

Emergence of potential biomarkers of response to anti-angiogenic anti-tumour agents

Agnieszka Michael*[#], Kate Relph* and Hardev Pandha*

Running title

Anti-angiogenic biomarkers

*Postgraduate Medical School, Oncology, University of Surrey

Daphne Jackson Road, Manor Park

Guildford, Surrey GU2 7WG

[#] Corresponding author a.michael@surrey.ac.uk

Tel: 01483 688503

Fax: 01483 688558

Keywords – Biomarker, VEGF, tumour

Minireview

Abstract

Anti-angiogenic agents targeting tumour vasculature have an established place in clinical practice and new data is constantly emerging. However, despite rapid clinical uptake, a very large number of questions regarding these agents remain unanswered. One of the main hurdles in clinical practice is lack of accurate and feasible ways of assessing response to drug beyond tumour reduction on conventional imaging. This review summarises recent development in the field of biomarkers of response to anti-VEGF drugs.

Introduction

The regulatory approval of agents targeting tumour vasculature such as Bevacizumab and oral multi-receptor tyrosine kinase inhibitors which include VEGF (Vascular Endothelial Growth Factor) receptors as one of their targets, has changed clinical practice swiftly and significantly. One potential attraction of anticancer agents designed against specific target molecules would be the logical design of biomarker assays based on some biological aspect of that target. However, despite rapid clinical uptake, a very large number of questions regarding these agents remain unanswered. The question marks start at the precise nature of the anti-tumour effect and differential efficacy in otherwise similar patients/tumours, mechanisms of synergy with cytotoxic agents, optimal dose and scheduling with other targeted agents through to pre-selection of patients most likely to benefit from treatment and, probably most importantly at this time, accurate and feasible ways of assessing response to drug beyond tumour reduction on conventional imaging.

Vascular Endothelial Growth factor (VEGF or VEGF-A) is a potent proangiogenic growth factor expressed by most cancer cells and some tumour stromal cells. It belongs to the platelet-derived growth factor family (PDGF) together with other dimeric glycoproteins such as VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF)¹.

The available anti-VEGF agents act through various mechanisms. Bevacizumab and VEGF-Trap for example are antibodies that target the VEGF ligand. Other agents target VEGF receptors or downstream signalling pathways and these include small molecule inhibitors (Sunitinib, Sorafenib, Axitinib and Pazopanib), antibodies *IMC-1121b* and ribozymes-angiozyme. Bevacizumab treatment has been associated with

antitumour responses in most tumours and improves survival in patients with colorectal and lung cancer when administered with standard chemotherapy^{2,3}. Broad spectrum small molecule inhibitors such as Sunitinib and Sorafenib have shown high activity in renal cell carcinoma and are now accepted first and second line treatments^{4,5}. The mechanism of action for antiangiogenic therapies is very complex and incompletely understood. The anti-VEGF agents are thought to have direct anti-vascular effects⁶, they inhibit blood-borne endothelial cell precursors⁷ and lead to tumour microvessel ‘normalisation’ (pruning the immature and leaky vessels, actively remodelling remaining vasculature) enabling efficient delivery of cytotoxic treatments⁸. Beyond these observations predominantly from murine models, no reliable validated serum or tissue biomarkers of response to anti-VEGF treatment have emerged. However, there are many potential candidates based on both murine and human models. The purpose of this review is to summarise recent developments in this field.

The possible approaches to identify markers of response to anti-angiogenic treatment can be divided into four major groups: markers isolated and analysed from tumour tissue, blood –based markers, imaging based criteria for response and patient factors such as changes on blood pressure.

Tumour derived markers.

The obvious candidate when trying to define and predict the response to anti-VEGF treatments would be VEGF expression. VEGF is overexpressed in the majority of tumours^{9,1} but it is also present in stromal cells and vascular endothelium (see figure 1)^{9,10,11}. It can be easily detected by immunohistochemistry, in situ hybridisation, quantitative immunassays, Western blotting or reverse-transcriptase polymerase chain

reaction *RT-PCR*. All of these methods require tumour tissue which is usually available before treatment but it is not always practical to obtain tissue during and after treatment, restricting their clinical utility. As VEGF is also expressed by benign tissue the interpretation of tissue expression and its correlation to clinical outcome has to be treated cautiously. Immunohistochemistry based studies can, concurrently, also assess other markers such as microvascular density (MVD), perivascular changes, cell proliferation, apoptosis and functional as well as genomic abnormalities.

