Biomarkers of angiogenesis for the development of antiangiogenic therapies in oncology: tools or decorations?

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SUMMARY

Since 2004, four antiangiogenic drugs have been approved for clinical use in patients with advanced solid cancers, on the basis of their capacity to improve survival in phase III clinical studies. These achievements validated the concept introduced by Judah Folkman that the inhibition of tumor angiogenesis could control tumor growth. It has been suggested that biomarkers of angiogenesis would greatly facilitate the clinical development of antiangiogenic therapies. For these four drugs, the pharmacodynamic effects observed in early clinical studies were important to corroborate activities, but were not essential for the continuation of clinical development and approval. Furthermore, no validated biomarkers of angiogenesis or antiangiogenesis are available for routine clinical use. Thus, the quest for biomarkers of angiogenesis and their successful use in the development of antiangiogenic therapies are challenges in clinical oncology and translational cancer research. We review critical points resulting from the successful clinical trials, review current biomarkers, and discuss their potential impact on improving the clinical use of available antiangiogenic drugs and the development of new ones.

KEYWORDS biomarker, imaging, monitoring, therapy, tumor angiogenesis

REVIEW CRITERIA

The information for this Review was compiled by searching the PubMed, MEDLINE and ASCO databases for articles published until 30 September 2007. Electronic early-release publications were also included. The following search terms were used: "tumor angiogenesis" in association with: "diagnosis", "prognosis", "monitoring", "prediction", "surrogate marker", "molecular marker", "biomarker", "imaging", "clinical study". When possible, primary sources have been quoted.

CME

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Learning objectives

Upon completion of this activity, participants should be able to:

1 Identify 4 antiangiogenesis drugs approved since 2004 for human use in patients with solid cancers.
2 List markers of clinical progression of cancer.
3 Describe tumors targeted by the 4 approved anti-angiogenesis drugs in clinical trials.
4 Discuss challenges in the development of validated biomarkers of antiangiogenic activity, as well as their potential role in cancer treatment.
5 Identify functional imaging techniques for assessing antiangiogenesis drugs.

Competing interests

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INTRODUCTION

Tumor angiogenesis is a tumor microenvironmental process that promotes tumor-cell survival, growth, invasion and metastasis, and its inhibition is emerging as a new therapeutic approach to control tumor progression.[1,2] Hundreds of molecules with antiangiogenic activity in preclinical models have been reported, and many of them have entered clinical testing in oncology.[3] Initially, antiangiogenic molecules were discovered on the basis of their ability to inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. Subsequently, the identification of molecular mediators of angiogenesis, such as vascular endothelial growth factor (VEGF), opened the possibility of selectively targeting specific pathways.[4] To date, four antiangiogenic drugs have

Table 1 Phase II and III clinical trials with bevacizumab, sorafenib, sunitinib and temsirolimus in renal cell carcinoma.

Drug	Target	Phase II	Phase III	End point	Biomarker
Bevacizumab (Avastin®) (humanized A4.6.1)	VEGF	Randomized, double-blind: HR for PFS: 2.55 at 10 mg/kg; $P<0.001$[94]	First line: bevacizumab + IFNα vs IFNα PFS: bevacizumab plus IFNα (10.2 months; HR = 0.63; $P<0.0001$); PFS: placebo/IFNα (5.4 months)[13]	PFS	None
Sorafenib (Nexavar®) (BAY43-9006)	VEGFRs PDGFRα/β c-KIT FLT-3 Raf	Randomized discontinuation: PFS: 6 months vs 1.2 months.[17]	First line: sorafenib vs placebo: ORR: 10 % vs 2 % PFS: 5.5 months vs 2.8 months (HR 0.44%, 95% CI 0.35–0.55%).[18]	PFS	None (Patients with greater PFS tended to have higher pretreatment VEGF levels)[19]
Sunitinib (Sutent®) (SU11248)	VEGFRs PDGFRβ FLT-3 c-KIT c-RET	Not randomized, single arm: OR: 34% (CI 25–44%), PFS: 8.3 months (95% CI 7.8–14.5 months).[20]	First line: sunitinib vs IFNα ORR: 31% vs 6% ($P<0.001$) PFS: 11 months vs 5 months (HR 0.42, CI 0.32–0.54; $P<0.001$).[22]	ORR	None (Responding patients tended to have low levels of sVEGFR3 and greater decrease upon treatment)[23]
Temsirolimus (Torisel®) (CCI-779)	mTOR	Randomized: 25, 75, or 250 mg PFS: 6.3 months (25 mg), 6.7 months (75 mg), and 5.2 months (250 mg); 5.8 months overall.[25]	First line: temsirolimus vs IFNα vs temsirolimus plus IFNα OS: temsirolimus 10.9 months (HR, 0.73; 95% CI 0.58–0.92; $P=0.008$); IFNα, 7.3 months; temsirolimus plus IFNα, 8.4 months, PFS ($P<0.001$).[26]	OS PFS	None

Abbreviations: c-KIT, stem cell factor receptor; c-RET, glial cell-line-derived neurotrophic factor receptor; FLT, Fms-like tyrosine kinase; HR, hazard ratio; IFN, interferon; OR, odds ratio; ORR, overall response rate; OS, overall survival; PDGFR, platelet-derived growth factor receptor; PFS, progression-free survival; VEGFR, vascular endothelial growth factor receptor.

been approved for human use in solid tumors: Avastin® (Genentech, San Francisco, CA; bevacizumab),[5] Nexavar® (Bayer Aktiengesellschaft, Leverkusen-Bayerwerk, Germany; sorafenib),[6] Sutent® (C.P. Pharmaceuticals International, New York, NY; sunitinib),[7] and Torisel® (Wyeth Corporation, Madison, NJ; temsirolimus).[8] The successful clinical development of these drugs is in contrast to the plethora of molecules that failed to replicate in clinical trials the efficacy that was seen in preclinical models.[3,9] This high failure rate should stimulate a critical reappraisal of the strategies used to test these drugs in the clinic: what can we learn from these trials to avoid future failures? What are the critical factors in the development of antiangiogenic drugs? What are the end points we should aim at? Could biomarkers of angiogenesis and drug activity help in this process? We will review the design of the studies that led to the approval of these antiangiogenic drugs, discuss the potential value of biomarkers of angiogenesis, review current candidate markers and methodologies, and provide some rationale for future developments.

