Coagulation, angiogenesis and cancer

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Kinetics of angiogenic biomarkers during sorafenib treatment in patients with metastatic renal cell cancer

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Abstract

Purpose
Bay 43-9006/sorafenib is a small molecule multi targeted tyrosine kinase receptor inhibitor, including VEGFRs, with efficacy in patients with advanced renal cell cancer (RCC). In this study we studied the change in angiogenic biomarkers during sorafenib treatment and possible correlations with clinical outcome.

Patients and methods
This single center biomarker analysis study was part of an international multicenter open label, noncomparative phase III study, evaluating the activity of the multi target kinase inhibitor sorafenib as first line (patients not suitable for cytokine therapy) or second line (after progression on IFN-α, or IL-2) therapy, in patients with metastatic RCC. Before and at several time-points during sorafenib therapy we measured plasma and platelet levels of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), soluble VEGF receptors (sVEGFR)-1 and -2, and basic fibroblast growth factor (bFGF) and correlated the observed changes with time to progression (TTP) and median survival (mS).

Results
We demonstrated that VEGF but not PlGF or sVEGFR-1-2 is sequestered in platelets. We measured an increased platelet VEGF concentration at baseline in RCC patients, compared to healthy volunteers, but no further increase of platelet VEGF concentrations during sorafenib therapy was observed, despite higher VEGF plasma levels. This suggests that already at low VEGF plasma concentrations platelets are maximally saturated and that sorafenib does not affect the balance between plasma and platelets concentrations. Except for VEGFR-2, no correlation was found between baseline plasma levels of VEGF, PlGF, sVEGFR-1, and bFGF and clinical outcome. In most patients we observed a durable increase in plasma VEGF and PlGF and a decrease in sVEGFR-1,-2 levels during long-term sorafenib treatment. The changes in these biomarker levels were not correlated with TTP and mS and were comparable between patients with disease progression on therapy and patients with stable or responding disease.

Conclusion
Several preclinical studies demonstrated that changes in angiogenic biomarker levels in plasma during VEGFR targeted therapy is a class effect, is tumor independent, may reflect optimal VEGFR inhibition, but cannot be considered as a predictive marker for tumor response or clinical benefit. The absence of correlations between angiogenic biomarker changes during sorafenib therapy and clinical outcome are in agreement with these preclinical observations.
Introduction

The FDA approval of sorafenib, sunitinib, temsirolimus and bevacizumab has had a huge impact on the available therapeutic options for advanced renal cell cancer (RCC) and will probably result in the design of new combined treatment modalities for RCC. Besides the classical prognostic markers for advanced RCC, new validated biomarkers are needed to determine the optimal biologic dose, and to predict the outcome of targeted therapy and the development of drug resistance. Circulating levels of vascular endothelial growth factor (VEGF), placenta growth factor (PIGF), soluble VEGF receptors (sVEGFRs) and basic fibroblast growth factor (bFGF) in sunitinib- or sorafenib-treated patients are potential markers to predict outcome of VEGFR targeted treatments.

Several studies demonstrated changes in circulating VEGF, PIGF, and sVEGFR-1, -2 and -3 levels during treatment with VEGFR inhibitors in animal tumor models and in human. MF-1 and DC101, both VEGFR-1, and -2-inhibiting monoclonal antibodies, induced a rapid increase in plasma VEGF levels in normal and tumor-bearing mice. Because the increase in VEGF levels was dose-dependent with maximum values at optimal anti-tumor activity doses of DC101 or MF-1, plasma VEGF levels may be a surrogate marker for VEGFR targeted therapy.

Clinical studies with tyrosine kinase inhibitors, AZD2171 (axitinib), SU11248 (sunitinib) and sorafenib, all targeting VEGFR-1-3, demonstrated increased plasma levels of VEGF and PIGF and decreased in plasma levels of sVEGFR-2 during treatment. Although a correlation of changes in plasma VEGF and sVEGFR levels with objective tumor response was demonstrated for sunitinib, the changes were not discrete enough to allow prediction of response on an individual patient basis.

In the present study, we serially measured VEGF, PIGF, bFGF, and sVEGFR-1, -2 levels in plasma and platelets of advanced RCC patients at several time points during treatment with sorafenib. We correlated baseline values and changes during treatment for the various markers with disease outcome (TTP), overall survival, response rate and the development of resistance during therapy.

