consistently alter MVD, evidence suggests that microvascular structure may be “normalized” as angiogenesis is inhibited [30,31,33,34,75]. This parameter can be measured through techniques such as vascular casting or vascular contrast, which typically require large samples of tissue for evaluation. Evaluation of vessel structure during a lead-in phase with neo-adjuvant delivery of the angiogenesis inhibitor alone may help distinguish what effects are attributable to the angiogenesis inhibitor.

2.2 Proteomic analysis

The field of proteomics was developed to allow the simultaneous evaluation of numerous peptide biomarkers. The concept evolved from the theory that the pattern of expression of a number of peptides in biologic samples might provide a better diagnostic tool than the evaluation of single proteins [76–78]. Advances in the field of proteomics such as 2D gel electrophoresis, mass spectrometry technologies, and protein array technologies such as surface-enhanced laser-desorption/ionization time-of-flight (SELDI-TOF) mass spectroscopy allow the generation of a descriptive “fingerprint” of polypeptide expression in serum samples [76]. The spectra generated through this analysis can be compared to the spectra of other subjects to generate a pattern predictive of the presence of cancer [79–82], disease stage [83, 84], and therapeutic effect [78,85].

Direct tissue profiling of small biopsy sections can yield full-scale proteomic analysis revealing relevant biomarkers or patterns [86,87]. In tissue samples, techniques such as laser capture microdissection can allow collection of specific cell subtypes. This powerful technology may allow the generation of specific expression profiles from primary tumor cells, metastatic tumor cells, and endothelial cells that will allow assessment of response to each cell type to therapy [88,89]. Additionally, by creating a fingerprint for each cell type and how they are altered in response to therapy, intermittent evaluation of serum profiles may allow an assessment of response in each cell line through shedding into the vascular compartment. The continued introduction of numerous technological advancements such as artificial intelligence-based pattern recognition algorithms will allow a more rapid and sensitive detection of patterns that will assist in detecting and monitoring cancers [78,90,91]. New methods like linear ion trap quadrupole mass spectrometers enhance quantification abilities [92,93] and capillary electrophoresis mass spectrometry is allowing better evaluation of large data sets, more precise differentiation of proteins and more rapid monitoring of data quality [94].

Recent studies have investigated the use of proteomic techniques to identify proteins associated with tumor endothelial cells [95–99]. The study that to date best represents this application defined 15 proteins differentially upregulated in tumor endothelium of lung metastases from breast primaries compared to normal rat lung [100]. Another study noted differential expression of four proteins in glioma vasculature not found in normal brain tissue [101]. Some of these proteins have been validated using specific antibodies to label tumor and normal tissue [100–102].

Since proteomic profiles can be generated from tissue as well as biological fluids, various limitations may apply. With tissue samples, the same concerns that exist for MVD often apply here, namely sampling error, invasiveness of the procedure, and the requirement for multiple biopsies to compare prior to and after therapy. The use of this technique for evaluation of biological fluids minimizes these concerns, however it remains unclear if the use of serum proteomic profiling will allow an accurate estimation of angiogenic state within a tumor. Furthermore, it is unclear if a signature or pattern representative of altered angiogenic state will apply across multiple primary sites (variable leak into the plasma compartment), histologies, grades of tumor, and different total tumor burdens.

2.3 Gene expression profiling

Technologies such as DNA microarrays and serial analysis of gene expression (SAGE) are creating opportunities to investigate gene expression in tumors [103]. This implies the potential to use tumor biopsies, circulating tumor cells, circulating endothelial cells, and whole blood to identify new surrogate markers of angiogenesis. Multiple genes have been implicated in angiogenesis by gene expression profiling for a number of tumors [77]. St Croix et al. purified and concentrated colon cancer endothelial cells and used SAGE to identify 79 genes that were

upregulated or downregulated. Among those discovered were novel tumor endothelial markers found to be overexpressed in other tumors [104,105], some with potential therapeutic implications [106,107]. SAGE has also been employed to identify genes in brain tumors and breast cancers [108–110]. Some investigators have proposed the use of gene expression databases using similarities of ordered gene lists approach for comparisons and pooling of data to increase identification of genes involved in angiogenesis or the response to these agents [76,111,112]. However, the large scale combination of these data sets would be limited by technical and statistical problems.