CLINICAL STUDIES OF APPROVED ANTIANGIOGENIC DRUGS

The efficacy of anticancer therapies is commonly evaluated by measuring direct effects on the tumor by use of the Response Evaluation Criteria In Solid Tumors (RECIST) scale,[10] and by determining the impact of treatment on disease progression (i.e. progression-free survival [PFS] and/or time to progression [TTP]), survival (i.e. overall survival (OS) or time to death) or quality of life.[11] Bevacizumab, sorafenib, sunitinib and temsirolimus were approved on the basis of these conventional end points, demonstrating that biomarkers *per se* were not essential for the development of this new class of drugs. To identify factors that led to these successful clinical developments, we will briefly review the studies that permitted the approval of these drugs (Table 1).

Bevacizumab

Bevacizumab is a humanized anti-VEGF monoclonal antibody that prevents VEGF from binding to its receptors, thereby inhibiting angiogenesis.[5,12] Evidence of antitumor activity was first observed in a randomized, double-blind, phase II trial that compared placebo with bevacizumab (3 mg/kg or 10 mg/kg) in pretreated patients with renal cell carcinoma (RCC). Bevacizumab prolonged TTP in the high-dose group but not in the low-dose group. A recent, randomized, double-blind, phase III study compared bevacizumab and interferon (IFN)-α2a with placebo and IFN-α2a as first-line therapy, and showed that the bevacizumab

combination improved PFS compared with IFN-α2a alone.[13] In patients with advanced colorectal cancer (CRC), bevacizumab in combination with chemotherapy extended TTP and OS leading to approval for clinical use.[14] Pretreatment circulating levels of VEGF did not predict response to bevacizumab in CRC.[15] Subsequently, bevacizumab was shown to improve PFS in metastatic breast cancer, OS in patients with CRC who had received previous treatment and, in combination with chemotherapy, OS in patients with non-small-cell lung cancer.[16]

Sorafenib

The small-molecule-kinase inhibitor sorafenib targets wild-type and mutated B-Raf, VEGFR2, VEGFR3, PDGFR-β, c-KIT, FLT-3 and p38.[6] It induces growth arrest and apoptosis of endothelial cells and some tumor cell types. Activity was first reported in a phase II randomized discontinuation trial in patients with RCC, whereby PFS was prolonged compared with placebo (24 weeks versus 6 weeks). Patients were treated for 12 weeks (run-in treatment) and at the end of that period those with stable disease were subsequently randomized to sorafenib or placebo.[17] In a phase III, randomized, double-blind, first-line placebo-controlled trial, sorafenib prolonged PFS of patient with metastatic RCC and was approved by the FDA and European Medicines Evaluation Agency (EMEA) for the treatment of advanced and metastatic RCC.[18] Retrospective analysis showed that high basal VEGF levels (>131 pg/ml) correlated with a poor prognosis and a trend towards greater PFS benefit in sorafenib versus placebo-treated patients.[19]

Sunitinib

Sunitinib is a tyrosine-kinase inhibitor (TKI) that targets VEGFR1, VEGFR2 and VEGFR3, PDGFR-α/β, FLT-3, c-KIT and c-RET.[7] Sunitinib showed evidence of activity in a phase I trial in many tumors, including thyroid and neuroendocrine cancers, soft tissue sarcoma, gastrointestinal stromal tumor and RCC.[20] In a phase II trial in patients with cytokine-refractory RCC, the overall response rate was 34% and median PFS was 8.3 months.[21] A phase III trial in patients with metastatic RCC showed that sunitinib improved PFS and overall response rate compared with IFNα as first-line therapy, and sunitinib was subsequently approved for the treatment of advanced and metastatic RCC.[22] Retrospective analysis from a phase II trial in bevacizumab-refractory RCC, suggests that circulating levels of VEGF and sVEGFR3 might be predictive of response to sunitinib: responding patients had lower basal levels of sVEGFR3 ($P<0.0318$) and a trend towards a greater decrease upon treatment levels ($P<0.10$) than nonresponding patients.[23]

Temsirolimus

Temsirolimus inhibits mTOR, an Akt-target kinase downstream of VEGFR2, which controls cell proliferation, cellular metabolism and survival.[24] Activity was demonstrated in a phase I trial in patients with RCC, and was confirmed in phase II studies, improving outcomes when administered either as single agent or in combination with IFNα.[25] A three-arm, randomized, phase III trial compared temsirolimus, IFNα and a combination of the two drugs as first-line treatment in aggressive RCC. Patients who received temsirolimus alone had longer OS and PFS than did patients in either of the other two treatment arms.[26]

Other studies of antiangiogenic agents

Some of the kinases targeted by sunitinib and sorafenib such as PDGFR, p38, FLT-3, c-KIT, c-RET, are active in cancer cells and promote tumor-cell proliferation, survival and motility. mTOR, the temsirolimus target, is often activated in cancer and has a central role in regulating metabolic events, in particular protein synthesis, which is essential for tumor growth.[24] Thus, in addition to antiangiogenic effects, these drugs may have direct effects on cancer cells that contribute to their global antitumor activities.

The use of anti-VEGF agents in CRC has provided varied results. While bevacizumab, in combination with irinotecan, fluorouracil and leucovorin induced a significant increase in OS in patients with advanced, previously untreated CRC,[14] the pan-VEGFR TKI vatalanib (PTK787), combined with the FOLFOX4 regimen, did not produce a survival benefit. In two phase I trials, vatalanib decreased vascular permeability and vascularity, as measured by dynamic contrast-enhanced (DCE) MRI in patients receiving ≥750 mg/d.[27] Although this effect occurred only in patients with liver metastases, it was interpreted as validation of vatalanib activity and was used to support the case for further clinical development. The phase III trial, however, failed to demonstrate efficacy and survival advantage. The reason for this negative result is unclear. A possible explanation might be that the dose and schedule determined in the early trials on

the basis of DCE MRI (pharmacodynamic biomarker) and applied to the phase III study were not optimum.