Patients and methods

Patients

Forty-five patients with metastatic RCC, with and without previous cytokine-based therapy (IFN-α or IL-2) were enrolled in this study after giving informed consent. The main inclusion criteria were: 18 years of age or older; histological confirmation of RCC; stage IV disease with presence of measurable lesions; adequate Karnofsky performance status (PS of 0-2); absence of severe comorbid conditions and adequate hematologic renal and hepatic function. Low Karnofsky performance status (<80%), high lactate dehydrogenase activity (>1.5 times upper limit of normal), low serum hemoglobin levels (<lower limit of
normal), high corrected serum calcium levels (>10 mg/dL) and absence of nephrectomy were risk factors. Each patients was then assigned to one of three risk groups, those with no risk factors (low-risk), those with one or two risk factors (intermediate-risk) and those with three or more risk factors (high-risk) according to Motzer et al. Pre-treatment patient characteristics, best response, date of progression and date of death or last follow up were recorded for all patients.

In 16 healthy volunteers, without sorafenib treatment, we also measured VEGF concentrations in PECT plasma and platelets. These data were used to make a comparison with baseline VEGF levels in RCC patients. The study was approved by the institutional review board of the Academic Medical Center and all patients gave written informed consent.

**Study design and treatment**

This biomarker analysis single center study was part of an international multicenter open label, noncomparative phase III study, evaluating the activity of the multi target kinase inhibitor sorafenib (Nexavar®) as first line (patients not suitable for cytokine therapy) or second line (after progression on IFN-α, or Il-2) therapy, in patients with metastatic RCC. The primary endpoint of this biomarker study was to the correlation of base line values and changes of various biomarkers during sorafenib therapy with disease outcome (TTP), overall survival, response rate and the development of sorafenib resistance. Patients were treated with 400 mg oral sorafenib bi-daily (bid) on a continuous basis. Dose reduction to limit toxicity was allowed to 200 mg bid. Sorafenib was provided by Bayer (Bayer Healthcare AG, Leverkusen, Germany), the sponsor of the trial.

**Objective clinical response, time to progression and overall survival**

Tumor assessments with CT or MRI scans of abdomen and thorax were performed at baseline (within 4 weeks before start of the therapy) and every eight weeks thereafter. Assessment of a response was confirmed by a second response evaluation at 4 weeks after initial documentation.

Response and progression were defined by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Clinical objective responses were characterized using the baseline sum of longest diameters (LD) for all lesions.

Adverse events were graded with the use of the Common Terminology Criteria (CTC) for adverse events version 3.0. Response was also assessed by severity of adverse events. All patients who received at least one dose of sorafenib were evaluated for safety and compliance. Biochemical profiles - based on a previous analysis - were obtained throughout the study as well. All laboratory test were performed at the Academic Medical Center. TTP was calculated from the date of first dose of sorafenib to the investigators assessment of progression. Overall survival was calculated from the data of the first dose...
Kinetics of angiogenic biomarkers of sorafenib to death. Patients who have not died at the time of analysis were censored at their last assessment date.

Assessment of biomarkers levels
Blood samples for determination of platelet counts, circulating angiogenic biomarkers, (VEGF, PI GF, bFGF and sVEGFR-1 and -2), and the concentration of these biomarkers in platelets, were harvested at day 0 before sorafenib administration, and at days 15, 56 and 112, during sorafenib treatment. From each patient venous blood was taken with a microperfuser (diameter 1 mm; Microflex, Vycon, Ecouen, France) and divided into different tubes. To avoid artificial ex vivo platelet activation blood was collected in an open system, drop by drop without using a tourniquet, and harvested in PECT medium, a 400 μL solution containing: prostaglandin E$_1$ (94 nmol/l), Na$_2$CO$_3$ (0.63 mmol/l), EDTA (90 mM) and theophylline (10 mM). In addition, blood was collected in tubes filled with EDTA or citrate using a tourniquet (Becton Dickinson vacutainers systems, Breda, The Netherlands). Blood collected in the PECT tubes was immediately placed on ice in contrast to the citrate and EDTA blood which were held at room temperature. For the circulating biomarkers, platelet depleted PECT plasma was prepared by spinning for 60 min at 1700 g at 4°C within 1 h after collection. The citrate samples were centrifuged within 30 min for 15 min at 1000 g to obtain plasma. EDTA blood was used to measure the total number of platelets and to measure VEGF, PI GF, sVEGFR-1 and -2 and bFGF concentrations within platelets. For this procedure, platelets were destroyed by a combination of Triton (2% Triton X-100), sonication during 15 sec on ice (microtip, Branson, amplitude 50%) and centrifuging during 5 min at 14,000 rpm in a micro-centrifuge. Platelet depleted PECT plasma was used to measure VEGF, PI GF, sVEGFR-1 and -2. Citrate plasma was also used to measure VEGF, PI GF, sVEGFR-1 and -2. BFGF-levels were measured in EDTA-plasma as recommended by the manufactory. All samples were tested for VEGF, PI GF, sVEGFR-1 and -2 and bFGF using commercially available sandwich enzyme–linked immunosorbent assays (ELISAs) from R&D Systems (Quantikine, R&D Systems, Abingdon, UK). The VEGF ELISA assay measured the VEGF-165 and VEGF-121 levels. The sVEGFR-1 ELISA and the sVEGFR-2 ELISA assays both measured the extracellular (soluble) domains of the VEGFR. All ELISA assays were validated for their intended purpose. VEGF, PI GF, and bFGF measurements were performed in undiluted samples. For sVEGFR-1, we used undiluted samples instead of 20-fold dilution as recommended by the manufactory. The samples for the measurements of sVEGFR-2 were diluted 5-fold as recommended by the manufactory.

