British Journal of Cancer (2011) 104, 1144–1150

www.bjcancer.com

Levels of circulating $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells correlate with outcome in metastatic renal cell carcinoma patients treated with tyrosine kinase inhibitors

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BACKGROUND: Predicting the efficacy of antiangiogenic therapy would be of clinical value in patients (pts) with metastatic renal cell carcinoma (mRCC). We tested the hypothesis that circulating endothelial cell (CEC), bone marrow-derived $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell or plasma angiogenic factor levels are associated with clinical outcome in mRCC pts undergoing treatment with tyrosine kinase inhibitors (TKI).

METHODS: Fifty-five mRCC pts were prospectively monitored at baseline (day 1) and day 14 during treatment (46 pts received sunitinib and 9 pts received sorafenib). Circulating endothelial cells ($CD45^{-}CD31^{+}CD146^{+}$7-amino-actinomycin (7AAD)$^{-}$ cells) were measured in 1 ml whole blood using four-color flow cytometry (FCM). Circulating $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells were measured in progenitor-enriched fractions by four-color FCM. Plasma VEGF, sVEGFR2, SDF-1α and sVCAM-1 levels were determined by ELISA. Correlations between baseline CEC, $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells, plasma factors, as well as day 1–day 14 changes in CEC, $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor, plasma factor levels, and response to TKI, progression-free survival (PFS) and overall survival (OS) were examined.

RESULTS: No significant correlation between markers and response to TKI was observed. No association between baseline CEC, plasma VEGF, sVEGFR-2, SDF-1α, sVCAM-1 levels with PFS and OS was observed. However, baseline $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cell levels were associated with PFS ($P=0.01$) and OS ($P=0.006$). Changes in this population and in SDF-1α levels between day 1 and day 14 were associated with PFS ($P=0.03$, $P=0.002$). Changes in VEGF and SDF-1α levels were associated with OS ($P=0.02$, $P=0.007$).

CONCLUSION: Monitoring $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells, plasma VEGF and SDF-1α levels could be of clinical interest in TKI-treated mRCC pts to predict outcome.

British Journal of Cancer (2011) **104,** 1144–1150. doi:10.1038/bjc.2011.72 www.bjcancer.com
Published online 8 March 2011

Keywords: circulating endothelial progenitor cells; circulating endothelial cells; biomarker; angiogenesis; metastatic renal cell carcinoma; tyrosine kinase inhibitors

Metastatic renal cell carcinoma (mRCC) is generally resistant to chemotherapy and hormonal therapy and marginally sensitive to immunotherapy. Insights on the genetics and biology underlying RCC especially the role of the von Hippel-Lindau tumour-suppressor gene (*VHL*), have provided the rationale to target this pathway in VHL-deficient RCC and supported antiangiogenic strategies in this disease (Rini and Small, 2005). Sunitinib is a multitargeted tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor A receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), stem cell factor receptor (KIT), glial cell-line-derived neurotrophic factor receptor (rearranged during transfection), FMS-like tyrosine kinase-3 (FLT3) and the receptor for macrophage colony-stimulating factor (CSF-1R) (Mendel *et al*, 2003). Sorafenib is a potent inhibitor of Raf-1, a member of the RAF/MEK/ERK signalling pathway, and of TKI receptors including VEGFRs, PDGFRs, FLT3 and KIT (Wilhelm *et al*, 2004). Both antiangiogenic drugs have shown clinical activity in metastatic clear-cell RCC and have been approved worldwide for the treatment of metastatic RCC (Escudier *et al*, 2007; Motzer *et al*, 2007).