What can we learn from these studies? First, all four approved drugs demonstrated activity in RCC and target the VEGF/VEGFR pathway, albeit to different extents. RCC is the prototype cancer with VEGF-driven angiogenesis owing to frequent mutations in the *von Hippel Lindau* (*VHL*) tumor suppressor gene, a negative regulator of hypoxia-inducible-factor-1 (HIF-1) activity and VEGF expression.[28] These findings suggest that the match between the targeted pathway and the cancer chosen may be relevant or possibly critical to the success of these drugs. Second, biomarkers are dispensable when clinical results obtained in phase I/II trials are already convincing. By contrast, when the tested agent doesn't achieve tumor control during an early stage of clinical development, a pharmacodynamic biomarker reporting biological activity would constitute an essential requirement to pursue clinical development. The use of a putative pharmacodynamic biomarker that is irrelevant to the main biological activity of the drug or representative of activity in only a specific patient subpopulation, however, might compromise further development.

BIOMARKERS OF ANGIOGENESIS

Biomarkers are defined as molecular, cellular or functional measurable parameters indicative of a particular genetic, epigenetic or functional status of a biological system.[29] In cancer, biomarkers can be used for diagnosis, staging, prognosis and treatment selection. Biomarkers should be repeatable, reproducible, and measurable through minimally invasive procedures. A biomarker has prognostic value when it is indicative of the natural course and outcome of the disease, regardless of the treatment, and acquires predictive value when its presence correlates with the clinical response to a particular treatment. Pharmacodynamic markers reflect the effect of a given drug on the host or the tumor, regardless of whether the effect might or might not be correlated to any beneficial antitumor effect. A pharmacodynamic marker becomes a surrogate marker of drug activity if its presence or modulation correlates with the clinical response. The potential value of different types of biomarkers (e.g. diagnostic, prognostic, predictive) should be tested and validated through appropriately devised studies and correlated with PFS and OS. Many biomarkers of angiogenesis have been proposed and investigated, but none has yet been validated for routine clinical use. A brief summary of the main biomarkers reported is provided in Table 2.[30–32]

Circulating angiogenic factors and related molecules

Pretreatment blood levels of VEGF have been tested in many studies. In general, elevated levels are indicative of a poor prognosis, and therefore prognostic, but do not predict response to antiangiogenic drugs, including bevacizumab.[15,33,34] Rising levels of circulating factors (e.g. VEGF and placenta growth factor) were observed in response to antiangiogenic drugs or chemotherapy, possibly reflecting treatment-induced tumor hypoxia.[7,35–37] The practical utility of using drug-induced increases in circulating factors as surrogate biomarkers remains to be demonstrated, and their use might be confounded by increases associated with tumor resistance or escape.

Microvessel density and endothelial-signaling events

Microvessel density (MVD) at regions of intense angiogenesis (i.e. 'hot spots') has prognostic but not predictive value in many cancers.[38] MVD does not provide information on the functionality (perfusion) of the tumor vessels. MVD of primary tumors in patients with metastatic CRC did not predict response to bevacizumab.[34] The phosphorylation statuses of ERK and AKT in tumor endothelial cells have been explored as biomarkers of antiangiogenic therapy. Phosphorylation of both kinases was observed in angiogenic vessels and was attenuated by treatment with SU6668.[39] Since tissue-based biomarkers provide direct information on events at tumor site, they are highly valuable; however, they are impractical as pharmacodynamic biomarkers for routine clinical use and might be applied only to selected studies.

Circulating cells

Increased numbers of circulating endothelial cells (CEC) and bone-marrow-derived circulating endothelial cell progenitors (CECP) are observed in the blood of patients with cancer.[40,41] In mice, CECP—mobilized by tumor-derived factors that include VEGF—have proangiogenic activities.[1] Their increased frequency correlates with angiogenesis, and the levels of these cells return to normal following antiangiogenic treatments.[42] In patients with cancer, elevated CEC numbers returned to normal after tumor removal

Table 2 Major biomarkers and techniques for monitoring angiogenesis in preclinical and clinical studies.

Biomarkers	Technique for monitoring angiogenesis	Examples	Comments
Molecular			
Circulating angiogenic factors	ELISA, WB, proteomics, Luminex Multiplex or FACS array technologies	VEGF; FGF-2; MMP-9; IL-8; IL-6; HGF	Prognostic value in many cancers
EC-derived molecules	ELISA, WB, proteomics, antibodies arrays	sVEGFR1, sVEGFR2, sVEGFR3; sTie-2, VCAM-1	Limited to 'known' molecules
Circulating proteins or peptides	ELISA, WB, proteomics, antibodies arrays	Endostatin; tumstatin	Promising approach to identify novel molecules in serum or tumor tissues
Signaling events	Immunohistochemistry, Immunofluorescence	Phospho-Erk; Phospho-Akt	Limited feasibility in clinical practice
Biological			
Microvascular density Endothelial cell proliferation/death	Immunohistochemistry, Immunofluorescence	$CD31^+$, $CD34^+$, $VEGFR2^+$, $CD105^+$ vessels Ki67/CD31; Tunel/CD31	Prognostic value in many cancers Limited feasibility in clinical practice
CEC or CECP	Flow cytometry, Veridex technology	EC: $CD45^-$; $CD31^+$, $CD146^+$, $CD144^+$; $VEGFR2^+$ CECP: CD133 $CD34^+$; $CD144^+$; $VEGFR2^+$	Promising approach. Nonstandardized protocols. Labor intensive
Functional			
Functional imaging	DCE-MRI DCE-CT PET Power (color) Doppler (ultrasound) Contrast-enhanced ultrasound	Gadolinium chelate tracers Iodine-based tracers $H_2{}^{15}O$ tracer Microbubbles (Sonovue®, Bracco Diagnostics Inc., Plainsboro, NJ)	Allows measurement of MTT, rBF, rBV, pO_2, pH, and vascular permeability. Evidence of modification by therapy. Used in many studies, but no standard protocols available yet. Inexpensive, easy and safe techniques to monitor blood flow Allows quantitative measurement of blood flow
Molecular imaging	Tracer coupled to mAb or peptide against a vascular target, detected by PET or ultrasound	Targeting EDB+-fibronectin Targeting αVβ3 integrin	May improve specificity and sensitivity of functional vascular imaging. Complementary to dynamic measurements

Abbreviations: CEC, circulating endothelial cells; CECP, circulating endothelial cell precursors; DCE-CT, dynamic contrast-enhanced computed tomography; DCE-MRI, dynamic contrast enhanced magnetic resonance imaging; EC, endothelial cell; EDB, extra domain B; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorting; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; IL, interleukin; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MTT, mean transit time; rBF, relative blood flow; rBV, relative blood volume; Tie-2, tyrosine kinase with Ig and EGF homology domains; VCAM, vascular cell adhesion molecule; VEGFR, vascular endothelial growth factor receptor; WB, Western blotting.