One of the first studies with Bevacizumab in rectal cancer provided some great insight into changes in tumour physiology such as blood perfusion, permeability, microvascular density and interstitial fluid pressure (IFP) in correlation with systemic responses measured by VEGF levels in blood, numbers of circulating endothelial cells and progenitor cells as well as tumour responses¹². In a study by Willet et al. (2004) twelve days after Bevacizumab infusion, five out of six patients showed significant decreases in tumour blood perfusion (40-44% $P < 0.05$) and blood volume within the tumour (16-30% in four of the five patients $P < 0.05$). This was accompanied by significant decrease in tumour MVD histologically (29-59% in five patients analysed $P < 0.05$). IFP was also reduced and was thought to be caused by 'normalisation' of the blood vessels within the tumour. However, these results did not correlate with the levels of circulating VEGF.

MVD was initially suggested by many groups as a surrogate measure of angiogenesis especially in colorectal cancer, but there are, however, many examples of positive association between VEGF expression and prognosis^{13,14} as well as reports of lack of any prognostic significance¹⁵. MVD as a tissue- based biomarker is only suitable for tumours that are easily accessible pre- and post treatment and is unlikely to find its

way into routine clinical practice. Apart from the changes in tumour vasculature the tumour cell apoptosis was also measured in situ¹⁶. Twelve days after Bevacizumab administration a significant increase in tumour cell apoptosis was seen in the biopsy tissue. Other surrogate markers measured in the tumour tissue include α -smooth muscle actin and angiopoietin-2 expression, both detectable and decreasing after Bavacizumab administration but again only measured in patients with easily accessible tumours¹⁶.

A broad –based profile of angiogenic factors and cytokines has recently been proposed as a measure of response to antiangiogenic treatment. A study presented by Hanrahan et al¹⁷ looked at correlation of a profile of cytokine and angiogenic factors (C/AF) with response to vandetanib (oral inhibitor of VEGFR, EGFR and RET) in patients with non small cell lung cancer. The C/AF consisted of 33 factors including VEGF, FGF (fibroblast growth factor), EGF (epidermal growth factor), HGF (hepatocyte growth factor), E-selectin, ICAM-1, MMP-9, multiple chemokines and interleukins. Plasma of patients treated with vandetanib was collected at baseline, on day 8, 22 and 43 of treatment. Several of the C/AF factors were found to be of prognostic value with low HGF and interleukin 2 receptor (IL-2R) predictive of benefit to antiangiogenic treatment but not chemotherapy. There were also significant gender differences in progression free survival (PFS) for patients benefiting from vandetanib. This is a very new concept of assessment of response to anti-VEGF treatment which warrants further evaluation.

Blood-based markers

VEGF levels can also be measured in body fluids. Patients with large tumour burden and widespread metastatic disease have increased levels of circulating VEGF;

there is a negative association between VEGF expression and prognosis^{18,19}. Unfortunately detection and precise measurement of VEGF may be inaccurate due to a number of factors. The immunoassays used to detect circulating VEGF usually use capture antibodies which only detect free VEGF. A significant amount of VEGF is bound to plasma proteins such as α_2 -macroglobulin. Some of the antibodies are only specific for single VEGF isoforms. VEGF can also be released from the platelets and leukocytes during blood sampling and handling²⁰⁻²². Two main isoforms of VEGF that are detectable in circulation are VEGF₁₂₁ and VEGF₁₆₅. Some of the available assays only detect VEGF₁₂₁ or only VEGF₁₆₅ whilst others measure the sum of both isoforms²². At present there is no evidence that any of the isoforms has any advantages over the other. The serum VEGF concentration increases with clotting duration and temperature²³ and the VEGF levels found in serum are to a large extent representative of VEGF released from platelets during clotting rather than a tumour secreted protein^{20,24}. The difficulties associated with the exact measurement of circulating VEGF levels, lack of international validated approach defining the standard operating procedures for blood handling and processing and the wide variety of commercial kits used for detection of circulating VEGF, makes circulating VEGF an unreliable biomarker for routine practice or for treatment decisions in clinical trials currently.