In addition to identification of genes involved in angiogenesis, expression profiling also provides other potential applications to the investigation of angiogenesis inhibitors. With the use of dynamic contrast imaging as a guide, areas of tumors thought to be active from an angiogenic standpoint can be targeted for biopsy [113–115]. Techniques such as laser capture microdissection allow comparisons of expression profiles in regions of tumors thought to be of interest [113,116–119]. By evaluating which proteins are expressed in regions of tumors with significant angiogenic activity, tumors from protocol candidates may be screened for expression to help determine the relative angiogenic state of their tumor. Once gene expression profiles of tumors responsive to angiogenic therapy or combination angiogenic and cytotoxic therapy are known, an initial tissue biopsy could help determine which patients would benefit prior to initiation of therapy. Finally, by evaluating changes in tumors which are a result of angiogenic therapy or the combination of angiogenic therapy and radiation, the mechanisms of the additive and synergistic responses may be better understood. These techniques require the ability to safely and accurately target a specific region of a tumor based on imaging.

2.4 Skin biopsies

Angiogenesis is known to play an important role in wound healing, and the effect of anti-angiogenic treatment on wound healing has been studied in clinical trials [120]. Because angiogenesis is required for wound healing, skin biopsies may be helpful in evaluating the response to anti-angiogenic agents or in titrating agents to an appropriate dose by performing serial punch biopsies of the skin, each time removing the previously biopsied site (Reviewed in [121]). Clearly, the same ethical issues for multiple biopsies apply in this instance as well. In addition, it is not entirely clear that inhibition of angiogenesis in a well-vascularized skin wound correlates with that observed in the heterogenous environment of a tumor. Few studies have performed this technique.

Zhang et al. evaluated anti-angiogenic effects of MEDI-522, a monoclonal antibody to $\alpha v\beta 3$ integrin, in a phase I dose-escalation study. Pre-treatment and post-treatment punch biopsies of the skin were assessed for vascular area, endothelial cell proliferation and apoptosis, and $\beta 3$ integrin levels. None of the above parameters after MEDI-522 treatment were found to be significantly different when compared to the pre-treatment tissue in spite of adequate drug presence identified in the vasculature by immunohistochemistry [122]. Mundhenke et al. evaluated the response to endostatin in patients by serial skin biopsies. While no significant changes were noted in vascular density or blood vessel maturity in biopsied skin, it is important to note that changes were also not appreciated in tumor biopsies after endostatin therapy [123]. Lockhart et al. used a similar approach to assess wound healing and the activity of a matrix metalloproteinase inhibitor. Rather than focusing on specific angiogenic parameters, they recorded wound healing by visual wound assessment, reporting a statistically significant difference in time to target healing level between treatment and control groups [124].

These studies show that this method is practical and well-tolerated. With continued investigation, this innovative procedure or similarly designed methods might play a role in assessment of individual response to anti-angiogenic drugs at various dose levels.

3 Minimally invasive measures: circulating markers

Identifying circulating markers of angiogenesis that could assist in diagnosis, staging, treatment response and follow-up is being aggressively pursued. Such markers would have the advantage of being minimally invasive allowing repetitive sampling throughout treatment and follow-up without the ethical and technical implications of multiple biopsies. In addition, sampling for these minimally invasive markers would not disrupt tumor physiology. There are currently three main categories of markers being investigated as minimally invasive measures of angiogenic state including growth factors and cytokines, cell surface molecules, and circulating endothelial and endothelial precursor cells.

3.1 Growth factors and cytokines

Multiple angiogenesis growth factors and cytokines in blood and urine have been investigated in a range of tumor histologies. For example, elevated VEGF, FGF, and HGF in various biological fluids have been associated with staging, progression, and prognosis [125–139]. Similar to most markers of angiogenesis, studies in this area have yielded conflicting results [140–142]. Various factors may reflect the varying results obtained with these markers, including microenvironmental variations such as vascular permeability altering the quantity of protein that intravasates into the blood stream [65]. Another possible confounding factor includes tumor heterogeneity in regards to elaboration of these factors.

Recent studies have shown promising results for use of growth factors as a marker of anti-angiogenic response in a variety of treatment conditions ([143,144]. Drevs et al. described a time- and dose- dependent reduction in soluble VEGFR-2 with once-daily oral AZD2171 in 36 patients with solid tumors and liver metastases. Increases in VEGF and PlGF were detected after treatment, but there was no suggestion of a dose relationship [145]. This increase in VEGF after anti-angiogenic treatment has been reported often since first noted in 2003 [14,146].