Data analysis
Baseline characteristics for continuous variables were expressed depending on the distribution of the data, as mean (SD) or median (range). The primary outcome was the protein plasma level (biomarker- level) correlated and analyzed with SPSS version 12.0 (SPSS, Gorinchem, The Netherlands) and GraphPad Prism software (GraphPad Prism, San
Diego, CA, USA). Statistical significance was established at p<0.05. Survival distributions and TTP graphs were estimated using Kaplan-Meier methodology.

Results

Patient characteristics
Forty five patients were included in the study. The median age was 59 years; 76% were male and 87% of the patients had clear cell histology. Sixty seven percent had undergone nephrectomy and 93% had two or more metastases. Twenty seven percent of the patients had received radiation therapy and 33% percent immunotherapy. The distribution of patients according to prognostic risk categories was: 33% favorable, 53% intermediate, and 13% poor.

Figure 1: Kaplan Meier plots. A) Survival curves of patients treated with sorafenib. B) TTP curves of patients treated with sorafenib. Continuous line is median survival or TTP, dotted lines are the 95% confidence intervals.
Disease outcome
The overall response rate for the 45 patients was 16%, one patient with CR, seven patients with PR. Twenty nine patients (64%) had a SD and 8 patients (18%) had PD. The median survival was 480 days (median 15.7 months 95% CI, 13.8 to NA). The median TTP was 162 days (median 5.3 months, 95% CI, 3.6 to 7.0) (Figure 1).

Baseline biomarker levels in plasma and platelets and relation with clinical parameters
To determine the influence of continuous sorafenib treatment on the kinetics of various angiogenic biomarkers and the distribution of these markers in plasma and platelets, we measured these biomarkers under different conditions and at several time-points. Under the minimal platelet activation condition (harvest without tourniquet and collection in PECT medium) we measured significantly lower VEGF levels compared to levels in citrate plasma (Table 1). The concentrations of PIgF, sVEGFR-1 and -2 in PECT plasma were similar to those in citrate plasma. This means that platelet activation and release of its content during the harvest procedure only affects VEGF plasma levels. This is also in line with the observation that VEGF was detectable in platelets and PIgF and sVEGFR-1 and -2 were not (Table 1). The level of bFGF was measured in EDTA plasma because the ELISA for bFGF was not compatible with citrate and PECT plasma. Of the baseline values of the various biomarkers, only sVEGFR-2 demonstrated a correlation with TTP (r=0.4; p=0.01) (Figure 2) but not with survival (not shown).

Figure 2: Correlation between biomarkers at baseline and TTP. Significant correlation was found between sVEGFR2 at baseline and TTP (r=0.4; p=0.02).
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Significant changes in median plasma levels of VEGF, PlGF, sVEGFR-1, and -2, were observed at all time points after the start of sorafenib treatment but not bFGF (Table 2). The maximal changes were already found at day 15 after the start of sorafenib and lasted for the period of treatment up to 112 days. These values returned to baseline levels after discontinuation of sorafenib treatment (data not shown). These data demonstrate that increased levels of VEGF, PlGF (median, 2- and 2.5-fold, respectively) and decreased levels of sVEGFR-1 and -2 (median decline 25% and 26%, respectively) are durable phenomena for the period of sorafenib treatment. A significant correlation was found between the changes in VEGF and PlGF levels and between changes in PlGF and its sVEGFR-1 receptor (Figure 3), but not between sVEGFR-1 and -2, and their ligand VEGF.

Table 2: Plasma levels of the different biomarkers. Levels of VEGF, PlGF, sVEGFR-1 and -2 measured in EDTA plasma. All at different time points during treatment with sorafenib (median values [interquartile range]). The difference between baseline (day 0) and day 15 biomarkers levels were statistically different for VEGF (p<0.0001), PlGF (p<0.0001), sVEGFR-1 (p=0.001) and -2 (p=0.001). All time points after two weeks of treatment did not show any significant difference.