Tumour vascularisation is dependent on the sprouting of blood vessels with migration of endothelial cells and the recruitment of mobilised bone marrow-derived (BMD) cells (Asahara *et al*, 1999). Circulating endothelial cells (CECs) are mature cells detached from vessel walls and high levels are observed in clinical diseases

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Received 12 October 2010; revised 13 January 2011; accepted 7 February 2011; published online 8 March 2011

hallmarked by vascular insult including cancer (Blann *et al*, 2005; Goon *et al*, 2006). Bone marrow-derived cells include several categories of hematopoietic and vascular progenitors recruited to sites of tumour neovascularisation in both cancer-bearing animals (Lyden *et al*, 2001) and humans (Peters *et al*, 2005). One subset, circulating endothelial progenitor cells (referred to as CEP), was reported to incorporate into tumour neovessels (Lyden *et al*, 2001; Spring *et al*, 2005; Nolan *et al*, 2007) and pre-clinical studies have identified a critical role for these cells in promoting the angiogenic switch and metastatic progression (Gao *et al*, 2008). Although the precise role of CEP as well as their phenotypic and functional definition is still debated (Purhonen *et al*, 2008; Yoder and Ingram, 2009), numerous studies have highlighted the importance of interactions between hematopoietic ($VEGFR1^{+}$) and endothelial ($VEGFR2^{+}$) BMD progenitor subsets in disease progression (Lyden *et al*, 2001; Purhonen *et al*, 2008; Yoder and Ingram, 2009), and vascularisation of metastatic lesions (Kaplan *et al*, 2005).

In clinical practice, the use of these vascular-targeted therapies is challenged by the absence of validated biomarkers allowing to predict response to treatment or toxic effects, select optimal dosage, or elucidate potential mechanisms of action and resistance. Among candidate biomarkers of antiangiogenic drugs, circulating BMD progenitors (including CEP) and CEC measurements have raised considerable interest (Jubb *et al*, 2006). However, because of technical difficulties for their measurement, reliable data on their actual prognostic or predictive value in cancer patients (pts) undergoing antiangiogenic treatment are lacking (Strijbos *et al*, 2008). Also, despite the fact that circulating levels of angiogenic growth factors generally indicate poor prognosis, their significance in terms of predicting antiangiogenic drug efficacy and clinical benefit is unclear.

In this study, we investigated whether the levels of CEC, of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell subset as well as of several proangiogenic/endothelial plasmatic factors (VEGF, sVEGFR-2, SDF-1α, sVCAM-1), measured before and after 2 weeks of therapy, are associated with clinical outcome in mRCC pts undergoing TKI therapy.

PATIENTS AND METHODS

Patients and blood sample collection

All pts had mRCC and were treated at the Institut Gustave Roussy, France, from October 2006 to January 2009. Patients received either sunitinib or sorafenib as antiangiogenic treatment. Before treatment, all pts had a detailed history, physical examination and baseline laboratory parameters. Pretreatment baseline tumour status was evaluated with CT scans of the brain, chest, abdomen and pelvis. Data collected included standard demographics and disease characteristics, first date of treatment, best response to treatment and date of progression, date of death or last follow-up. Tumour evaluation was performed after 12 weeks of sunitinib or after 2–3 months of sorafenib. Responses were documented according to Response Evaluation Criteria in Solid Tumours (RECIST). Patients were followed by their physician (BE) every 4–6 weeks. Informed consent was obtained for all pts. This prospective study was approved by our institutional review board.

Peripheral blood sampling was performed at baseline day 1 (before treatment initiation) and at day 14 of antiangiogenic therapy: 2 ml of whole blood was collected in Cellsave Preservative tubes (Immunicon, Huntingdon Valley, PA, USA) for CEC analysis, and 10 ml whole blood was collected in standard heparin tubes for $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cell and plasmatic protein analysis.

Measurement of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells

$CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells were measured in 10 ml of progenitor-enriched whole blood according to a four-color FCM assay previously reported (Farace *et al*, 2007; Taylor *et al*, 2009). Ficoll-gradient mononuclear cells were enriched in progenitor cells using the RosetteSep antibody cocktail (StemCell Technologies Inc., Vancouver, Canada). Progenitor-enriched mononuclear cells were distributed into control and test tubes and treated with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Staining was performed with monoclonal antibodies CD45-FITC (clone T29/33, DakoCytomation, Glostrup, Denmark), CD34-APC (clone BIRMA-K3, Dako Cytomation), KDR-PE (clone 89106, R&D Systems, Minneapolis, MN, USA) and 7AAD (BD Biosciences, San Jose, CA, USA). Control tubes included a control PE tube (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) performed to measure accurately background noise and to adjust the gates precisely. Cells were acquired on a FACSCalibur (BD Biosciences). Data were analysed using CELLQuest 3.2 software (BD Biosciences). Results were expressed as the percentage of $VEGFR2^{+}$ cells among circulating $CD34^{+}$ progenitor ($CD45^{dim}CD34^{+}7AAD^{-}$ and $CD45^{-}CD34^{+}7AAD^{-}$) cells. The multigating strategy used to identify circulating $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells by four-color FCM is shown in Supplementary Figure S1.