Because most anti-angiogenic agents are combined with other modalities, it is important to understand how other therapies perturb the levels of these growth factors as well. Similar correlations have also been reported in anti-angiogenic therapies combined with other treatments [147]. Evaluations of these growth factors with radiation have yielded interesting results, with a clear time dependence of the kinetics of these markers after therapy and a correlation with outcome. Chan et al. found significant predictive value in the kinetics of urine VEGF levels in patients treated with one month of radiation for various tumor types. Increasing serum levels of VEGF after radiation were correlated with eventual failure or progression [127]. Ria et al. found decreases in serum FGF-2, VEGF, and HGF following radiation to primary or metastatic tumors of various histologies, with a correlation between radiation dose and decreases in serum FGF-2 and VEGF [148]. Others have found the higher pretreatment serum VEGF levels to be predictive of poor outcome following combined chemotherapy and radiation [149,150].

More data regarding the kinetics and utility of these minimally invasive markers are needed to successfully incorporate them into clinical use. Many ongoing trials continue to include these important markers, hopefully leading to firmer conclusions about their utility in diagnosing, staging and following patients treated with standard and anti-angiogenic therapies.

3.2 Endothelial cell surface molecules

Endothelial cells release various molecules into the circulation, implying the potential for angiogenesis markers related to cellular adhesion. Some soluble molecules (i.e. sVCAM-1, sICAM-1, sFLT-1) have been elevated in patients with cancers, relative to normal controls or patients with benign neoplasms [151–156]. Shariat et al. found that circulating sVCAM-1

levels increased incrementally from healthy controls to prostate cancer patients with localized disease and then to those with lymphatic spread and metastasis. In a pre-operative model, elevated plasma sVCAM-1 and VEGF were both associated with biochemical progression [136]. Since inflammatory processes in the vasculature may cause elevation of cell surface markers, inflammatory response to radiation therapy may alter the kinetics of these molecules [157,158]. As with other potential markers, cell surface markers for angiogenesis may be more useful in the pretreatment and surveillance setting due to perturbations during therapy by other concurrent therapies such as cytotoxic chemotherapy and radiotherapy.

3.3 Circulating endothelial and endothelial precursor cells

Tumor angiogenesis involves locally derived circulating endothelial cells (CECs) and bone marrow-derived endothelial precursor cells (EPCs) [159]. As tumors grow, pro-angiogenic molecules recruit nearby tumor endothelial cells, perivascular cells, and circulating EPCs to the vascular bed [160,161]. Anti-angiogenic agents have been shown to inhibit EPC mobilization. [162]. A sufficiently strong correlation between CECs and EPCs and angiogenesis appears to support the potential use of these cells to monitor anti-angiogenic effects [163].

Measurement of CECs and EPCs has been reported in vascular surgery patients and following myocardial infarction [164,165]. Recently, Norden et al. treated imitinab-resistant gastrointestinal stromal tumor patients with sunitinib. In this series, the presence of VEGF bearing CECs and monocytes differentiated patients with progressive disease and those who exhibited a clinical response [144]. Another potential marker from endothelial cells is VE-Cadherin. One study measured elevated circulating VE-Cadherin RNA levels in breast cancer patients and pregnant women but found none in healthy controls [166]. As VE-Cadherin is an endothelial-specific gene, it is proposed that this may be a marker for CECs. Measuring CECs and EPCs in cancer patients undergoing anti-angiogenic therapy may allow titration of dose to the desired effect. However, before these markers will be effectively incorporated into clinical use, further characterization of these cells' response to other modalities is needed to understand the complex interactions which appear to be present. For example, Furstenberger et al. described an elevation of CECs and EPCs in 10 patients with locally advanced breast cancer. Neoadjuvant chemotherapy resulted in a decrease in CECs and an increase in EPCs [167].

4 Non-invasive markers: imaging

The application of existing imaging technologies to the measurement of metabolism, oxygenation, and perfusion is a field of intense research (Table 4) [62,121,168–170]. Imaging offers the distinct advantage of being able to serially evaluate anatomical and physiological processes in tumors without disrupting tumor tissue. To use these non-invasive methods, a better understanding of the invasive correlates of the images and information obtained with these technologies is needed. In addition, if the various imaging modalities available are to be effectively incorporated into widespread clinical trials, standardization is necessary.

4.1 PET

Positron emission tomography (PET) has played an ever increasing role in the staging of malignancy and assessment of response to anticancer therapy. The use of fluoro-deoxy-D-glucose (FDG) PET to evaluate the response to anti-angiogenic therapy or radiation has been described in variety of tumor types and disease sites [121,171–175]. This is complicated by the fact that hypoxia, theoretically induced by anti-angiogenic agents, increases the expression of the Glut-1 glucose transporter and the uptake of FDG into tumor cells ([176,177]. Other variables, such as the proportion of metabolism due to aerobic versus anaerobic mechanisms

may also affect the accumulation of FDG [178]. The effects of the tumor microenvironment on FDG uptake are incompletely understood and complicate the quantitation of FDG accumulation and comparisons between tumors in different subjects.

