CHAPTER 5

CD133\(^+\) CIRCULATING HEMATOPOIETIC PROGENITOR CELLS PREDICT FOR RESPONSE TO SORAFENIB PLUS ERLOTINIB IN NON-SMALL CELL LUNG CANCER PATIENTS

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ABSTRACT
BACKGROUND: Blood-based biomarkers may be particularly useful for patients selection and prediction of treatment response for angiogenesis inhibitors. Circulating endothelial cells (CECs) and hematopoietic progenitor cells (HPCs) might play a role in tumor angiogenesis and in tumor growth. Measurement of CECs and HPCs in the blood of patients could be a simple, non-invasive way to monitor or predict responses to treatment.

METHODS: (VEGFR2+) CECs, (CD133+) HPCs, plasma vascular endothelial growth factor (VEGF) and erythropoietin were measured in blood from 25 non-small cell lung cancer (NSCLC) patients before and during treatment with sorafenib plus erlotinib (SO/ER). In order to assess the drug specificity of changes in CECs and HPCs, 18 patients treated with bevacizumab plus erlotinib (BV/ER), and 10 patients with erlotinib (ER) monotherapy were studied. Response was measured in all patients groups by Response Evaluation Criteria in Solid Tumors (RECIST) .

RESULTS: At D7, SO/ER treated patients showed a 3-fold increase in CECs (P<0.0001) comparable to BV/ER treated patients (P<0.01), and the CECs did not change with erlotinib treatment (P=0.8). At day 7, CD133+/HPCs decreased with SO/ER treatment (P<0.0001). HPC numbers did not change with either BV/ER or erlotinib. In SO/ER treated patients pre-treatment CD133+/HPCs were significantly lower in responders (P = 0.01) and pre-treatment CD133+/HPC numbers lower than the median correlated with a longer time to progression (P = 0.037).

CONCLUSION: Pre-treatment CD133+/HPCs are a promising candidate biomarker to further explore for use in selecting NSCLC patients that might benefit from SO/ER treatment.
CHAPTER 5

INTRODUCTION

Anti-angiogenic agents used to target tumor neovascularization can be classified into two groups: agents binding the vascular endothelial growth factor (VEGF), such as the humanized monoclonal antibody (Moab) bevacizumab or the soluble decoy receptor VEGF Trap and secondly the receptor tyrosine kinase inhibitors (TKIs) targeting the VEGF receptor family, such as sunitinib and sorafenib.

Bevacizumab has proven efficacy against several solid tumors [1], amongst others against non-small cell lung cancer (NSCLC). A phase III study by Sandler et al [2] showed a survival benefit in advanced stage NSCLC patients when bevacizumab was combined with chemotherapy in first-line setting. This was recently confirmed by Manegold et al [3]. Moreover, a phase I/II trial combining the epidermal growth factor receptor (EGFR) inhibitor erlotinib or chemotherapy with bevacizumab resulted in higher response rates and longer median progression free survival (PFS) in both bevacizumab containing arms [4].

The orally available anti-angiogenic TK-inhibitor, sorafenib is a multitargeted inhibitor of raf-kinase, VEGFR1, VEGFR2, VEGFR3, PDGFR-β, Flt3, and c-kit which has been approved in the treatment of both advanced renal cell carcinoma and hepatocellular carcinoma. There is emerging evidence of clinical antitumor activity in NSCLC [5, 6].

Although antitumor activity is achieved by these anti-angiogenic drugs either as monotherapy or in combination with chemotherapy or with other targeted agents, it remains unclear which patients will benefit most from treatment [7]. Biomarkers that potentially predict treatment response, in order to guide patient selection and/or monitor early response, are currently being investigated [8, 9].

Particularly attractive biomarkers might be circulating endothelial cells (CECs), as these may reflect damage to the tumor vasculature, or circulating hematopoietic progenitor cells (HPCs), as they have been proposed to reflect a pro-angiogenic cell population, which may respond to increased hypoxia by effective anti-angiogenic therapy [10-12].