Measurement of CECs

Circulating endothelial cells were measured in 1 ml whole blood by four-color FCM according to a method we previously established (Jacques *et al*, 2008). Immunofluorescent staining was performed with monoclonal antibodies CD31 FITC (clone WM59, BD Pharmingen, San Diego, CA, USA), CD146 PE (clone P1H12, BD Pharmingen), CD45 APC (clone T29/33, DakoCytomation). An IgG-PE control was performed in 0.5 ml of whole blood (CD45-APC/CD31-FITC/mouse IgG1-PE/7AAD) to measure background noise and to adjust the gates precisely. All of the cells contained in the IgG-PE control tube and in the CEC test tube were acquired, representing approximately 2.5×10^6 events and 5×10^6 events, respectively. Data were analysed using CELLQuest 3.2 software. Using this method, we found that median CEC levels were 6.5 ml^{-1} (0–15 ml^{-1}) in healthy adults ($n = 20$) and 16.0 ml^{-1} (0–179 ml^{-1}) in pts with metastatic carcinoma ($n = 125$) ($P < 0.001$) (Jacques *et al*, 2008).

Plasmatic factors

Plasma levels of VEGF, sVEGFR-2, sVCAM-1 and SDF-1α were determined using commercial ELISA kits (R&D Systems). Plasma samples were assayed in duplicates. Optical density values were considered significant if found to be at least twice as high as background noise.

Statistical analysis

Correlation between markers and clinical response to treatment (progressive *vs* non-progressive) were tested using the Wilcoxon–Mann–Whitney test. The Wilcoxon signed-rank test was used to test differences between marker levels at baseline and day 14. Overall survival (OS) was calculated from the start of treatment to the date of death or the last follow-up (censored data). Progression-free survival (PFS) was calculated from the start of treatment to the date of disease progression, death or the last follow-up (censored data).

Overall survival and PFS rates were estimated using the Kaplan–Meier method for survival curves. The relationships between survival and the different markers were tested using the log-rank test. The hazard ratios yielded by the Cox model were provided.

Values at baseline and day 14 were dichotomised according to the third quartile cut-off. As levels of $CD45^{dim}CD34^{+}VEGFR2^{+}$ cells in normal individuals and certain pts are very low (Taylor *et al*, 2009) and close to the detection limit of the method used, a

cut-off at a low or even at the median value might not have allowed to discriminate pts with the highest risk *vs* pts with a lowest risk because of an overlap between these two groups. We therefore decided to select a threshold at two-thirds of the values and to compare the third of the pts with the highest values with the two-thirds remaining with lower values.

Variations between baseline and day 14 were classified as increased, decreased or stable. All tests were two-sided and a *P*-value <0.05 was considered statistically significant. The statistical analysis was performed using SAS software (Release 9.1; SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics and baseline levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells and plasma proangiogenic factors

A total of 55 pts with mRCC were included in this study: 46 (84%) pts received sunitinib and 9 pts (16%) received sorafenib. Tumour histology (43 pts had clear cell renal carcinoma *vs* 12 with non-clear cell), clinical characteristics at baseline and response to treatment are presented in Table 1. A majority of pts received TKIs as first-line therapy (38 out of 55). No patient reached a complete response after treatment. The partial response rate to treatment was 19% (10 pts). Stable disease was achieved in 28 pts (53%) and progression was observed in 15 pts (28%). Two pts were not evaluable for response because of early cessation because of toxicity. Kaplan–Meier curves for PFS and OS for the 55 pts are presented in Supplementary Figure S2. Median PFS and median OS were 6 and 21 months, respectively.