or following complete remission after chemotherapy.[41,43] Bevacizumab reduced the frequency of viable CEC and CECP in patients with rectal cancer,[36] while ZD6474, a VEGFR2/EGFR TKI, caused an increase in mature CECs but not CECP, possibly reflecting therapy-induced endothelial cell detachment from tumor vessels.[44] In patients with metastatic gastrointestinal stromal tumors, those demonstrating a clinical benefit had greater increases in CECs. Sunitinib was reported to cause a greater increase in CEC in patients with gastrointestinal stromal tumor, and was associated with clinical benefits compared with patients with progressive disease.[45] Further studies are needed to establish the potential clinical value of CEC and CECP as biomarkers of angiogenesis.[40] The low frequency of these cells in samples, the sophisticated methodology required for their detection, and the difficulty in standardizing protocols may limit the widespread application of this approach in clinical practice. Peripheral blood lymphocytes have been used to demonstrate drug effects on selected signaling pathways. Monitoring ERK phosphorylation in lymphocytes obtained from sorafenib-treated patients and from lymphocytes stimulated *in vitro* with a phorbol ester provided useful information on the dose necessary to achieve ERK inhibition *in vivo*.[46]

Functional genomics and proteomics

Approaches based on genomics and proteomics have provided insights into the mechanistic

processes of angiogenesis, and facilitated the identification of novel candidate therapeutic targets and allowed the monitoring of drug activities.[47] Gene-expression profiling was used to identify genes expressed in tumor endothelial cells,[48,49] determine transcripts induced by VEGF,[50] define the angiogenic stage of endothelial cells,[51] and characterize CEC in patients.[52,53] Specific gene-expression signatures have been reported in endothelial cells and blood lymphocytes in response to antiangiogenic drugs, such as the VEGFR2 inhibitor SU5416, endostatin, and brivanib alaninate, a VEGFR2/FGFR-1 inhibitor.[54–56] This approach should be further explored for its potential to monitor tumor angiogenesis and antiangiogenic drug activity in the clinic. Proteomics-based approaches, although still in their infancy, have been used to identify angiogenesis-related proteins that are expressed in tumor vessels,[57] tumor interstitial fluid,[58] and cultured endothelial cells,[59] and to profile the secretome of endothelial cells.[60] Proteomic techniques have been applied to profile the substrate selectivity of antiangiogenic drugs, such as SU6668,[61] and to study therapy-induced effects on endothelial cells.[62] Proteomic analysis of serum proteins is considered a promising approach to identify angiogenesis biomarkers.[63,64] While promising, these approaches still face many challenges before entering routine clinical consideration, including the need to obtain high-quality material and the requirement of costly and sophisticated technologies.

IMAGING TECHNIQUES

Functional imaging approaches have been included in several antiangiogenesis studies.[65–67] In general, imaging approaches rely on the intravenous injection of a contrast agent that enhances the vascular and tumoral structures and on the acquisition of sequential images before, during and after injection. The time-concentration curve of the tracer allows the semi-quantitative deduction of several parameters: the mean transit time (MTT) of the contrast passing from the arterial to the venous circulation;[68] the relative blood volume in the tissue studied (rBV); and the relative blood flow (rBF), which corresponds to the maximal slope of the curve at the arterial phase (calculated as rBV/MTT). When the contrast agent diffuses from the intravascular into the extravascular space, a plateau phase is observed, which can be used to estimate vascular permeability, K^{trans} (Figure 1A). Quantitative parameters can be calculated by fitting a time concentration curve by use of recognized pharmacokinetic models[69] adapted to each imaging modality. Calculation of the parameters of the curves for each pixel in the region of interest allows parametric images of