Soluble components of VEGF receptors can be detected in peripheral blood. A recently reported study by Rini et al showed a possible application of soluble VEGFR-3 (VEGF receptor -3) and VEGF-C levels as a prognostic marker of response to anti-VEGF treatment²⁵. In this study sixty one patients with metastatic renal cell carcinoma were treated with Sunitinib, a tyrosine kinase inhibitor with anti-VEGF activity. VEGF-A isoform 165 and 121 were measured together with VEGF-C, soluble VEGF receptor

–sVEGFR-3 and placental growth factor (PlGF). Plasma samples were collected on days 1, 14, and 28 of cycle 1 and days 1 and 28 of cycles 2 through to 4. The levels of these markers were analysed using validated ELISA kits. Significant changes in the mean plasma levels of VEGF-A, sVEGFR-3 and PlGF were observed within the first cycle of sunitinib treatment. After 28 days VEGF-A levels increased 2.8-fold (range 0.4-13.6) and PlGF levels increased 3.9-fold (range 0.8-20.4), whereas sVEGFR-3 levels decreased by 37.6% and VEGF-C levels decreased by 22.7%. The correlative analysis showed that patients with baseline sVEGFR-3 and VEGF-C levels less than the median baseline values (sVEGFR-3-47000pg/ml and VEGF-C 722,1pg/ml) had longer progression free survival (PFS) than patients with higher levels. There was no correlation between plasma VEGF or PlGF levels and PFS²⁵.

Soluble VEGFR-2 (sVEGFR-2) levels have also been evaluated in a clinical study in patients with gastrointestinal stromal tumours (GIST)²⁶. The levels of VEGF and sVEGFR2 were consistently modulated by treatment; however there was no clear correlation with clinical response. Patients with metastatic breast cancer as well as patients with neuroendocrine tumours treated with sunitinib also showed decrease in plasma of soluble VEGFR-2 and VEGFR-3^{27,28}. Renal cell cancer treatment with pazopanib also leads to a significant decrease in soluble VEGFR-2 and is associated with tumour response (p-0.00002)²⁹. Soluble KIT (tyrosine kinase transmembrane receptor) appears to correlate well with clinical responses in breast cancer and in GIST^{27,28,30}.

Circulating Endothelial Cells

VEGFR-2 is one of the receptors expressed on endothelial cells^{31,32}. Circulating endothelial cells (CEC), as opposed to the soluble VEGF receptors, have also been suggested as a surrogate marker of anti-angiogenic activity. CECs are mature endothelial cells sloughed off the vessel wall as a result of a vascular damage. A population of circulating endothelial cells which is thought to be derived from the bone marrow is often referred to as circulating endothelial progenitors (CEP). CECs and CEPs are distinct from circulating tumour cells (CTC), which can also be measured in the plasma. Increased plasma levels of CECs have been reported in cancer patients and the levels appear to increase in response to VEGF^{33,34}. The available assays to enumerate CECs and CEPs are not straightforward and are subject to many potential errors associated with sampling, preparation and analysis³⁵. One possible reason for conflicting results in reported studies is the changing immunophenotype of endothelial cells that is dependent on the type of injury to the vessel. The majority of assays define the CECs as positive for CD146, CD31 and negative for CD45 marker. CEPs are also positive for CD133. There is however some data indicating that CD146 is homogeneously expressed on vascular endothelium but not on viable CECs³⁶. The currently available assays are based on manual or automated immunomagnetic isolation or flow cytometry. This system could be used in general hospitals and once validated could potentially be of great value. Data emerging in the last 2 to 3 years is encouraging but by no means clear. Although in many studies CECs levels correlate with tumour progression/response to treatment, same data indicates that the changes depend on the type of antiangiogenic agent and on the tumour type³⁷.