While FDG PET plays an important role in modern oncology, the development of new tracers for PET imaging promises to improve the understanding of the physiologic state of individual tumors. Physiologic variables such as tumor blood flow, metabolism, and hypoxia can be measured with PET utilizing various specialized tracers. For example, tumor hypoxia can be imaged by utilizing nitroimidazole tracers that are absorbed into tissues and trapped in their reduced state [179–183]. Cher et al. showed that ^{18}F-FMISO PET provided a noninvasive assessment of hypoxia in glioma that was prognostic for treatment outcomes in the majority of patients, and hypothesized that it may have a future role in monitoring anti-angiogenic treatment [184]. The use of ^{18}F-FMISO PET has been incorporated into clinical trials of chemoradiation in conjunction with tirapazamine, a toxin targeting hypoxic cells [185–187] To our knowledge, this has not yet been included in human clinical trials with anti-angiogenic agents.

Tumor blood flow can be measured with the use of H^{15}_2O as a tracer. As water is freely diffusible, this tracer will rapidly equilibrate and is metabolically inert. A number of laboratory studies have assessed this technique in combination with anti-angiogenic agents [181,188, 189]. Clinical studies with radiation, chemotherapy or anti-angiogenic agents have incorporated this technique as a measure of tumor perfusion [62,171,174,190–193]. It remains to be determined whether H^{15}_2O PET imaging will play a significant role in assessing response to anti-angiogenic agents in the clinic. Another tracer developed to measure vascular volume is $C^{15}O$ which binds to the hemoglobin of red blood cells. A combination of H^{15}_2O and $C^{15}O$ may be the best method to reflect vessel density changes [194].

Design and manufacture of PET probes that bind to signaling intermediates or are ligands for angiogenic receptors are under investigation and may help to better define the angiogenic state of tumors [195–203]. Examples include radiolabeled peptides that bind to VEGFR [204,205] and also to the $\alpha_v\beta3$ integrin receptor expressed on endothelial cells [206,207]. These approaches are being actively evaluated in the laboratory, and it is unclear what role they will play in the clinic.

Limitations of PET imaging include the relatively poor anatomic resolution compared to CT and MRI and the requirement of a radioactive isotope generated in a cyclotron. Additionally, attempts to quantify PET data require an ability to correct for the attenuation of emitted photons in tissue [181,208]. Registration of PET images to computed tomography (CT) or magnetic resonance imaging (MRI) can significantly improve the diagnostic accuracy of PET [209, 210], partially correcting for decreased anatomic resolution of PET by integrating the anatomic data of CT.

4.2 MRI

Dynamic contrast-enhanced MRI (DCE-MRI) is currently the most useful method for assessing early changes in tumor vasculature in clinical trials [211,212]. The technique involves the acquisition of magnetic resonance images before, during, and after the delivery of contrast to evaluate physiologic parameters such as perfusion and capillary permeability [213]. DCE-MRI can distinguish malignant and benign tissue based on differences in the function of tumor microvasculature [214]. A number of contrast agents can be used to perform DCE-MRI, including low molecular weight agents, high molecular weight agents, and agents that accumulate at sites of angiogenesis (reviewed in [168,215]. The choice of contrast agents for DCE-MRI depends on the physiologic process to be evaluated [211].

Low molecular weight agents rapidly diffuse into the extracellular fluid space, with as much as 12–45% of the contrast media passing into the extracellular space during the first pass [216]. Tissue perfusion and blood volume can be measured using T1 and T2-weighted MR imaging sequences with low molecular weight contrast enhancement [217]. In addition, regions of tumors with necrosis and fibrosis have differing vascular function which can be visualized with these techniques [214].

Tumor vasculature is highly permeable to macromolecules including high molecular weight contrast agents [218]. These agents can be used to assess changes in vascular permeability in tumors after treatment with radiation [201] or anti-angiogenic therapy.

Agents designed to image tumors by binding to angiogenic molecules have been evaluated in preclinical studies including $\alpha_v\beta_3$ integrin and E-selectin antibody contrast agents [122,219–223]. These agents may be difficult to image due to the low concentration of their target in tumors, typically below the detection level for MRI [224]. Solutions to this problem would include agents designed to accumulate at sights of angiogenesis through modulation or amplification [170].