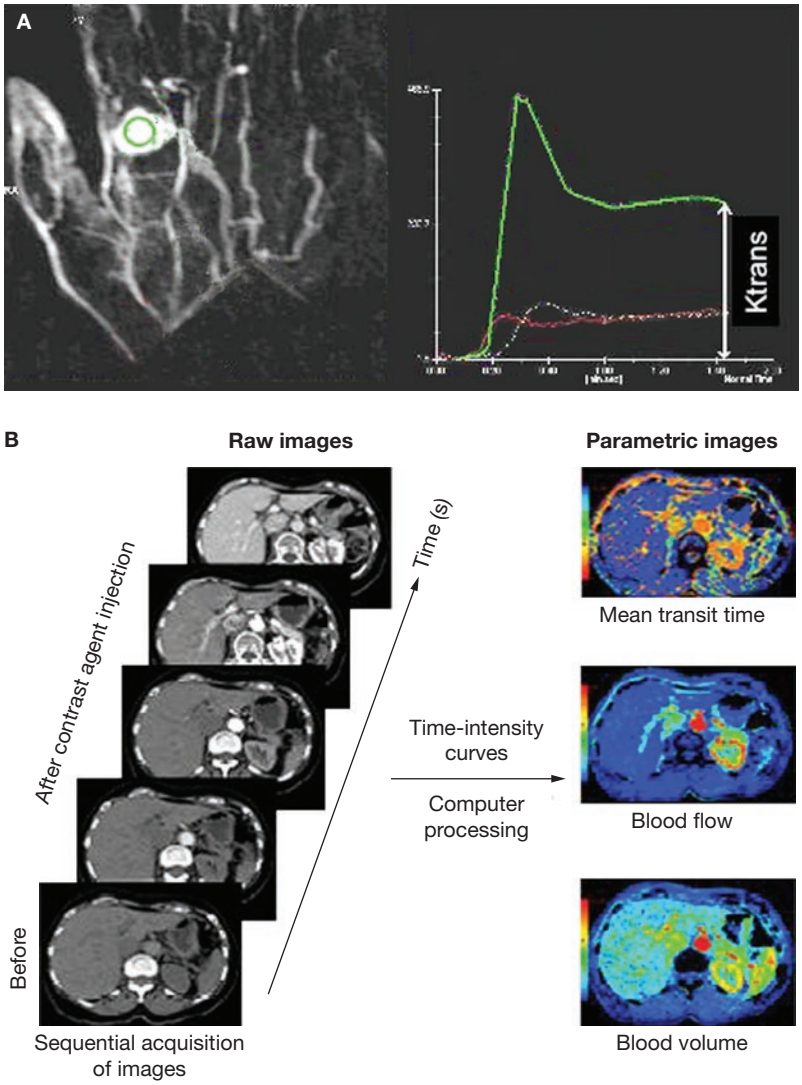


Figure 1 Dynamic contrast-enhanced MRI and dynamic contrast-enhanced CT of a hypervascular tumor. (**A**) Dynamic contrast-enhanced MRI of a hypervascular tumor, located in the right hand. The left panel represents the raw data image with the regions of interest for determining the time-intensity curves. The right panel shows the time-intensity curves: the green curve represents tumor perfusion, with a strong enhancement of the arterial phase, a plateau due to extravascular diffusion of the contrast agent, and slight increase due to the recirculation of the contrast agent (second pass); the red curve and the white curve represent the arterial and venous references, respectively. K^{trans} can be estimated after recirculation of the contrast agent and represents vascular permeability. (**B**) Dynamic contrast-enhanced CT. After sequential acquisition of the raw images over time (left panels), the time-intensity curves of the different perfusion parameters can be obtained for each pixel of the images or any regions of interest. Parametric images representing time-intensity values for each pixel over time will be calculated from these curves (right panels). (Images courtesy of Dr J-Y. Meuwly, CHUV, Lausanne).

the tissue to be created for each variable studied. In addition, imaging-based techniques can be used to monitor tissue hypoxia and acidosis.[67]

Dynamic contrast-enhanced MRI

This technique uses gadolinium-chelate-based agents that cause a decrease of T1 (time of realignment of hydrogen nuclei in the magnetic field) at low concentration and a decrease of T2 (time of loss of transverse magnetism) at high concentration.[70] T2 imaging is used early during the arterial input to calculate MTT, rBV and rBF, while T1 imaging is used at later time points to estimate K^{trans} (Figure 1A). DCE-MRI has been used in multiple studies to monitor the effects of antiangiogenic drugs.[71] The area under the curve and K^{trans} have been used to monitor treatment-induced changes in vascularity and permeability.[65,72] While K^{trans} has been used to measure biological activity (i.e. a pharmacodynamic marker) its value in predicting clinical response (i.e. a surrogate marker) remains to be demonstrated. The main limitations of DCE-MRI include quantification and reproducibility. Quantification by use of pharmacokinetic modeling necessitates calibration to determine the relationship between concentration of the contrast agent and signal intensity;[73] however, there is no simple relationship between contrast concentration and signal-intensity, especially at high contrast concentration. The nonlinear signal-intensity correlation is probably responsible for much of the variability in measurements observed with this technique. In addition, data acquisition is highly dependent on the machine characteristics, making comparisons across different platforms difficult.

Dynamic contrast-enhanced CT

This imaging technique uses iodine-based contrast agents to generate a linear relationship between contrast concentration and measured signal. It allows calculation of MTT, rBV, rBF and K^{trans} (Figure 1B). DCE-CT has not yet been systematically included in clinical studies, in part because of the lack of common protocols and methodologies for data acquisition, standardization and analysis.[74,75] There are some reports, however, that suggest the feasibility of this approach.[76] DCE-CT was used to assess tumor vascularity in rectal cancer and measure perfusion changes after chemoradiotherapy. The results revealed that tumors with high pretherapy rBF values tended to have a poor response to therapy.[77] The routine use of DCE-CT might be limited by the high X-ray exposure required.

Positron-emission tomography

$H_2{}^{15}O$ PET is considered the reference technique for quantification of rBF and vascular permeability.[70,78] Injected radioactive water can freely diffuse in the tissue, and a time-concentration curve reflecting radioactive oxygen accumulation can be determined by PET scanning. The requirement for a nearby cyclotron for probe preparation, owing to the short half-life of radioactive oxygen, is a major obstacle to the widespread clinical use of this technique. PET scanning by use of ^{11}C-methionine uptake coupled with DCE-MRI revealed a correlation between vascularity and methionine uptake in brain gliomas.[79] Combining imaging techniques could allow studies to concomitantly monitor the direct effects of antiangiogenic drugs on tumor vasculature and their secondary effects on tumor metabolism. In addition, PET can be applied to measure tumor hypoxia.[80]

Contrast-enhanced ultrasound

Contrast-enhanced ultrasound is an attractive technique to measure perfusion. The contrast agent consists of phospholipid-based microbubbles that encapsulate an inert gas, which when exposed to an ultrasound pulse, generate nonlinear resonances that allow enhanced representation of the vasculature.[81] Semiquantitative perfusion measurements can be derived from the time-concentration curve, while quantitative measurements can be obtained by local destruction of the microbubbles with a high-pressure acoustic pulse and measurement of the following signal recovery by low pressure acoustic pulses[82] (Figure 2). The time-intensity curve in each region of interest depends on perfusion and is fitted to a kinetic model to determine volume flow rate.[82] The accuracy of contrast-enhanced ultrasound for microvascular perfusion measurement and perfusion changes following therapy has been documented both in experimental models[83,84] and in patients with cancer.[85–87] Contrast-enhanced ultrasound has several advantages over DCE-MRI and DCE-CT: measured values strictly refer to the intravascular compartment and are not confounded by extravascular diffusion; repeat measurements can be taken with equipment that is easily accessible and has limited costs; and patients are not exposed to ionizing radiation.

In conclusion, imaging techniques allow measurement of tumor vascular parameters and have been employed in many clinical studies, but their impact on decisions during drug development has remained modest. This outcome might change if imaging analyses can be associated with analyses of molecular and biological biomarkers and compared with PFS and OS to test their value as surrogate markers. Future developments will include molecular-imaging-based approaches that allow expression levels or activity of specific molecular targets, such as vascular integrins, VEGFRs or matrix metalloproteinases, to be monitored.[88] Since the number of targets on angiogenic endothelial cells is small, only PET and single-photon-emission CT techniques have sufficient sensitivity for targeted imaging.[65]

BIOMARKERS OF ANGIOGENESIS: TOOLS, NOT DECORATIONS

In spite of the many candidate biomarkers of angiogenesis tested in preclinical and clinical studies, there are no established methods in routine clinical use to monitor angiogenesis or predict response to antiangiogenic drugs. Since the four antiangiogenic drugs were developed without the aid of biomarkers, one wonders whether biomarkers are needed at all. The ideal match between RCC, a tumor dependent on VEGF-driven angiogenesis, and drugs targeting the VEGF pathway, was key to the success of these trials and such success stories might be hard to repeat in the future. Indeed, many drugs with demonstrated potent antiangiogenic activity in preclinical studies failed to show efficacy in clinical trials and were abandoned.[3] Most importantly, many basic questions related to the assessment of tumor angiogenesis and monitoring antiangiogenesis therapies have remained unanswered. Thus, in spite of the initial success in the field, biomarkers of angiogenesis are desperately required (Box 1). Validated surrogate markers for monitoring angiogenesis, and drug activity, predicting response, defining optimum biological dose, designing combination therapies, and identifying resistance to antiangiogenesis therapies would be of great benefit and would, in addition, facilitate translational research.