No studies are currently available assessing the effects of anti-angiogenic agents in NSCLC patients on CECs and HPCs. In this study we evaluated 1) changes in CECs, defined as CD34bright/CD45/VEGFR2+ and HPCs defined as CD34bright/CD45dim/CD133+ in NSCLC patients treated with anti-angiogenic therapy with sorafenib plus erlotinib (SO/ER) 2) we investigated the specificity of these changes by comparing these with a control group of patients receiving bevacizumab plus erlotinib (BV/ER) or monotherapy erlotinib and 3) we correlated changes of the biomarkers with treatment response in the SO/ER patient group.
MATERIALS AND METHODS

PATIENT AND STUDY DESIGN

From November 2007 to October 2008: 25 chemotherapy-naive patients with advanced NSCLC who were treated at the VU University Medical Center in a phase II trial combining erlotinib (150 mg/day) and sorafenib (400 mg twice daily) were enrolled in this study [13].

For comparison a cohort of 18 NSCLC patients treated with bevacizumab (15 mg/kg intravenously every 21 days) plus erlotinib (150 mg/day orally) in a phase II trial [14] and a control group of 10 patients treated with whole brain radiotherapy (WBRT) plus concurrent erlotinib for brain metastases from NSCLC in phase I trial were evaluated [15]. These control group patients received erlotinib 1 week prior to, and during WBRT (30 Gy in 10 once-daily fractions) and continued maintenance erlotinib (150mg/day) until unacceptable toxicity or disease progression.

Prior to study entry, all patients provided written informed consent in accordance with national and institutional guidelines which strictly adhere to the principles of the Declaration of Helsinki and ICH/ Good Clinical Practice.

Peripheral blood (PB) was taken from all patients on 3 occasions: before start of treatment, day (D) 0, and 7 and 21 days after starting drug treatment, D7 and D21 respectively. Computed Tomography (CT) was performed before and 6 weekly after start of treatment to assess clinical response according to the Response Evaluation Criteria in Solid Tumors (RECIST) [16]. In the SO/ER treated patients responses incorporating primary tumor cavitations as proposed by Crabb et al [17] were also determined. Clinical responses after 6 weeks of treatment were used to examine a possible relation with VEGF and erythropoietin (EPO, in SO/ER treated patients only) levels and the cellular biomarkers.

EVALUATION OF CELLS AND PLASMA BIOMARKERS

Blood from SO/ER treated patients was collected in EDTA tubes and the circulating HPCs and CECs were measured using a full-blood flow cytometric method as previously described [18, 19]. (CD133+) HPCs were defined as CD34^{bright}/CD45^{dim} (and CD133^{+}) and CECs were defined as CD34^{bright}/CD45^{neg} and largely positive for VEGFR2. The antibodies used to perform analysis in this study included CD45-FITC, CD34-APC, CD133-PE, VEGFR2-PE and viability marker 7-AAD. Cell populations were measured by flow cytometry (FACSCalibur, BD Bioscience) in a total volume of 1.5 mL full blood and appropriate IgG isotypes were used as a control. Circulating cells from the comparison groups (monotherapy erlotinib and BV/ ER) were assessed with CD45-FITC, CD34-APC and 7-AAD. Cell populations of the total white blood cells (WBC) were calculated as
number/mL of blood using blood cell count measured on a Sysmex. EDTA blood was used for the collection of plasma which was stored at −80°C until further analysis. Plasma VEGF and EPO levels were measured in duplicate with the enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis).

**STATISTICAL ANALYSES**

Circulating cell populations (numbers/mL), plasma levels of VEGF (pg/mL), and EPO (mIU/mL) were expressed as median (range). Continuous variables between groups were compared with Mann-Whitney U test. To compare the blood-based parameters at pre-treatment with those during treatment (D0, D7, and D21) the Wilcoxon Signed Rank test was used. Time to progression (TTP) was determined as the time period from start of treatment to the time of documented disease progression with a minimum follow-up period of six months. Overall survival (OS) was the time between the first day of treatment and the date of death or the date on which patients were last known to be alive. Correlations of the circulating cells with TTP and overall OS were determined with the Kaplan-Meier method and its associated log-rank statistic. Responders were defined as those achieving partial response (PR) according to RECIST and non-responders as those with stable disease (SD) or progressive disease (PD) after 6 weeks of treatment. Differences were considered statistically significant when \( P < 0.05 \) (two-tailed). Since this was an exploratory study, no correction for multiple testing was done. Statistical analyses were performed with SPSS (SPSS for Windows 15.0, SPSS, Inc., Chicago, IL) software program.