Some difficulties in the use of DCE-MRI to assess angiogenesis have become apparent. Studies evaluating the correlation of microvessel density and DCE-MRI have found conflicting results [213,225]. This discrepancy may be due to a lack of correlation between vessel permeability and perfusion. Some regions of the body are better candidates for evaluation with DCE-MRI due to technical considerations such as immobilization. Registration of images can be difficult for non-stationary organs, and lack of immobilization resulting in motion artifact may cause difficulties in image sequence registration [226]. Additional concerns for DCE-MRI imaging include the injection rate and the timing of sequence timing [213].

Quantitation of data obtained with DCE-MRI is complex and requires consideration of multiple variables corrected with standardized values taking into account the patient's weight and cardiac output. Technical limitations and physiologic considerations that affect the accuracy of quantitation with DCE-MRI have been described in detail [211,214].

Anti-angiogenic agents have been shown to reduce tumor vascular permeability in pre-clinical studies [227,228]. Several clinical trials evaluating alterations in DCE-MRI parameters following therapy with anti-angiogenic agents have been completed with promising results [61,229–232]. Liu et al. showed a decrease in vascular parameters measured by DCE-MRI after anti-angiogenic therapy that indicate utility as an indicator of drug pharmacokinetics [233]. However, questions remain about the optimal timing of DCE-MRI evaluation in relation to delivery of anti-angiogenic drugs [214] in order to appropriately evaluate for response. Regardless, recent clinical trials have demonstrated that measurements obtained from DCE-MRI correlate with plasma concentration of anti-angiogenic agents and can potentially predict clinical response after anti-angiogenic treatment [61,234–236].

4.3 CT

Functional CT imaging may provide an evaluation of tumor blood flow, blood volume, and permeability [237–239]. Many of the concepts used to evaluate these variables with MRI can be extended to CT. The procedure involves injection of a contrast agent followed by serial evaluations at various time points to assess these physiologic endpoints ([65]. Advantages of CT include a linear relationship between signal and contrast concentration allowing for simple quantification and widespread availability. However, lack of experience and technology with CT and concerns about recurrent exposure to ionizing radiation have limited its progress as a marker.

The procedure has been used to evaluate the angiogenic state of tumors and compared favorably to MVD [240–242]. Ma et al. found that sixteen-slice spiral CT perfusion imaging was significantly associated with tumor angiogenesis and reflected MVD measurement and cyclin D1 expression in untreated peripheral lung cancer patients less than 1 week before surgery [243]. Other trials have investigated angiogenic evaluation with functional CT for different malignancies before and after anti-angiogenic therapy with both mixed results [244–247]. Comparisons of contrast-enhanced dynamic CT and DCE-MRI show similar results can be obtained with either modality [248].

4.4 Ultrasound

Ultrasound is playing an ever increasing role in the staging of primary tumors and metastatic disease in a variety of malignancies including lung, gastrointestinal, and urologic malignancies [249–254]. The ability to apply ultrasound probes in proximity to these tumors in concert with numerous technical advancements allows accurate determination of tumor depth and lymph node involvement.

In addition to anatomic imaging, ultrasound can evaluate tumor blood flow and the tumor microvasculature. Advancements such as color Doppler and power Doppler allow assessment of response to anticancer therapy with an assessment of tumor blood flow and microvascular anatomy [255]. Color Doppler imaging allows quantitation of blood flow through computerized image analysis [256]. Unfortunately, the low velocity of capillary flow is typically not evaluable by Doppler sonography [257]. The use of blood pool ultrasound contrast agents has allowed the investigation of smaller vessels, including those measuring 30 to 60 μm[170].

Ultrasound has also been evaluated for molecular imaging using ultrasound contrast agents designed to bind to specific ligands such as the $\alpha_v\beta_3$ integrin [258]. Analysis of the amount of bound targeted ultrasound contrast through ultrasound induced microbubble destruction may allow quantitation of contrast binding [170] and can demonstrate vessels as small as 70 μm [121]. Advances such as these may allow an evaluation of the density or function of specific receptors in tumors that an anti-angiogenic therapy may target.