Table 1 Description of patient characteristics, treatment and outcome ($n=55$)

	No.	%
Male	43	78
Age (years)		
Median (range)	58	(34–86)
ECOG performance status		
0	23	42
1	28	51
2	4	7
Histology		
Clear cell	43	78
Non-clear cell	12	22
Metastatic sites		
1	13	24
2	26	47
>3	16	29
Treatment		
Sunitinib	46	84
Nexavar	9	16
Best response		
PR	10	19
SD	28	53
PD	15	28
MSKCC category		
Low	17	31
Intermediate	36	65
Poor	2	4

Abbreviations: ECOG = Easter Cooperative Oncology Group; MSKCC = Memorial Sloan-Kettering Cancer Center; PD = progressive disease; PR = partial response; SD = stable disease.

Levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell, plasma VEGF, sVEGFR-2, SDF-1α and sVCAM-1 were monitored at baseline and at day 14 (Table 2). Circulating endothelial cells were identified as CD31^{+}CD146^{+}CD45^{-}7AAD^{-} viable events in whole blood by four-color FCM (Jacques *et al*, 2008). In the present cohort of mRCC pts, the median CEC level at baseline was 13 ml^{-1} (range 0–119 ml^{-1}) (Table 2). We analysed VEGFR-2 (KDR) expression in both CD45^{dim}CD34^{+}7AAD^{-} and CD45^{-}CD34^{+}7AAD^{-} progenitor cell subsets. At baseline, the CD45^{-}CD34^{+}VEGFR2^{+}7AAD^{-} subset represented $<0.005\%$ of circulating CD34^{+} progenitor cells. However, the median level of the CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} subset was of 0.5% of circulating CD34^{+} progenitor cells (range 0–24.3%). Median levels of plasma VEGF, sVEGFR-2, SDF-1α and sVCAM-1 at baseline were 151 pg ml^{-1} (range 0–1706 pg ml^{-1}), 9523 pg ml^{-1} (range 5410–17 680 pg ml^{-1}), 2726 pg ml^{-1} (range 1210–3948 pg ml^{-1}) and 673 ng ml^{-1} (range 279–1610 ng ml^{-1}), respectively (Table 2).

Changes in levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell and plasma proangiogenic factors under treatment

Absolute counts of CEC did not significantly change between day 1 and day 14 ($P=0.12$) (Table 2). Also, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels were not significantly modified between day 1 and day 14 (0.5 *vs* 1.7%, $P=0.08$). As expected, TKI treatment induced an increase in plasma VEGF levels (median values: 151 *vs* 273 pg ml^{-1}, $P<0.0001$), which was associated with a concomitant decrease in plasma sVEGFR2 levels (9523 *vs* 6229 pg ml^{-1}, $P<0.0001$). Both SDF-1α and sVCAM-1 plasma levels significantly increased at day 14 (2726 *vs* 2931 pg ml^{-1}, $P<0.0001$, 673 *vs* 720 ng ml^{-1}, $P=0.04$).

Association between levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells and plasma proangiogenic factors and clinical outcome

For all markers, values at baseline and absolute variations between day 14 and baseline values were compared between non-progressive (stable and partial responses, $n=38$) and progressive ($n=15$) pts. No significant association between CEC, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell, plasma VEGF, sVEGFR-2, SDF-1α, sVCAM-1 values and clinical response to TKI treatment was observed (Table 3).

No significant correlation was observed between baseline CEC, plasma VEGF, sVEGFR-2, SDF-1α, sVCAM-1 levels and PFS or OS (Table 3). However, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels at day 1 were associated with PFS and OS. Patients with a CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell level at baseline $>2\%$ had a higher risk of progression (HR = 2.5, $P=0.01$) (Figure 1A) and had poorer prognosis compared with those pts whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels at baseline were $\leqslant 2\%$ (HR = 3.3, $P=0.006$) (Figures 1B).