Monitoring angiogenesis

While many parameters reflecting specific aspects of angiogenesis can be measured in patients, none has been unambiguously validated. It might be necessary to combine different approaches in order to obtain robust quantification of angiogenesis. Thus, future studies should be designed to test multiple surrogate biomarkers in parallel.

Monitoring drug activity

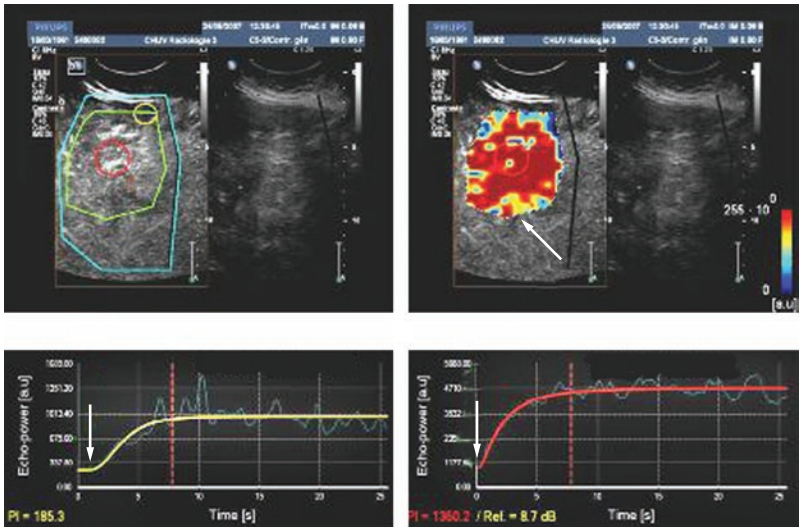
Biomarkers of efficacy are less relevant in phase III studies aimed at validating treatments, because improved TTP or OS are the critical end points at this stage of development. Biomarkers will be most useful as surrogate end points in phase I and phase II studies to facilitate the identification of active versus inactive drugs, and make the transition to phase III clinical studies more rapid (Figure 3). If a drug does not provide evidence of antitumor activity on the basis of standard criteria at an early stage of development, there are three possibilities: first, the drug has no effect on the target; second, the drug modulates the target, but does not elicit the desired biological activity; third, the drug exerts a biological activity, but this does not translate into a therapeutic effect. Nowadays, biological activity is considered the minimal requirement to proceed with drug development when clinical responses are not measurable. Therefore, monitoring drug effects through a pharmacodynamic biomarker, is the only way to obtain valuable information for 'go/no–go' decisions in phase I/II trials. This approach might possibly ensure that biologically active drugs reach large, phase III trials, while preventing the progression of biologically inactive drugs.

Predicting response

Not every patient responds equally well to antiangiogenic drugs, which is not surprising considering the multiplicity of angiogenic mechanisms and the intrinsic heterogeneity of tumor biology. In clinical practice, a biomarker able to predict response to therapy would be extremely valuable. Such a biomarker could act as a surrogate marker of clinical outcome and would be useful for identifying those patients responding to therapy.

Definition of optimum biological dose

The activity and toxicity profiles associated with antiangiogenic drugs are different from those associated with chemotherapy. Antiangiogenic drugs are expected to cause cytostasis, rather that cytotoxicity and tumor regression. The optimum biological dose for these drugs might be lower than the maximal-tolerated doses. Moreover, standard criteria used to define doses for cytotoxic drugs do not apply to this class of drugs. Pharmacodynamic biomarkers could provide



Healthy tissue Tumor tissue

Figure 2 Measurement of perfusion index in a hepatocellular carcinoma by contrast-enhanced ultrasound-based imaging. Microbubbles are injected intravenously and after reaching a steady-state they are destroyed by a high-pressure acoustic pulse (white arrow). A low acoustic pressure is applied to obtain a time-concentration curve in a region of interest determined by the user (red circle, region of interest in tumor tissue; yellow circle, region of interest in healthy liver tissue; the blue region indicates the areas of the image processed to compensate for respiration-induced liver movements to obtain a still image). Perfusion parameters may be automatically calculated by software or by postprocessing on a stand-alone work station, as in this example (software supplied by Bracco Research SA, Geneva).[91] A perfusion index representing relative blood flow (rBF) can be computed from the raw data. Upper left image—raw image. Upper right image—, parametric-map showing he perfusion index within the yellow region, which is indicated by the solid white arrow.

important information for the definition of a minimally active dose or a range of doses, which, in conjunction with clinical data, could define the dose escalation process and determine the optimum biological dose.

Design of combination therapies

Antiangiogenic drugs enhance the activity of standard chemotherapy, while combinations of drugs targeting complementary angiogenic pathways are expected to enhance responses. Early biological data may provide information that is useful to the selection of drugs and combination strategy. In the case of available antiangiogenic drugs, surrogate biomarkers could help to improve their use and to design novel combinatorial regimens.

Resistance to antiangiogenesis therapies

Patients treated with antiangiogenic drugs who show an initial antitumor response eventually show disease progression, suggesting that

Box 1 Relevance of biomarkers of angiogenesis to clinical oncology.

Biomarkers are molecular, cellular or functional parameters that are indicative of a particular genetic, epigenetic or functional status of a biological system. Biomarkers can be used for diagnosis, staging, prognosis and treatment selection, but these parameters should be reproducible, measurable and easy to repeat by use of minimally invasive procedures. A biomarker that is indicative of prognosis regardless of whether the treatment is of prognostic value. A prognostic marker acquires predictive value when its presence correlates with a response to a particular treatment. Pharmacodynamic markers reflect the effect of a given drug on the host or the tumor regardless of the antitumor effect. Such markers become a surrogate marker of drug activity if their presence (or modulation) correlates with the clinical response. No biomarker of angiogenesis has yet been validated for routine clinical use.