Ultrasound is being used increasingly in clinical trials to evaluate the vasculature in tumors, and seems to compare favorably with more established techniques [259,260]. Transcranial imaging has been shown to be effective for evaluating blood flow in high grade gliomas, with similar results as those obtained with perfusion MR imaging [259]. Ultrasound has effectively been used to evaluate the response of preclinical tumor models and human tumors to anti-angiogenic drugs [261–263]. As with any marker, conflicting results have been obtained with attempts at clinical translation. In one Phase I trial, ultrasound blood flow parameters in metastatic liver tumors were explored as a marker for PTK/ZK response. Only a non-statistically significant trend towards higher blood flow with increasing doses of drug was seen and no dose-related changes were noted for a calculated resistance index [264].

While ultrasound may provide an accurate prediction of blood flow, a major problem with the incorporation of Doppler imaging into a clinical trial is the dependence on experienced operators [265]. A lack of experience may lead to significant inter- and intra-observer variability in measurements, complicating longitudinal evaluations. Additionally, physical characteristics of the tissues through which tumors will be visualized can affect the quality of imaging. Benefits to this technology include the relatively low cost, portability, and non-invasive nature of the procedure [255].

4.5 Optical imaging

Technologies such as near-infrared spectroscopic diffuse tomography and orthogonal polarization spectroscopy are under evaluation for their utility in imaging angiogenic vasculature. Optical imaging generates images using measurements of visible or near-infrared light scattered across human tissues [266]. The technology is inexpensive and portable, but the consistent challenge has remained the limited penetration and intense scattering of light. Nevertheless, optical imaging is considered feasible in superficial tumors such as in the breast, eye and pediatric neoplasms [267]. Some trials investigating the sensitivity and specificity of optical imaging techniques measuring total hemoglobin and relative oxygenation to compare normal to malignant breast tissue [268] and to differentiate malignant from benign breast masses [269] have been performed with promising results [270].

5 Conclusion

Anti-angiogenic therapies are part of a growing body of molecularly targeted therapies for cancer. Such treatments are imposing changes on the process of drug development, evaluation and approval. Recent interpretations of FDA regulations allow for phase 0 clinical trials to be performed, which would involve developing assays to evaluate target modulation and tissue effects of a drug and to obtain preliminary pharmacokinetic data [36]. The benefits of this process cannot be realized without the use of accurate biomarkers.

Numerous candidate markers of angiogenesis have been identified, but the use of these markers in diagnosis, prognosis, and monitoring of treatment remains investigational and of uncertain utility. It is improbable that any one biomarker will provide all relevant clinical information in the setting of a trial of anti-angiogenic therapy alone or in combination with additional cytotoxic therapies. Rather, a combination of markers obtained from tissue, biological fluids, and imaging is more likely to result in a comprehensive understanding of the complex process of angiogenesis and any perturbations from therapy. Additionally, putative markers will probably vary with differences in tumor attributes (histology, size, proliferation rate, etc) and the treatment regimen employed. Future clinical trials of anti-angiogenic agents should seek to forward discovery of new biomarkers and to validate promising candidate markers and imaging modalities already described. Following this pattern in the progress of anti-angiogenic therapy will hopefully lead to better outcomes in cancer patients while establishing a model for investigation of future molecularly targeted therapies.

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Table 1

FDA-approved drugs with presumed anti-angiogenic mechanisms

Category	Drug name	Target/mechanism	Cancer indications	Current clinical trials[a]		References
				Phase (no.)	Tumor or histology	
Monoclonal antibodies	Bevacizumab (Avastin)	VEGF	mCRC, NSCLC	I (58); II (336); III (51); IV (5)	Numerous	[271–274]
	Cetuximab (Erbitux)	EGFR	mCRC, H & N	I (3); II (18); III (4) IV (1)	H & N, RCC, lung, pancreas, CRC, glioma	[275,276]
	Panitumumab (Vectibix)	EGFR	mCRC	II (2); III (1)	CRC, lung	[277,278]
	Trastuzumab (Herceptin)	EGFR, HER-2	Breast Cancer	I (3); II (8); III (2)	Breast, endometrial, gastric, lung, ovarian	[279]
Small-molecule tyrosine kinase inhibitors	Erlotinib (Tarceva)	EGFR	NSCLC, Pancreatic Cancer	I (16); II (44); III (4)	Numerous	[280,281]
	Gefitinib (Iressa)	EGFR	NSCLC	II (3)	Skin, RCC	[282]
	Imatinib (Gleevec)	bcr/abl, c-kit, PDGFR	CML, GIST	I (5); II (15); III (9); IV (1)	GIST, leukemia, melanoma, lung, prostate, liposarcoma	[283,284]
	Lapatinib (Tykerb)	EGFR, HER-2	Breast Cancer	I (1); II (1)	Breast, solid tumors	[285]
	Sorafenib (Nexavar)	VEGFR-2, -3, PDGFR-ß, Raf-1,	RCC	I (3); II (9); III (3)	GIST, melanoma, fallopian ovarian, RCC	[271]
	Sunitinib (Sutent)	VEGFR-2, PDGFR-ß	GIST, RCC	I (27); II (85); III (17)	Numerous	[286,287]
Other agents with anti-angiogenic properties	Bortezomib (Velcade)	Proteosome Inhibitor	MM, Mantle Cell Lymphoma	I (7); II (16); III (6)	MM, lung, pancreas, RCC, lymphoma	[288,289]
	Interferon Alfa-2b (Roferon A, Intron A)	Inhibits angiogenic factor expression	Hairy cell leukemia, CML, NHL, Melanoma, Kaposi's Sarcoma,	I (36); II (126); III (52); IV (3)	Numerous	[290,291]
	Temsirolimus (Torisel)	mTOR inhibitor	RCC	I (22); II (36); III (4)	Numerous	[287,292]
	Thalidomide (Thalomid)	Unknown	MM	I (29); II (149);	Numerous	[293]