No significant association between day 1 and day 14 changes in CEC, sVEGFR-2, sVCAM-1 levels was observed. Patients whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels were stable ($<-2\%$ or $\leqslant +2\%$) and pts whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels increased ($\geqslant 2\%$) between baseline and day 14 had a lower risk of progression compared with pts whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels decreased over the same period ($<2\%$) (HR = 0.3 and 0.5, respectively, $P=0.03$) (Table 3). The variation in VEGF levels between day 1 and day 14 levels was correlated with OS: pts whose VEGF values increased more than 270 pg ml^{-1} between day 1 and day 14 had a poorer OS (HR = 4.9, $P=0.02$) (Figure 2). The

Table 2 Median levels of CEC, $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ cells and plasmatic factors at baseline and day 14

	Median levels at		
Markers	**Day 1**	**Day 14**	**Changes day 1–day 14 P^a**
CEC (ml^{-1})	13 ($n=55$) (0–119)[b]	17 ($n=51$) (0–157)	0.12
$CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ cells (%)	0.5 ($n=52$) (0–24.3)	1.7 ($n=48$) (0–28.4)	0.08
VEGF ($pg\,ml^{-1}$)	151 ($n=54$) (0–1706)	273 ($n=49$) (1–3765)	<0.0001
sVEGFR-2 ($pg\,ml^{-1}$)	9523 ($n=54$) (5410–17 680)	6229 ($n=49$) (2609–10 393)	<0.0001
SDF-1α ($pg\,ml^{-1}$)	2726 ($n=54$) (1210–3948)	2931 ($n=49$) (1400–4433)	<0.0001
sVCAM-1 ($ng\,ml^{-1}$)	673 ($n=53$) (279–1610)	720 ($n=48$) (325–1796)	0.04

Abbreviations: CEC = circulating endothelial cells; SDF-1α = stroma-derived factor -1α; sVCAM-1 = soluble vascular cell adhesion molecule-1; VEGF = vascular endothelial growth factor; VEGFR2 = vascular endothelial growth factor receptor 2. [a]Wilcoxon signed-rank test. [b]Range.

Table 3 Levels of significance of associations between markers and clinical outcome (response, PFS, OS)

	Clinical outcome		
Markers	**Response P^a**	**PFS P^a**	**OS P^a**
Day 1			
CEC (ml^{-1})	0.47	0.69	0.87
$CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ cells (%)	0.12	**0.01 (0.005)**	**0.006 (0.001)**
VEGF ($pg\,ml^{-1}$)	0.12	0.62	0.30
sVEGFR-2 ($pg\,ml^{-1}$)	0.16	0.45	0.48
SDF-1α ($pg\,ml^{-1}$)	0.15	0.08	0.47
sVCAM-1 ($ng\,ml^{-1}$)	0.26	0.35	0.10
Changes day 1–day 14			
CEC (ml^{-1})	0.27	0.53	0.75
$CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ cells (%)	0.83	**0.03 (0.05)**	0.07
VEGF ($pg\,ml^{-1}$)	0.75	0.48	**0.02 (0.02)**
sVEGFR-2 ($pg\,ml^{-1}$)	0.38	0.87	0.92
SDF-1α ($pg\,ml^{-1}$)	0.18	**0.002 (0.01)**	**0.007 (0.009)**
sVCAM-1 ($ng\,ml^{-1}$)	0.65	0.09	0.78

Abbreviations: CEC = circulating endothelial cells; OS = overall survival; PFS = progression-free survival; SDF-1α = stroma-derived factor -1α, sVCAM-1 = soluble vascular cell adhesion molecule-1; VEGF = vascular endothelial growth factor; VEGFR2 = vascular endothelial growth factor receptor 2. [a]Log rank test. Values in brackets present levels of significance of associations in the 43 patients with metastatic clear cell renal carcinoma.

variation in SDF-1α levels between day 1 and day 14 was correlated with both PFS and OS (Table 3). Patients whose SDF-1α values increased between 0 and 600 $pg\,ml^{-1}$ and pts whose SDF-1α values increased more 600 $pg\,ml^{-1}$ between day 1 and day 14 had a lower risk of progression (HR = 0.3 and 0.2, respectively, $P=0.002$) and a lower risk of death (HR = 0.3 and 0.6, respectively, $P=0.007$) compared with pts with decreased SDF-1α values (Figures 3A and B).