Detection and monitoring angiogenesis
A still unmet need in the field is the ability to detect and reliably monitor angiogenesis and antiangiogenic drug activities in patients. Such a test would greatly facilitate the clinical testing and development of new drugs and improve the use of available antiangiogenic drugs (e.g. optimize dosing, scheduling, and combination therapies).

Prognosis
Elevated parameters of angiogenesis are of prognostic value in certain cancers. Different parameters studied under the same conditions often give divergent results, thereby complicating the interpretation of their prognostic value.

Prediction of response
From a therapeutic point of view, the most valuable contribution of biomarkers of angiogenesis to clinical oncology is the determination of whether a patient is responding to a given antiangiogenic drug early in the course of treatment. Predictive markers are likely to differ depending on the biology of the tumor and of the drug used. Such biomarkers may allow the design of 'customized' therapies that maximize benefits and minimize costs.

Translational research
The possibility of measuring molecular, biological or functional parameters of tumor angiogenesis and monitoring the changes in these parameters in patients during tumor progression or therapy is instrumental to both the clinical validation of mechanisms originally described in experimental models and to the generation of novel mechanistic hypotheses to be pursued in experimental models. Thus, surrogate biomarkers of angiogenesis contribute to 'bench-to-bedside' and 'back-to-bench' translational research.

tumors develop resistance to this class of drugs.[14] Resistance under anti-VEGF therapy was demonstrated experimentally.[89] It will be important to detect resistance during treatment so patients can be given the most appropriate second-line treatment. The mechanisms responsible for resistance to antiangiogenic drugs are likely to be complex and are yet to be defined.[1]

Translational research

Our understanding of the mechanisms of angiogenesis and their modification by antiangiogenic treatments in human cancer are still rudimentary. Analysis is mostly focused in understanding the role of individual molecules or pathways, while we lack an integrated view and understanding of the functional association between apparently distinct events and their modification during angiogenesis and antiangiogenesis therapy. For example, a growing amount of evidence indicates that tumors react to therapy by upregulating angiogenic factors and mobilizing bone-marrow-derived CECP.[7,35–37,90] In spite of the obvious clinical relevance of these observations, we still have little knowledge of how tumors adapt to, and possibly escape from, angiogenesis inhibition. To address these and many other outstanding questions, it will be important to associate clinical studies not only with pharmacodynamic measurements, but also with relevant preclinical experimental models.

In short, biomarkers of angiogenesis should still be considered essential elements of clinical drug development. In phase I/II trials they are needed as pharmacodynamic biomarkers to validate the supposed biological effect of the drug, and define the optimum biological dose and schedule. At late stages of development (phase III) or in clinical practice, biomarkers are needed to identify responding patients early in the course of treatment (predictive biomarkers), to detect patients developing resistance and to guide the development of novel combination therapies.

The natural intra-patient and inter-patient variability of candidate biomarkers currently under investigation should be taken into account during future studies. Data collection and analysis should help to identify and quantify these variabilities and other putative confounding factors. In the case of simple biomarkers (e.g. serum measurements, blood pressure), the natural variability can be easily determined by taking multiple measurements. In the case of more complex analyses, such as DCE-MRI, multiple assessments could be very burdensome and financially prohibitive.

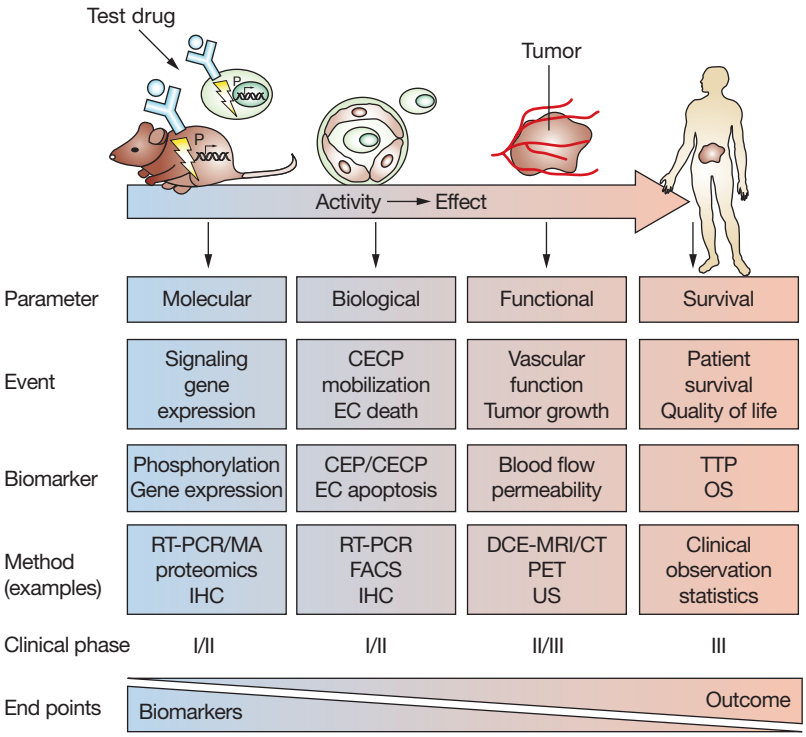


Figure 3 Rationale for the use of biomarkers in the development of antiangiogenic therapies. Abbreviations: EC, endothelial cell; CEC, circulating endothelial cells; CECP, circulating endothelial cell progenitors; CT, computed tomography; DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; FACS, fluorescence activated cell sorting; IHC, immunohistochemistry; OS, overall survival; RT-PCR, reverse transcription-polymerase chain reaction; PET, positron emission tomography; TTP, time to progression; US, ultrasound.

CONCLUSIONS AND FUTURE DEVELOPMENTS

Currently, the use and the understanding of antiangiogenic therapies in oncology are at different stages of development: on the one hand, four drugs extending patient survival in some cancers are available for clinical use, while on the other we are not able to sufficiently monitor the activity of these drugs, identify those patients responding to them or predict therapy outcome. Improved clinical use of these drugs and the successful development of new ones will depend heavily on our ability to monitor angiogenesis and drug activity in patients. How can we move on from here?