**RESULTS**

**PATIENT CHARACTERISTICS AND TREATMENT RESPONSE**

In total 53 NSCLC patients in three study groups were investigated. Patients treated with SO/ER included in this study (14 males and 11 females) had a median age of 60 years (range 41-78) at the start of treatment. The clinical response of the patients was assessed using RECIST criteria \[16\]. Thirteen out of the 25 SO/ER treated patients had developed cavitations in the primary tumor at week 6 of evaluation. For these patients responses incorporating the cavitations were also correlated with the circulating cells and plasma parameters. Patients treated with SO/ER had a median TTP of 6 months (range 0.82 – 12.7) and an OS of 12.4 months (range 2.3 – 16.0). Patients treated with BV/ER had a median TTP of 6.8 months (range 3.2 - 10.3) and an OS of 6.9 months (range 5.4 - 8.3). Further patient and tumor characteristics are summarized in Table 1.
Table 1. Patient Characteristics and Response to Treatment at 6 Weeks

<table>
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<tr>
<th>Characteristic</th>
<th>SO+ER N (%)</th>
<th>BV+ER N (%)</th>
<th>ER N (%)</th>
</tr>
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<tbody>
<tr>
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<td>25</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
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<tr>
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<tr>
<td>Female</td>
<td>11 (56)</td>
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<td>4 (22)</td>
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<td>2 (20)</td>
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<td>Response at 6 weeks corrected for cavitations</td>
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<td>6.8 (3.2-10.3)</td>
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<td>Median survival, months (95% CI)</td>
<td>12.5 (11.7-13.2)</td>
<td>6.9 (5.4-8.3)</td>
<td>6.0 (0-12.8)</td>
</tr>
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</table>


1 One patient had no evaluation for treatment response because of early off study due to treatment related toxicity.
2 One patient had no evaluation for treatment response because of early death unrelated to treatment and disease.
DEFINITIONS OF HPCs AND CECs POPULATIONS
The FACS analysis of a blood sample of a representative patient from the study is shown in Figure 1. As described previously for renal cell cancer patients [19], two distinct CD34+ cell populations, which differ in the expression of the leukocyte marker CD45 and progenitor marker CD133, were identified in the NSCLC patients. HPCs are the CD45^{dim}/CD34^{bright} cells which are largely CD133^{pos}. The cutoff value for CD133 positivity in HPCs was determined based on isotype control and was adjusted for every individual sample. CECs are CD34^{bright}, which are almost uniformly positive for VEGFR2, but lack both markers CD45 and CD133 as previously defined [18, 19] (Figure 1).

MARKED DECREASE OF HPCs DURING TREATMENT WITH TK-INHIBITOR SORAFENIB
The total white blood cell count (WBC) showed little change during treatment with SO/ER, BV/ER or monotherapy erlotinib (Figure 2A). Although no leucopenia was observed during any of the treatment regimens we found a marked decrease of HPCs (Figure 2B) after seven days of treatment with SO/ER (median from 1345 at D0 to 511 cells/mL on D7, \( P < 0.001 \)) which decreased further after 3 weeks of treatment (to median of 356 cells/mL, \( P < 0.0001 \)). The subpopulation of CD133^{+}/HPCs also showed a similar decrease (from 1086 cells/mL at D0 to 361 on D7, \( P < 0.0001 \)) to 264 on D21 (\( P < 0.0001 \)). In the patient groups, treated with either BV/ER or monotherapy erlotinib, the HPC numbers did not change significantly (Figure 2B).