<table>
<thead>
<tr>
<th>Treatment in days</th>
<th>RCC patients 0 Plasma levels (pg/ml)</th>
<th>RCC patients 15 Plasma levels (pg/ml)</th>
<th>RCC patients 56 Plasma levels (pg/ml)</th>
<th>RCC patients 112 Plasma levels (pg/ml)</th>
</tr>
</thead>
</table>
Figure 3: Correlation between changes in biomarkers after two weeks of treatment with sorafenib. Significant correlation was found between the changes in VEGF and PlGF levels ($r=0.3$, $p=0.03$) and between the changes in PlGF levels and VEGFR-1 levels ($r=0.4$, $p=0.02$) but not between changes in sVEGFR-1 and VEGFR-2 and VEGF (data not shown).
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Figure 4: Correlation between changes in biomarkers after two weeks of treatment with sorafenib and TTP and mS. No correlation was found between the changes in VEGF, PlGF, sVEGFR-1 and -2 levels and TTP or mS (data only shown for VEGF-levels)
To explore potential relationships between changes in biomarker levels and disease outcome, linear regression analyses were performed. Because of the low number of sorafenib induced responses and because TTP and mS better reflect treatment outcome, we correlated TTP and mS with changes in plasma biomarkers. No correlation was found between changes in VEGF, PlGF, sVEGFR-1,-2 levels and TTF or mS (data shown for VEGF, Figure 4).

Changes in biomarker levels after two weeks treatment in a subgroup of 8 patients with RECIST responses compared to eight patients with progressive disease (PD) were not different. In 15 patients on sorafenib treatment, we harvested blood for biomarker analysis at the moment that disease progression was documented. In these patients the change in biomarker levels was persistent and not different compared to patients with stable or responding disease (Figure 5). These data demonstrate that changes in VEGF, PlGF, sVEGFR-1,-2 levels and TTF or mS (data shown for VEGF, Figure 4).

Changes in biomarker levels after two weeks treatment in a subgroup of 8 patients with RECIST responses compared to eight patients with progressive disease (PD) were not different. In 15 patients on sorafenib treatment, we harvested blood for biomarker analysis at the moment that disease progression was documented. In these patients the change in biomarker levels was persistent and not different compared to patients with stable or responding disease (Figure 5). These data demonstrate that changes in VEGF, PlGF, sVEGFR-1,-2 levels and TTF or mS (data shown for VEGF, Figure 4).

**VEGF levels in plasma versus platelets**

The median VEGF levels in PECT plasma from 16 healthy controls were, although significantly, only slightly different compared to baseline levels of patients with RCC (17 pg/ml vs. 20 pg/ml, respectively, p=0.0008). Levels in platelets were significantly different
between these two groups (3.4 pg/10^6 platelets vs. 5.6 pg/10^6 platelets, respectively, p=0.0004). The increase in VEGF plasma levels during sorafenib treatment did not result in a further increase in platelet VEGF levels. These data suggest that already at low VEGF plasma concentrations platelets are maximally saturated and that sorafenib does not affect the balance between plasma and platelet VEGF levels (Table 3).

**Table 3: VEGF levels measured in healthy volunteers and RCC patients. VEGF-levels measured in PECT plasma and in platelets in healthy persons compared to RCC patients at baseline, and followed at 15, 56 and 112 days of treatment with sorafenib (median (interquartile range)). Significant difference between the VEGF levels in PECT plasma in healthy persons and RCC patients at baseline (p=0.0008) and between the VEGF-levels in platelets in healthy persons compared to RCC patients (p=0.0004). Significant difference in VEGF levels after two weeks of sorafenib treatment (p<0.0001) but no significant difference after treatment of sorafenib after two weeks or in the platelets.**

<table>
<thead>
<tr>
<th>Treatment in days</th>
<th>Healthy persons</th>
<th>RCC patients</th>
<th>RCC patient 15</th>
<th>RCC patient 56</th>
<th>RCC patient 112</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (pg/10^6 platelets)</td>
<td>3.4 [2.8-4.4]</td>
<td>5.6 [3.8-6.7]</td>
<td>5.3 [4.1-7.1]</td>
<td>5.6 [4.3-7.3]</td>
<td>5.8 [4.5-7.7]</td>
</tr>
</tbody>
</table>

**Discussion**

In this clinical study, we assessed the kinetics of various angiogenic biomarkers during sorafenib treatment and tried to correlate these results with clinical outcome in patients with metastatic RCC. Because platelets are a well known reservoir for VEGF, which may affect plasma VEGF concentration by release from platelets during the harvest procedure and which phenomenon may be responsible for the high variability in VEGF measurements, we minimized platelet activation by collecting blood samples without tourniquet and in PECT medium. We detected both at baseline and at multiple time-points during long-term sorafenib treatment, significantly lower VEGF levels in PECT plasma compared to citrate plasma. The levels of PIGF, and sVEGFR-1, -2 were not different between PECT and citrate plasma. This was in line with the observation that in contrast to VEGF, PIGF and sVEGFR-1, -2 are not detectable in platelets.