First, we should take advantage of the availability of these drugs to study how individual biomarkers behave in patients in response to treatment, how changes in these parameters

Box 2 Rationale for the use of biomarkers in the development of antiangiogenic therapies (also refer to Table 2 and Figure 3).

Quantification of tumor angiogenesis and the measurement of antiangiogenic drug activities in patients remain unresolved issues. Many approaches have been tested in experimental models and clinical studies, but to date none has been validated for routine use in patients. It is not clear which biomarker best represents angiogenesis and, as a matter of fact, whether there is an optimum biomarker of angiogenesis. The intrinsic complexity of tumor angiogenesis, and the multiple regulatory mechanisms of and adaptations to angiogenesis during therapy, suggest that, in fact, multiple and different biomarkers will be necessary to obtain a comprehensive representation of angiogenesis and its therapeutic modulation, with different markers being required depending on the tumor of interest, its stage, the drug being tested, the question being asked and the clinical stage of development (phase I/II/III). One can distinguish three kinds of biomarkers:

i) Molecular biomarkers
These markers consist of molecules such as growth factors (e.g. VEGF, FGF), cell surface receptors (e.g. VEGFR2, integrins), downstream signaling molecules (e.g. ERK, Akt) and their modifications (e.g. activation, phosphorylation), or transcripts of single or multiple genes (e.g. signatures) and their modifications. Molecular biomarkers are indicative of the molecular events associated with angiogenesis or drug activity;

ii) Biological biomarkers
In order to obtain initial information on the effects of these molecular events on angiogenesis, it will be necessary to monitor basic characteristics of endothelial cell function, such as cell proliferation and death. Angiogenesis-associated cells (e.g. circulating endothelial cells and circulating endothelial cell progenitors) in blood may reflect these changes and serve as easily accessible biomarkers of biological effects. Molecular and cellular biomarkers are important in phase I/II studies, where demonstration of target modification and drug activity is the main goal.

iii) Functional biomarkers
Modification of tumor perfusion is likely to reflect significant changes in physical or functional state of the tumor vasculature. However, acquisition and meaningful interpretation of vascular perfusion and permeability data is challenging, since changes may be only transient, occur late after drug administration, or may not necessarily be representative of drug effects (e.g. some tyrosine-kinase inhibitors concomitantly target tumor and stromal cells). Measurement of perfusion-related parameters (rBV, MTT, rBF, K^{trans}) by imaging technique is more useful at late phases of drug development (phase III studies) when evidence of antivascular activity is for the aim. In addition, imaging techniques can be used to monitor the effects on tumor biology (tumor regression, metabolism).

Clinical end points (e.g. overall survival and progression-free survival) are used to validate the impact of the tested drug on disease progression and patient survival. Data generated by different classes of biomarkers should be compared with data available on their relative value and suitability for monitoring angiogenesis or dissecting mechanisms of drug action. For example, vascular disrupting agents cause a transient shut down of the tumor vasculature in both experimental models[92] and patients,[93] while they concomitantly cause acute mobilization of circulating endothelial cell progenitors from the bone marrow and recruitment to the viable tumor rim remaining after therapy, thereby contributing to tumor regrowth.[90] This positive-feed back loop suggests that a combination of antivascular and antiangiogenic drugs may result in better therapeutic response.

relate to each other, and how they correlate with treatment outcome. Improved knowledge of biomarker biology will be essential for selecting biomarkers useful for monitoring treatments in the clinic and dissecting mechanisms in translational studies.

Second, a range of biomarkers will be necessary, since a single approach will not be sufficient to comprehensively monitor angiogenesis or antiangiogenic activities at different stages of drug development. Analyses performed on tumor biopsies or blood-circulating cells will demonstrate the biochemical or biological activities of biomarkers (i.e. pharmacodynamic biomarkers) that can then be used in phase I and phase II studies to provide the evidence of drug activity that is essential for further development. More demanding imaging-based techniques will be relevant in phase II and III studies, where demonstration of antitumor activity is needed (Box 2). Molecular or cellular biomarkers could also be useful in phase III trials or in clinical practice to identify patients responding or developing resistance to treatment.

Third, biomarkers should be chosen by considering the biology of the tumor and the putative mechanism of action of the tested drug. Molecular biomarkers may comprise molecules belonging to pathways known to be deregulated in the analyzed cancer and be 'downstream' of the drug target (or possibly be the target itself). The time course of the drug and dose dependence of response to the drug, or possible feedback mechanisms should also be considered. Thus, clinical trials need to be designed in such a way that the choice of the surrogate biomarkers and their analyses are coordinated with the tumor type, the drug, the schedule of treatment, and any additional pharmacodynamic or pharmacokinetic data generated during the study process.

In conclusion, in spite of the indisputable success of angiogenesis and antiangiogenesis research in deciphering mechanisms, delivering new drugs and introducing a new therapy paradigm in clinical oncology, many important questions remain unanswered. New questions are emerging, such as how tumors adapt to and escape from antiangiogenic therapy. To accelerate the validation of available biomarkers and the discovery of new ones, and to address questions emerging from clinical studies, it will be important to associate relevant preclinical models to clinical studies. The development of biomarkers of angiogenesis represents a unique opportunity for clinical oncologists and laboratory scientists to join forces to address relevant questions and design meaningful studies.

KEY POINTS

- Four antiangiogenic drugs, bevacizumab, sorafenib, sunitinib and temsirolimus, have been approved for clinical use on the basis of results from randomized phase III clinical trials without significant contributions from biomarkers
- No validated biomarkers of angiogenesis or antiangiogeneic activity are available for routine clinical use
- Biomarkers of angiogenesis might be useful for monitoring angiogenesis, assessing drug activity and distinguishing between active and inactive drugs, predicting clinical outcome and response to therapy, defining the optimum biological dose, facilitating development of combination therapies, and rapidly identifying resistance to treatment
- Biomarkers under consideration for clinical use include circulating cells, proteins (e.g. angiogenic factors, angiogenesis-associated molecules; protein expression profiles), nucleic acids (e.g. gene-expression patterns) and functional parameters (e.g. tumor perfusion, metabolism)
- The association of laboratory investigations with clinical trials will be instrumental for the validation of biomarkers of angiogenesis and for improving the design, monitoring and evaluation of antiangiogenic treatments

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