In pre-clinical models treatment with a targeted VEGFR-2 antibody caused a dose-dependent reduction in viable CEPs that paralleled its anti-tumour activity³⁸. In human studies the data is accumulating and CECs are increasingly thought to reflect the disease status as well as response to antiangiogenic treatment. Metastatic breast cancer patients treated with bevacizumab and erlotinib show clear changes of CECs levels comparing to baseline, this is most pronounced in the first 3 weeks of treatment and appears to predict response to treatment³⁹. A study of patients treated with letrozole and bevacizumab showed that CECs are a biomarker of progression as an increase in CECs at week 3 compared to week 0 predicted worse PFS (0.015)⁴⁰.

Patients with hepatocellular carcinoma (HCC) who responded to treatment with Bevacizumab showed a dramatic drop in CEC levels⁴¹. Similarly the CEC levels show a significant decrease in patients with metastatic renal cell cancer⁴², GIST malignancies and colorectal cancer^{26, 37}. On the other hand patients with pancreatic cancer responding to bevacizumab showed no correlation with CEC levels⁴³. Interestingly CEP levels appear to increase in response to treatment with anti-angiogenic agents^{34,42}. An analysis of 4 phase II studies by Duda et al³⁷ suggested that CEC and CEP kinetics depend on the type of antiangiogenic agent and therefore their biomarker value is differential. Sunitinib induced a sustained decrease in CEPs but not CECs in patients with HCC, bevacizumab caused increase in CEPs but not CECs in ovarian cancer, and cediranib did not significantly change neither CEPs nor CECs in glioblastoma patients³⁷.

In summary CEC and CEPs are likely to be helpful in future studies but more extensive research is clearly required.

Imaging based biomarkers

An idea that new blood vessels developing within the tumours can be captured by imaging techniques has been around for a long time. The tumour supplying blood vessels are often dilated and tortuous with abnormal branching patterns, dead ends and lack of structure typical for other organs such as presence of arterioles, capillaries and venules. The permeability of the new blood vessels is also often much higher. Microvessel density has been previously mentioned and can be assessed in biopsy samples. A method of measuring microvessel density by various imaging techniques would offer a less invasive and more acceptable alternative to the patients. Imaging techniques can in theory measure blood volume and blood flow as well as differences in blood vessel permeability, vascular size and amount of oxygen in the tissue⁴⁴. The various methods used so far are: magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) ultrasound and other methods such as near-infrared optical imaging.

An ultrasound technique is relatively cheap and safe, when used with a contrast agent that can target blood vessels, it can be potentially very effective. A novel approach uses contrast-enhancing microbubbles conjugated with antibodies targeting newly formed endothelial cells. Willmann et al⁴⁵ used microbubble technique and managed to demonstrate microbubble binding to VEGF receptors on tumour cells *in vitro* and tumour associated endothelium *in vivo*. A validation of this innovative method in clinical trials is still pending.

MRI techniques have been used extensively in preclinical and clinical studies. MRI allows assessment of vascular changes over time, is safe to use as it does not involve ionizing radiation but it is expensive. T1-weighted images are sensitive to the

low concentration of gadolinium –based contrast and therefore MRI images can give information not only on blood flow and tumour changes but also blood vessel permeability. CT scan on the other hand can give more quantitative data on blood flow due to the linear correlation between the CT image intensity and concentration of the contrast agent. PET scanning can give quantitative data and is very sensitive to minute concentrations of tracer molecules⁴⁴ .

One of the first human studies evaluating dynamic contrast-enhanced MRI (DCE-MRI) was a study reported by Morgan et al⁴⁶. The study looked at dose-related changes in the contrast-enhancement parameters in DCE-MRI as a biomarker of response to an oral VEGF receptor tyrosine kinase inhibitor in the context of phase I trial. The study showed a statistically significant relationship between reduction in contrast enhancement and disease response to the drug suggesting that this method needs further evaluation and could potentially be used as an anti-VEGF treatment biomarker of response. Other studies also confirmed relationship of disease response and contrast uptake but without a correlation to response rate (see figure 2). The results of the studies using DCE-MRI are certainly very encouraging but require validation and further studies are ongoing⁴⁷⁻⁴⁹. Although the initial data shows the feasibility of DCE-MRI based biomarkers, the complexity and demand on patient and staff as well as considerable expense do not seem to support its use as a practical biomarker used on a day-to-day basis.