ANTI-ANGIOGENIC TREATMENT -SPECIFIC INCREASE OF CECs
Patients treated with SO/ER showed an increase in the number of CECs which was also found in patients treated with BV/ER but not with monotherapy erlotinib (Figure 2C). SO/ER treated patients showed a 3-fold increase in CECs on D7 (median, from 41 to 124 cells/mL, \( P = 0.001 \)) and BV/ER treated patients more than a 2-fold increase in CECs (median, from 59 to 132 cells/mL, \( P = 0.002 \)). CECs during monotherapy erlotinib did not change after 7 days of treatment (median, from 48 to 51 cells/mL, \( P = 0.8 \)). The VEGFR2 positive fraction of CECs enumerated in SO/ER treated patients followed the same trend as total CECs (Figure 2D). In both patient groups treated with either a TKI or anti-VEGF Moab angiogenesis inhibitor CECs further increased after 3 weeks of treatment in contrast to patients treated with monotherapy erlotinib (Figure 3).

RESPONSE AND BIOMARKERS
In order to analyse possible relations of biomarker values with response we used RECIST criteria and RECIST criteria taking into account tumor cavitations as recently suggested by Crabb et al. [17]. CD133^{+}/HPCs were not significantly correlated with the response when RECIST was not adjusted for tumor cavitations. When the response was corrected
Figure 1 - Full blood analysis of circulating endothelial cells (CECs) and hematopoietic progenitor cells (HPCs) in a non-small cell lung cancer (NSCLC) patient.

A, detection of total CECs and HPCs based on marker expression of CD45 and CD34. CECs by CD45\(^{\text{neg}}\)/CD34\(^{\text{bright}}\) and HPCs defined by CD45\(^{\text{dim}}\)/CD34\(^{\text{bright}}\). B, expression of VEGFR2 and CD133 on CECs and HPCs as indicated by the boxes.

For cavitations, pre-treatment levels of CD133\(^+\)/HPCs but not the total HPCs, were significantly lower in responding (PR) patients compared to non-responding (SD+PD) patients treated with SO/ER (\(P = 0.01\) and \(P = 0.28\) respectively, Figure 4A). Nine out of 12 patients with lower than the median of 1086 CD133\(^+\)/HPCs/mL and 4 out 12 with higher than the median of CD133\(^+\)/HPCs showed a response (PR). Patients with low CD133\(^+\)/HPCs (lower than the median of 1086 cells/mL) had a significantly longer TTP than those with CD133\(^+\)/HPCs higher than the median pre-treatment levels which was again not seen for the total population of HPCs (\(P < 0.05\) and \(P = 0.334\) respectively, Figure 4B). TTP was calculated from RECIST not corrected for tumor cavitations as this is currently not validated. At the time of analyses CD133\(^+\)/HPCs did not correlate with OS. Pre-treatment CECs or changes in CECs after 7 or 21 days in patients treated with SO/ER did not correlate with TTP, OS or response (according RECIST) with or without adjustments for cavitations. The latter was comparable for patients treated with BV/ER.
Figure 2 - Blood cell populations during anti-angiogenic treatment.

A, the total white blood cell (WBC) levels show little change during treatment with sorafenib plus erlotinib (SO/ER) and bevacizumab plus erlotinib (BV/ER). B, whereas BV/ER and ER treatment do not show a decrease in viable hematopoietic progenitor cell (HPC) numbers, treatment with tyrosine kinase inhibitor (TKI) SO/ER shows a significant marked depression of the viable HPCs after D7 ($P < 0.001$) and D21 ($P < 0.001$). C, tyrosine kinase inhibitor (TKI) SO and monoclonal antibody (Mab) BV combined with ER lead to an increase in viable circulating endothelial cells (CECs) after D7 ($P < 0.01$ and $P < 0.01$ respectively) and further increased after D21 ($P < 0.001$ and $P < 0.01$ respectively), which was not observed with monotherapy ER. D, the viable CD133$^+$/HPCs and VEGFR2$^+$/CECs subpopulations measured in SO/ER patients showed a respective decrease and increase in parallel to the total population of HPCs and CECs. * $P < 0.05$, ** $P < 0.001$. 
PLASMA VEGF AND EPO LEVELS IN NSCLC PATIENTS