Category	Drug name	Target/mechanism	Cancer indications	Current clinical trials[a]		References
				Phase (no.)	Tumor or histology	
				III (39)		
	Lenalidomide (Revlamid)	Unknown	MM	I (9); II (19); III (5)	Numerous	[294]

mCRC Metastatic colorectal cancer, *NSCLC* non-small cell lung cancer, *H & N* head and neck cancer, *CML* chronic myelogenous leukemia, *GIST* gastrointestinal stromal tumor, *RCC* renal cell carcinoma, *MM* multiple myeloma, *NHL* non-Hodgkin's lymphoma

[a] www.clinicaltrials.gov

Table 2

Presumed anti-angiogenic agents currently in clinical trials[a]

Category	Drug name	Target/mechanism	Current clinical trials[a]	
			Phase (no.)	Cancers in trials
Target angiogenic factor	Volociximab (M200)	Anti-α5β1 integrin monoclonal antibody	II	Melanoma
	Aflibercept (VEGF TrapR1/R2)	VEGF decoy receptor	II, III	Ovary, lung
	PI-88	Mimics Heparan Sulfate, inhibits heparanase, binds FGF and VEGF	II	Prostate
	PTC299	VEGF 5′-UTR mRNA	I	Breast
	Ad-hIFN-β (BG00001)	IFN-β gene, adenovirus vector	II	CNS, CRC, mesothelioma
Target angiogenic signal: tyrosine kinase inhibitors	Cediranib (AZD2171)	VEGF	I, II, III	CRC, solid tumors, lymphoma
	Nilotinib (AMN107)	KIT, PDGFR, and Bcr-Abl	III	GIST
	Vatalanib (PTK787/ZK 222584)	VEGF	I, II, III	CRC, MM, metastatic tumors
	AMG 706	VEGF, PDGF, c-kit, Ret	II, III	Breast, NSCLC
	Pazopanib (GW786034)	VEGFR, c-kit, PDGF-R	II, III	RCC
	Axitinib (AG-013736)	VEGFR-1, -2, PDGF-βR	I, II	Lung, thyroid, CRC, melanoma, kidney, pancreas
	Semaxanib (SU5416)	Flk-1/KDR	I, II	Fallopian tube, breast, H & N, sarcoma, melanoma, kidney
	AG-6013736	VEGFR, PDGFR	II	Thyroid
	Brivanib (BMS-582664)	VEGFR, FGFR	II	HCC
	ABT-869	VEGFR, PDGFR	II	RCC
	Dasatinib (BMS-354825)	bcr/abl	I	Solid tumors
	Vandetanib (ZD 6474)	VEGFR, EGFR	I-III	Numerous
	RAF265	Raf, VEGFR-2	I	Metastatic melanoma
Target endothelial cells	TNP-470	Fumagillin analogue, binds MetAP2 causing EC arrest in G1 phase	I, II	Pancreas, Kaposi's sarcoma