Biomarkers based on patient related factors such as changes in blood pressure have been reported²⁸ there appears to be correlation with response to anti-angiogenic

agents however the changes are not reliable enough to be used in clinical practice as indicators of response.

Conclusion

The identification and validation of biomarkers indicating not only benefit of antiangiogenic therapy but capable of directly influencing clinical decisions on dosage and scheduling are urgently needed. It remains unclear as to the optimal biomarker for this purpose; most likely, the combination of data from tissue, blood and imaging will ultimately prove most useful. As well as response, biomarkers of resistance to antiangiogenic therapy also need to be considered. Resistance to primary therapy may be due to a number of factors including tumours switching to alternate pathways that are not targeted by that agent. Emerging data of elevated levels of SDF1 correlating with poor outcome in recurrent glioblastoma refractory to Bev, and elevated levels of IL-6 in hepatocellular carcinoma resistant to sunitib suggest specific alternate pathways exist, can be identified and characterised and themselves be targeted by other antiangiogenic agents.

Acknowledgements

Funding for all three authors is from the University of Surrey. There are no conflicts of interest.

References

- 1) Dvorak, H.F.. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002; 20: 4368-80.
- 2) Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*, 2004; 350: 2335-42.
- 3) Sandler, A., Gray, R., Perry, M.C., Brahmer, J., Schiller, J.H., Dowlati, A., Lilenbaum, R. & Johnson, D.H. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006; 355: 2542-50.
- 4) Escudier, B., Eisen, T., Stadler, W.M., Szczylik, C., Oudard, S., Siebels, M., Negrier, S., Chevreau, C., Solska, E., Desai, A.A., Rolland, F., Demkow, T., et al. Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med*, 2007; 356:125-34.
- 5) Motzer, R.J., Hutson, T.E., Tomczak, P., Michaelson, M.D., Bukowski, R.M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., Kim, S.T., Chen, I., Bycott, P.W., et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N Engl J Med* 2007; 356:115-24.
- 6) Kerbel, R. & Folkman, J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002; 2: 727-39.
- 7) Rafii, S., Lyden, D., Benezra, R., Hattori, K. & Heissig, B. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer* 2002; 2: 826-35.

- 8) Jain, R.K. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 2001; 7: 987-9.
- 9) Brown, L.F., Berse, B., Jackman, R.W., Tognazzi, K., Guidi, A.J., Dvorak, H.F., Senger, D.R., Connolly, J.L. & Schnitt, S.J. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol* 1995; 26: 86-91.
- 10) Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E.C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R.K. & Seed, B. Tumor induction of VEGF promoter activity in stromal cells. *Cell* 1998; 94: 715-25.
- 11) Hlatky, L., Tsiou, C., Hahnfeldt, P. & Coleman, C.N. Mammary fibroblasts may influence breast tumor angiogenesis via hypoxia-induced vascular endothelial growth factor up-regulation and protein expression. *Cancer Res* 1994; 54: 6083-6.
- 12) Willett, C.G., Boucher, Y., di Tomaso, E., Duda, D.G., Munn, L.L., Tong, R.T., Chung, D.C., Sahani, D.V., Kalva, S.P., Kozin, S.V., Mino, M., Cohen, K.S., et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 2004; 10: 145-7.
- 13) Bossi, P., Viale, G., Lee, A.K., Alfano, R., Coggi, G. & Bosari, S. Angiogenesis in colorectal tumors: microvessel quantitation in adenomas and carcinomas with clinicopathological correlations. *Cancer Res* 1995; 55: 5049-53.
- 14) Frank, R.E., Saclarides, T.J., Leurgans, S., Speziale, N.J., Drab, E.A. & Rubin, D.B. Tumor angiogenesis as a predictor of recurrence and survival in patients with node-negative colon cancer. *Ann Surg* 1995; 222: 695-9.