Patients treated with SO/ER showed a slight non-significant increase in plasma VEGF during 21 days of treatment (Figure 5A), which was similar in patients treated with monotherapy erlotinib (data not shown). BV/ER treated patients showed a significant decrease in plasma VEGF levels after 7 days (median, 134 pg/mL to 25 pg/mL, \( P = 0.002 \), data not shown), probably because the used ELISA-kit measures only free VEGF [20]. No correlation was found between pre-treatment plasma VEGF levels and treatment outcome. Moreover, we found no significant relationship between the (VEGFR2\(^+\)) CECs, (CD133\(^+\)) HPCs and pre-treatment plasma VEGF in any patient group (data not shown).

To investigate if plasma EPO levels can serve as a surrogate pharmacodynamic biomarker or may have a possible relation with CECs and HPCs the plasma EPO levels were determined in the SO/ER treated patients. Minor changes in plasma EPO were observed (median, from 6.5 on D0 to 6.6 on D7 to 7.8 mIU/mL on D21, Figure 5B) which were all in the normal range of EPO levels (3.1-14.9 mIU/mL). Pre-treatment plasma EPO levels showed no correlation with circulating cell numbers or patient response (data not shown).

**Figure 3 - Specificity of increase in circulating endothelial cells (CECs).**

Anti-angiogenic treatment with direct VEGFR tyrosine kinase inhibitor (TKI) sorafenib as well as indirect vascular endothelial growth factor (VEGF) withdrawal by monoclonal antibody (Mab) bevacizumab in combination with anti-epidermal growth factor receptor (EGFR) inhibitor erlotinib showed an increase in CECs after 7 days and 21 days of treatment. No change in CEC numbers was observed in control patients treated with TK inhibitor erlotinib. * \( P < 0.01 \), ** \( P < 0.001 \).

**DISCUSSION**

We monitored, to our knowledge for the first time, VEGFR2-positive circulating endothelial cells and CD133-positive hematopoietic progenitor cells in NSCLC patients treated with the anti-angiogenic agent SO combined with ER. The second objective of the study was to provide data on the specificity of the measured changes in CECs and HPCs
Figure 4 - Boxplots and Kaplan-Meier curves, according to peripheral blood circulating CD133+/ hematoopoietic progenitor cells (HPCs) in sorafenib plus erlotinib (SO/ER)-treated non-small cell lung cancer (NSCLC) patients.

A, mean pre-treatment numbers of CD133+/HPC of non-responders (n=11) and responders (n=13). Responses are according RECIST incorporated cavitations. * = P< 0.05. B, time to progression (TTP) according to Response Evaluation Criteria in Solid Tumors (RECIST) correlated with pre-treatment CD133+/HPCs and the total population of HPCs with a median cutoff value of 1086 cells/mL and 1345 cells/mL respectively. ‘Low’ indicates (CD133+)/ HPCs at baseline below the median, and ‘high’ indicates (CD133+)/ HPCs at baseline higher than the median.

for SO/ER treatment. For this purpose data from two smaller groups of patients were obtained. The most important findings we report here are: 1) VEGFR2+ CECs increase in an anti-VEGF treatment specific manner, with a similar 2- to 3-fold increase (on D7 or D21) after treatment with the VEGFR –TKI sorafenib or anti-VEGF antibody bevacizumab, both combined with the EGFR-TKI erlotinib, but not with erlotinib monotherapy (see Figure 2C). 2) CD133+/HPCs markedly decreased with >60% on D7 and about 75% on D21 of treatment with SO/ER. This effect is not seen during treatment with BV/ER or
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Figure 5 - Plasma vascular endothelial growth factor (VEGF) and erythropoietin (EPO) levels in non-small cell lung cancer (NSCLC) patients

A, free plasma VEGF levels (pg/mL) did not change significantly during SO/ER (n= 25). * P < 0.01.