In this small patient population, we found that a low baseline plasma VEGFR-2 level was associated with shorter TTP but not with mS. However, a correlation was not found between baseline plasma levels of the other biomarkers VEGF, PIGF, sVEGFR-1 and bFGF and TTP and mS. In the TARGET trial, a phase III trial comparing sorafenib with placebo in RCC patients, high baseline VEGF plasma levels were associated with worse outcome in the placebo group, but not in the sorafenib treated group, suggesting that sorafenib negates the negative impact of high baseline plasma VEGF levels on clinical outcome in RCC patients. Previously, it has been shown that circulating levels of soluble receptors, such as sVEGFR, cKIT, and HER-2/neu, are useful as potential biomarkers for cancer progression.
The majority of these studies showed positive correlations between disease and soluble receptor levels. However, recent clinical studies investigating sVEGFR-2 and cancer progression have been less clear and showed variable correlations with disease, such that the potential of sVEGFR-2 as a biomarker for tumor growth, remains unknown\textsuperscript{10,11}. In most patients, plasma levels of VEGF and PIGF increased and sVEGFR-1,-2 decreased significantly during treatment with sorafenib. The maximal changes of these biomarker concentrations could already be measured at 15 days after the start of sorafenib and lasted for the full period of treatment, measured till day 112. These biomarker changes during sorafenib treatment are comparable to the results in the TARGET trial\textsuperscript{5}. The increase in plasma VEGF levels and decrease in sVEGFR-2 levels by drugs targeting VEGFRs is now a well known phenomenon which has been described for several VEGFR inhibitors, like SU11248/sunitinib\textsuperscript{4}, AG-013736/axitinib\textsuperscript{3}, and PTK787/vatalanib\textsuperscript{12}. Our observation that VEGFR inhibition resulted in lower sVEGFR-1 levels as well, comparable to sVEGFR-2, has not been reported earlier. The similar effects on both VEGF and PIGF, and the correlation between these two in our study probably reflects sorafenib induced inhibition of both VEGFR-1 and -2, as VEGF is a ligand for VEGFR-1 and -2 and PIGF for VEGFR-1. The mechanism behind this phenomenon is unknown and it may not be tumor dependent because VEGF-upregulation by VEGFR-2 inhibition was also demonstrated in non-tumor bearing mice treated with a monoclonal antibody against VEGFR-2\textsuperscript{2}. A recent paper from the group of Kerbel demonstrated that in an animal tumor model, VEGFR inhibition resulted in similar VEGF and PIGF increases and sVEGF-2 decreases, in both tumor as well as in non-tumor bearing animals\textsuperscript{13}. The observed changes in angiogenic biomarker levels during treatment with small molecular VEGFR inhibitors can be considered to be a class effect of this group of targeted drugs, and is independent of the presence of tumor\textsuperscript{14}. This dose dependent phenomenon may probably be used as a marker for optimal VEGFR inhibition and the most efficacious antitumor dose, but not as a predictive marker of tumor response or clinical benefit. The data from the present clinical study are in agreement with these pre-clinical observations. No relationship was observed between changes in angiogenic biomarker levels and clinical outcome. In addition, patients with progressive disease showed comparable biomarker changes to patients with partial remissions. And patients with disease progression on therapy showed biomarker changes to be persistent and not different from non-progressive patients. To investigate effects of sorafenib on the VEGF pool in plasma compared to platelets, we measured VEGF in platelets at several time-points during sorafenib treatment. Before start of therapy, VEGF levels in platelets of RCC patients were already increased compared to healthy controls. During sorafenib treatment there was no further increase in platelet VEGF levels despite higher VEGF plasma levels. These data suggest that the platelet VEGF pool is saturated at already low plasma VEGF levels and is therefore not affected by sorafenib treatment.
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The results of this study confirm the preclinical observations that changes in angiogenic biomarker levels are a class effect of VEGFR targeting drugs and do not predict clinical benefit in patients with RCC.

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References


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