Category	Drug name	Target/mechanism	Current clinical trials[a]	
			Phase (no.)	Cancers in trials
	Squalamine lactate	Intracellular blockage of numerous pathways	II	Prostate, ovarian
	Recombinant tPA	Induces Angiostatin	II	Solid tumors
	Ad-rhEndo (E10A)	Endostatin gene transfer	I	Advanced Solid tumor
	ABT-510	Thrombospondin Analogue: inhibits VEGF, bFGF, HGF, IL-8	I	Solid tumors
	Col-3	MMP inhibitor	I	Metastatic cancer
	2-methoxyestradiol (Panzem)	Estradiol Metabolite	I, II	RCC, carcinoid, solid tumors
	Combretastatin A4 Phosphate	Tubulin	I	Solid tumors
	Bavituximab	Aminophospholipids monoclonal antibody	I	Solid tumors
Other agents with anti-angiogenic properties	AMG 386	Angiopoietins	II	Breast
	Tetrathiomolybdate	Copper Chelator	II	Breast, prostate, CRC, HCC, esophageal
	Celecoxib	COX-2 inhibitor	I-III	MM, head and neck, CRC, leukemia, lymphoma

mCRC Metastatic colorectal cancer, *NSCLC* non-small cell lung cancer, *H & N* head and neck cancer, *CML* chronic myelogenous leukemia, *GIST* gastrointestinal stromal tumor, *RCC* renal cell carcinoma, *MM* multiple myeloma, *NHL* Non-Hodgkin's lymphoma

[a] www.clinicaltrials.gov

Table 3

Summary of candidate markers reviewed

Marker/ method	Examples of methods described	Invasiveness	Advantages	Disadvantages
MVD	Immunostaining with CD31, CD34, CD105; structural analysis	Invasive	Potential prognostic indicator; variety of immunostaining methods; potential for structural evaluation of tumor vasculature	No clear association with other markers or AA response; invasive (sampling error; disruption of normal tumor biology; wound healing concerns with AA; ethical concerns)
Skin biopsy	Visual Scoring System; Immunostaining	Invasive	Accessible sample; no disruption of tumor biology; reproducible physiologic process with continuous observation; patient is their own control	Invasive (wound healing concerns with AA, ethical concerns); non-tumor tissue may not be representative of tumor tissue
Proteomics	2D GE; Mass Spectrometry; Protein Arrays; Cytometry	Invasive or minimally invasive applications	Ability to identify numerous proteins from multiple sample types; applications to multiple aspects of cancer management	Invasive (sampling error; disruption of normal tumor biology; wound healing concerns with AA; ethical concerns); uncertain correlation of biological fluids and angiogenic state
Gene profiling	Microarray; SAGE	Invasive or minimally invasive applications	Ability to identify genes from multiple sample types; applications to multiple aspects of cancer management	Invasive (sampling error; disruption of normal tumor biology; wound healing concerns with AA; ethical concerns); uncertain correlation of biological fluids and angiogenic state
Growth factors and cytokines	VEGF, HGF, FGF	Invasive or minimally Invasive applications	Ability to serially evaluate multiple aspects of cancer management	Inconsistent results; lack of understanding of kinetics; modification by other treatments;
Cell-surface markers	VCAM, ICAM, FLT	Invasive or minimally Invasive applications	Probably most useful in pretreatment and surveillance phases	Inconsistent results; lack of understanding of kinetics; modification by inflammation
CECs and EPCs	VEGF, VE-cadherin	Minimally invasive	Ability to serially evaluate multiple aspects of cancer management	Inconsistent results; lack of understanding of kinetics; modification by other treatments;

AA Anti-angiogenic agent

Table 4

Summary of imaging reviewed

Modality	Examples of specific methods described	Advantages	Disadvantages
MRI	DCE-MRI, antibody contrast agents (αvβ3 integrin, E-selectin)	Excellent anatomic information and spatial resolution; highly sensitive; multiple types of low-toxicity contrast; no radiation exposure	Expensive; motion effects; lack of targeted probes; longer procedure time
CT	Functional CT	Excellent anatomical information and spatial resolution; simple quantitation; direct proportion of contrast	Recurrent ionizing radiation exposure; contrast agent toxicity; lack of targeted probes
PET	FDG, [18]F-FMISO, [15]O H2O, [15]O CO, radiolabeled peptides	Whole body imaging; direct proportion to contrast; high sensitivity; high throughput; ability to use multiple tracers	Expensive with limited availability; limited spatial resolution; lack of anatomical information; short radionuclide half-life limited penetration; interoperator variability; lack of targeted probes
Ultrasound	Color Doppler; power Doppler; microbubbles; antibody contrast agents (αvβ3 integrin)	Sensitive applications; anatomical imaging; inexpensive and widely available; portable;	
Optical imaging	Near infra-red	Highly sensitive; inexpensive; portable	Poor tissue penetration allowing for superficial sites only; lack of anatomical information; technology still in early development