- 15) Jubb, A.M., Hurwitz, H.I., Bai, W., Holmgren, E.B., Tobin, P., Guerrero, A.S., Kabbinar, F., Holden, S.N., Novotny, W.F., Frantz, G.D., Hillan, K.J. & Koeppen, H. Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer. *J Clin Oncol* 2006; 24, 217-27.
- 16) Willett, C.G., Boucher, Y., Duda, D.G., di Tomaso, E., Munn, L.L., Tong, R.T., Kozin, S.V., Petit, L., Jain, R.K., Chung, D.C., Sahani, D.V., Kalva, S.P., et al. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. *J Clin Oncol* 2005; 23: 8136-9.
- 17) Hanrahan, E.O., Lin, H.Y., Du, D.Z., Yan, S., Kim, E.S., Lee, J.J., Ryan, A.J., Tran, H.T., Johnson, B.E. & V., H.J. Correlative analyses of plasma cytokine/angiogenic factor (C/AF) profile, gender and outcome in a randomized, three-arm, phase II trial of first-line vandetanib (VAN) and/or carboplatin plus paclitaxel (CP) for advanced non-small cell lung cancer (NSCLC). *JCO, ASCO Annual Meetings Proceedings Part I*, 2007; 25: 7593.
- 18) Chin, K.F., Greenman, J., Gardiner, E., Kumar, H., Topping, K. & Monson, J. Pre-operative serum vascular endothelial growth factor can select patients for adjuvant treatment after curative resection in colorectal cancer. *Br J Cancer* 2000; 83: 1425-31.
- 19) Davies, M.M., Jonas, S.K., Kaur, S. & Allen-Mersh, T.G. Plasma vascular endothelial but not fibroblast growth factor levels correlate with colorectal liver metastasis vascularity and volume. *Br J Cancer* 2000; 82: 1004-8.

- 20) Banks, R.E., Forbes, M.A., Kinsey, S.E., Stanley, A., Ingham, E., Walters, C. & Selby, P.J. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer* 1998; 77: 956-64.
- 21) Freeman, M.R., Schneck, F.X., Gagnon, M.L., Corless, C., Soker, S., Niknejad, K., Peoples, G.E. & Klagsbrun, M. Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis. *Cancer Res* 1995; 55: 4140-5.
- 22) Jelkmann, W. Pitfalls in the measurement of circulating vascular endothelial growth factor. *Clin Chem* 2001; 47: 617-23.
- 23) Webb, N.J., Bottomley, M.J., Watson, C.J. & Brenchley, P.E. Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. *Clin Sci (Lond)* 1998; 94: 395-404.
- 24) Verheul, H.M., Hoekman, K., Luykx-de Bakker, S., Eekman, C.A., Folman, C.C., Broxterman, H.J. & Pinedo, H.M. Platelet: transporter of vascular endothelial growth factor. *Clin Cancer Res* 1997; 3: 2187-90.
- 25) Rini, B.I., Michaelson, M.D., Rosenberg, J.E., Bukowski, R.M., Sosman, J.A., Stadler, W.M., Hutson, T.E., Margolin, K., Harmon, C.S., DePrimo, S.E., Kim, S.T., Chen, I. et al. Antitumor activity and biomarker analysis of sunitinib in patients with bevacizumab-refractory metastatic renal cell carcinoma. *J Clin Oncol* 2008; 26: 3743-8.
- 26) Norden-Zfoni, A., Desai, J., Manola, J., Beaudry, P., Force, J., Maki, R., Folkman, J., Bello, C., Baum, C., DePrimo, S.E., Shalinsky, D.R., Demetri, G.D. et al.

Blood-Based Biomarkers of SU11248 Activity and Clinical Outcome in Patients with Metastatic Imatinib-Resistant Gastrointestinal Stromal Tumour 2007a; 13: 2643-2650.