B, plasma EPO levels (mIU/mL) in NSCLC patients showed a slight increase during sorafenib treatment. (D21, * P < 0.01).

monotherapy erlotinib with whole brain radiotherapy. 3) The pre-treatment CD133+/HPC numbers predicted for response incorporating tumor cavitations to SO/ER therapy. In addition, TTP defined by RECIST was significantly longer in the patient group with a lower than median number of CD133+ HPCs.

CECs are a very rare cell population, normally defined as mature endothelial cells shed from the vasculature and are considered a useful biomarker of vascular damage [21-24]. In addition, the possibility to monitor circulating endothelial progenitor cells (CEPs) has been proposed as an attractive biomarker for anti-angiogenic agents after the seminal identification of circulating progenitors which could grow out to highly proliferative endothelial outgrowth cells (EOCs) [11, 25]. While originally CEPs were discriminated from mature CECs mainly by the presence or absence of CD133 progenitor markers, more recent data have cast serious doubts on this premise [26, 27].

The population of CECs that we have monitored has a phenotype consistent with a population containing the precursor for endothelial outgrowth cells, namely CD34+, VEGFR2+, but CD45− and CD133− (see Figure 1) according to Case et al [28] and Timmermans et al [28, 29]. However, whether CECs are a mixed mature/progenitor population will only be determined once these very rare cells can be sorted after a unique specific marker of the endothelial progenitor cell will have been identified [26].

In the current study, the CEC population did not predict for response to SO/ER or BV/ER therapy. Regarding the explanations for the lack of correlation between pre-treatment
values or increases in CECs and response one can only speculate. Our finding of an increase in CECs in SO/ER or BV/ER, but not monotherapy erlotinib treated patients is consistent with our earlier finding of a similar increase in renal cell cancer patients treated with the VEGFR TKI sunitinib [19]. Therefore the increase of this population of cells is likely a pharmacodynamic marker for VEGF/VEGFR signaling inhibitor therapy. Preclinical and early clinical data (phase I and phase II trials) have supported combined inhibition of VEGFR and EGFR pathways in NSCLC [30]. Activating EGFR mutations are predictive of response to EGFR inhibitors with reported response rates of up to 75%. In the specific group studied here 6 of 8 (75%) of patients with an activating EGFR mutation showed a response (PR). Of the WT EGFR group 6 out of 13 (46%) showed a response (PR). This is higher than rates reported for erlotinib monotherapy and implies a significant contribution of sorafenib on the clinical outcome with this combination treatment. The currently identified CD133+ progenitor cell population might be of help in future evaluation of this type of combination therapy. Unlike CECs, the total HPC population showed a marked decrease during SO/ER treatment in contrast to patients treated with BV/ER or monotherapy erlotinib with WBRT. Such a decrease of the HPCs also occurs in RCC patients treated with sunitinib [19] and might be related to the Flt-3 inhibitory effects of both TKI sorafenib and sunitinib since Flt3-signaling is required for HPC proliferation and mobilization into the circulation [31-33]. In this study the pre-treatment level of the CD133+/HPCs was significantly higher in the non-responders to SO/ER therapy, and the group with higher than median CD133+/HPC values showed a shorter TTP. Also, the decrease in CD133+/HPCs on D7 was much smaller in patients with longer TTP (data not shown), which may be a reflection of the lower HPC mobilization from the bone-marrow into the circulation in those patients. While the inhibition of circulating HPC numbers is very likely a pharmacological effect of sorafenib, the correlation with the antitumor effect (of the sorafenib plus erlotinib combination) may or may not be indicative of a role of this population in tumor growth. In contrast to hypothesized effects of populations of circulating endothelial progenitors, a pro-angiogenic role of HPCs as well as CD133+ subpopulations of HPCs recruited from the bone-marrow to hypoxic or tumor tissues [34, 35] is consistently reported. Thus there is wide agreement on a positive role of CD133+/CD34bright/CD45dim HPCs in (tumor) adult neovascularization. The ongoing debate is on the evidence for the existence in the adult of bone-marrow derived progenitors for potent long-term endothelial layer forming cells in addition to the progenitors for cell populations that promote tumor neovascularization indirectly by secreting growth factors. However, present studies point to a limited incorporation of bone marrow-derived cells into the endothelium of tumors [36]. Many recent studies and reviews discuss the properties of pro-angiogenic cell populations or “circulating angiogenic cells” in-depth
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[26-29, 37, 38]. A specific role of CD133+/HPCs has been suggested in pulmonary vascular remodeling in patients with obstructive pulmonary disease [39] or idiopathic pulmonary arterial hypertension [40]. Thus, it seems plausible that the production and release of cytokines, chemokines and proteolytic enzymes (VEGF, SDF-1, metalloproteases) by the lung tumor tissue will increase the mobilization of CD133+/HPCs from the bone-marrow, which may home to hypoxic tumor tissue and play a fundamental role in tumor neovascularization and progression [35, 41]. In this scenario part of the anti-tumor effect of sorafenib, but also sunitinib, may be related to the suppression of mobilization of (CD133+) HPCs from the bone-marrow. Other studies also suggest that high numbers of circulating CD133+/HPCs are related to a worse outcome in cancer patients. CD133 mRNA from peripheral blood cells, which is predominantly derived from the HPCs was shown to be an independent prognostic factor for patient survival [42].