- 27) Bello, C.L., Deprimo, S.E., Friece, C., Smeraglia, J., Sherman, L., Tye, L., Baum, C.M., Meropol, N.J., Lenz, H. & H., K.M. Analysis of circulating biomarkers of sunitinib malate in patients with unresectable neuroendocrine tumors (NET): VEGF, IL-8, and soluble VEGF receptors 2 and 3. *ASCO Annual Meeting Proceedings* 2006; 4045.
- 28) Deprimo, S.E., Friece, C., Huang, X., Smeraglia, J., Sherman, L., Collier, M., Baum, C., Elias, A.D., Burstein, H.J. & D., M.K. Effect of treatment with sunitinib malate, a multitargeted tyrosine kinase inhibitor, on circulating plasma levels of VEGF, soluble VEGF receptors 2 and 3, and soluble KIT in patients with metastatic breast cancer. *ASCO Annual Meeting Proceedings* 2006; 578.
- 29) Hutson, T.E., Davis, I.D., Machiels, J.H., de Souza, P.L., Baker, K., Bordogna, W., Westlund, R., Crofts, T., Pandite, L. & Figlin, R.A. Biomarker analysis and final efficacy and safety results of a phase II renal cell carcinoma trial with pazopanib (GW786034), a multi-kinase angiogenesis inhibitor 2008 Abstract no. 5046
- 30) Blackstein, M., Huang, X., Demetri, G.D., Casali, P.G., Garrett, C.R., Schöffski, P., Shah, M.H., Verweij, J., Baum, C.M. & E., D.S. Evaluation of soluble KIT (sKIT) as a potential surrogate marker for TTP in sunitinib malate (SU)-treated patients (pts) with advanced GIST. *ASCO Annual Meeting Proceedings* 2007; 10007.
- 31) Li, T.S., Hamano, K., Nishida, M., Hayashi, M., Ito, H., Mikamo, A. & Matsuzaki, M. CD117+ stem cells play a key role in therapeutic angiogenesis induced by

- bone marrow cell implantation. *Am J Physiol Heart Circ Physiol* 2003; 285: H931-7.
- 32) Peichev, M., Naiyer, A.J., Pereira, D., Zhu, Z., Lane, W.J., Williams, M., Oz, M.C., Hicklin, D.J., Witte, L., Moore, M.A. & Rafii, S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000; 95: 952-8.
- 33) Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M. & Isner, J.M. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *Embo J* 1999; 18: 3964-72.
- 34) Beaudry, P., Force, J., Naumov, G.N., Wang, A., Baker, C.H., Ryan, A., Soker, S., Johnson, B.E., Folkman, J. & Heymach, J.V. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. *Clin Cancer Res* 2005; 11: 3514-22.
- 35) Strijbos, M.H., Gratama, J.W., Kraan, J., Lamers, C.H., den Bakker, M.A. & Sleijfer, S. Circulating endothelial cells in oncology: pitfalls and promises. *Br J Cancer* 2008; 98: 1731-5.)
- 36) Duda, D.G., Cohen, K.S., di Tomaso, E., Au, P., Klein, R.J., Scadden, D.T., Willett, C.G. & Jain, R.K. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. *J Clin Oncol* 2006; 24: 1449-53.

- 37) Duda, D.G., Cohen, K.S., Ancukiewicz, M., di Tomaso, E., Zhu, A.X., Penson, R.T., Horowitz, N.S., Batchelor, T.T., Willett, C.G. & Jain, R.K. A comparative study of circulating endothelial cells (CECs) and circulating progenitor cells (CPCs) kinetics in four multidisciplinary phase II studies of antiangiogenic agents, 2008; Abstract no 3544-.
- 38) Shaked, Y., Bertolini, F., Man, S., Rogers, M.S., Cervi, D., Foutz, T., Rawn, K., Voskas, D., Dumont, D.J., Ben-David, Y., Lawler, J., Henkin, J., et al. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis; Implications for cellular surrogate marker analysis of antiangiogenesis. *Cancer Cell*, 2005; 7: 101-11.
- 39) Rugo, H.S., Dickler, M.N., Scott, J.H., Moore, D.H., Melisko, M., Yeh, B.M., Caravelli, J., Brogi, E., Hudis, C. & Park, J.W. Change in circulating endothelial cells (CEC) and tumor cells (CTC) in patients (pts) receiving bevacizumab and erlotinib for metastatic breast cancer (MBC) predicts stable disease at first evaluation 2005; abstract no. 525
- 40) Rugo, H.S., Dickler, M.N., Traina, T.A., Scott, J.H., Moore, D.H., Bruckner, J., Hudis, C. & Park, J.W. Change in circulating endothelial cells (CEC) predicts progression free survival (PFS) in patients (pts) with hormone receptor positive metastatic breast cancer (MBC) receiving letrozole (L) and bevacizumab (B), 2006; Abstract no 3039
- 41) Malka, D., Dromain, C., Farace, F., Horn, S., Pignon, J., Ducreux, M. & Boige, V. Bevacizumab in patients (pts) with advanced hepatocellular carcinoma (HCC): Preliminary results of a phase II study with circulating endothelial cell (CEC) monitoring, 2007 ; Abstract no. 4570.

- 42) Farace, F., Vielh, P., Plantade, A., Amsellem, S., Bouaita, L., Vassal, G., Bidart, J.M., Fizazi, K. & Escudier, B. Circulating endothelial cells and progenitor cells in metastatic renal cell cancer: Predictive value during anti-angiogenic therapy, 2007; Abstract no. 3555.
- 43) Iyer, R.V., Javle, M.M., Pande, A.U., Yu, J., Wilkinson, D., Rustum, Y. & Wallace, P. Levels of circulating endothelial cells (CECs) as early pharmacodynamic markers of activity of anti-angiogenic (AA) therapy in advanced pancreatic cancer (PC) 2006; Abstract no. 14080.
- 44) Miller, J.C., Pien, H.H., Sahani, D., Sorensen, A.G. & Thrall, J.H. Imaging angiogenesis: applications and potential for drug development. *J Natl Cancer Inst* 2005; 97: 172-87.
- 45) Willmann, J.K., Paulmurugan, R., Chen, K., Gheysens, O., Rodriguez-Porcel, M., Lutz, A.M., Chen, I.Y., Chen, X. & Gambhir, S.S. US imaging of tumor angiogenesis with microbubbles targeted to vascular endothelial growth factor receptor type 2 in mice. *Radiology* 2008; 246: 508-18.
- 46) Morgan, B., Thomas, A.L., Drevs, J., Hennig, J., Buchert, M., Jivan, A., Horsfield, M.A., Mross, K., Ball, H.A., Lee, L., Mietlowski, W., Fuxius, S., et al. Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. *J Clin Oncol* 2003; 21: 3955-64.
- 47) Evelhoch, J.L., LoRusso, P.M., He, Z., DelProposto, Z., Polin, L., Corbett, T.H., Langmuir, P., Wheeler, C., Stone, A., Leadbetter, J., Ryan, A.J., Blakey, D.C. &

Waterton, J.C. Magnetic resonance imaging measurements of the response of murine and human tumors to the vascular-targeting agent ZD6126. *Clin Cancer Res* 2004; 10: 3650-7.

48) Galbraith, S.M., Rustin, G.J., Lodge, M.A., Taylor, N.J., Stirling, J.J., Jameson, M., Thompson, P., Hough, D., Gumbrell, L. & Padhani, A.R. Effects of 5,6-dimethylxanthenone-4-acetic acid on human tumor microcirculation assessed by dynamic contrast-enhanced magnetic resonance imaging. *J Clin Oncol* 2002; 20: 3826-40.

49) Stevenson, J.P., Rosen, M., Sun, W., Gallagher, M., Haller, D.G., Vaughn, D., Giantonio, B., Zimmer, R., Petros, W.P., Stratford, M., Chaplin, D., Young, et al. Phase I trial of the antivascular agent combretastatin A4 phosphate on a 5-day schedule to patients with cancer: magnetic resonance imaging evidence for altered tumor blood flow. *J Clin Oncol* 2003; 21: 4428-38.