A very recent study showed that higher pre-treatment levels of CD133+/CD34+/CD45dim cells (called circulating progenitor cells in that study) were associated with worse outcome of sunitinib therapy in hepatocellular carcinoma [43]. Altogether these results fit with the concept of the pro-angiogenic capacity of HPCs in tumor neovascularization and might indicate a causative role of the suppression of these cells by the anti-angiogenic TKIs sorafenib and/or sunitinib to their anti-tumor effects and patient responses. Since this cell population is well defined it is also amenable to analysis at the RNA level in peripheral blood mononuclear cells.

In addition, plasma levels of VEGF and EPO (representing a larger repertoire of cytokines/growth factors) are known to be increased in mice or patients upon treatment with anti-VEGF therapy [19, 44] and have been early on considered candidate biomarkers for effectivity of this type of therapy. In this study, VEGF and EPO, were not predictive of response or TTP to SO/ER combination therapy. Dowlati et al found that VEGF was predictive for response in NSCLC patients treated with bevacizumab, but no relation was found with survival [45].

An important difficulty in defining or identifying biomarkers is the evaluation of the endpoint of the actual responses of the patients. RECIST is the most widely used method for assessing the responses of patients [16], but has been critically updated by Karrison et al [46] and Verweij et al [47]. In addition Crabb et al [17] suggest that response assessment might be improved in NSCLC patients, treated with angiogenesis inhibitors, by incorporating cavitation into volume assessment for target lesions potentially altering treatment outcomes. In that study marked pulmonary cavitation occurred in 24% of patients treated with the angiogenesis inhibitor cediranib plus chemotherapy which was not seen with chemotherapy alone. Our sorafenib data suggest that correcting for cavitations may be of importance in evaluating potential biomarkers in relation to response. In SO/ER treated patients 13 out of 25 patients had cavitations due to the
treatment. Incorporating these cavitations in response assessment altered the RECIST responses. Pre-treatment numbers of CD133⁺/HPCs were only prognostic for the response if corrected for cavitations.

In our study, several cell populations and plasma markers were evaluated to serve as a potential biomarker during anti-angiogenesis treatment. This introduces the potential problem of multiple testing which increases the risk to find false-positive relations. Clearly, our study was designed to explore associations that should be confirmed in an independent group of patients.

In conclusion, CECs increased in NSCLC patients treated with SO/ER and BV/ER, but not with erlotinib monotherapy. Thus this effect can be ascribed to the anti-angiogenic agents. The CD133⁺/HPCs decreased significantly in all patients treated with SO/ER and pre-treatment numbers were significantly lower in responding patients and pre-treatment CD133⁺/HPC numbers correlated with the TTP. CD133⁺/HPCs may therefore be considered for further investigation as a biomarker for selecting patients likely to benefit from SO/ER.

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