The analysis of associations between levels of CEC, $CD45^{dim}$ $CD34^{+}VEGFR2^{+}$ progenitor cells and plasma proangiogenic factors and clinical outcome was repeated in the 43 pts with metastatic clear cell carcinoma. As shown in Table 3, baseline $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cell levels were associated with PFS ($P=0.005$) and OS ($P=0.001$) in this specific histologic subtype. Similarly, changes in $CD45^{dim}CD34^{+}$ $VEGFR2^{+}7AAD^{-}$ progenitor cell levels and in SDF-1α levels between day 1 and day 14 remained associated with PFS ($P=0.05$, $P=0.01$). Changes in VEGF and SDF-1α levels were also associated with OS ($P=0.02$, $P=0.009$) in pts with metastatic clear cell carcinoma. Given the preponderance of sunitinib-treated pts, the analysis was finally repeated in pts with this specific subtype receiving this single treatment modality (i.e., sunitinib). All of the above associations remained significant in this small cohort of 34 pts (data not shown).

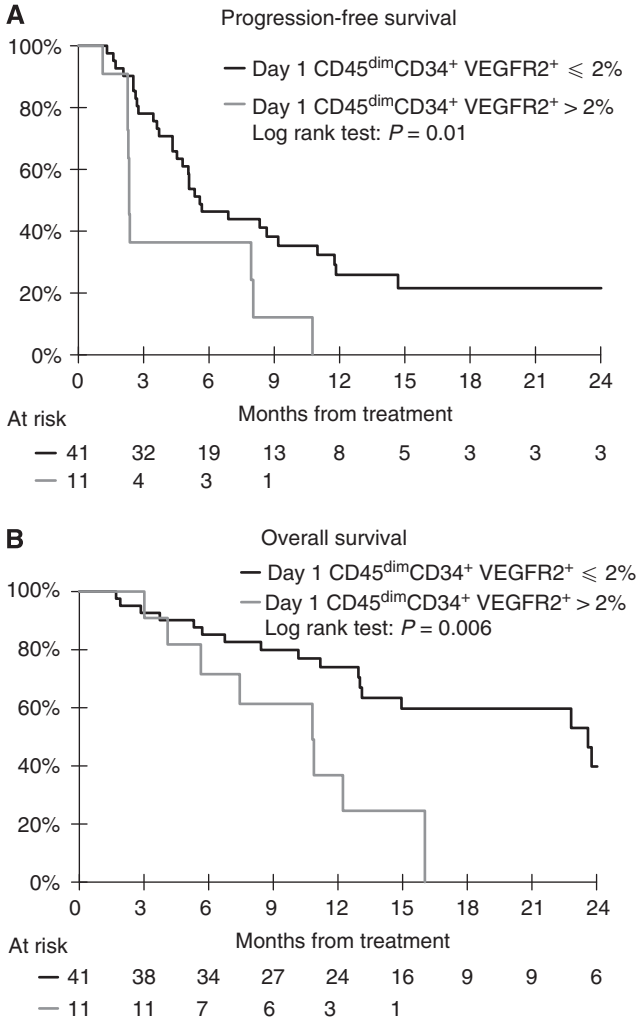

Figure 1 Progression-free survival and OS according to day 1 $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels. (**A**) Progression-free survival according to day 1 $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels. (**B**) Overall survival according to day 1 $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels.

DISCUSSION

Drugs targeting the VEGF pathway have made a major impact in the treatment of many types of cancer. Currently, multitargeted TKI, such as sunitinib or sorafenib, are considered as the standard of care for therapy in pts with mRCC. However, these agents clearly demonstrate therapeutic heterogeneity in terms of both efficacy and toxicity (Escudier *et al*, 2007; Motzer *et al*, 2007).

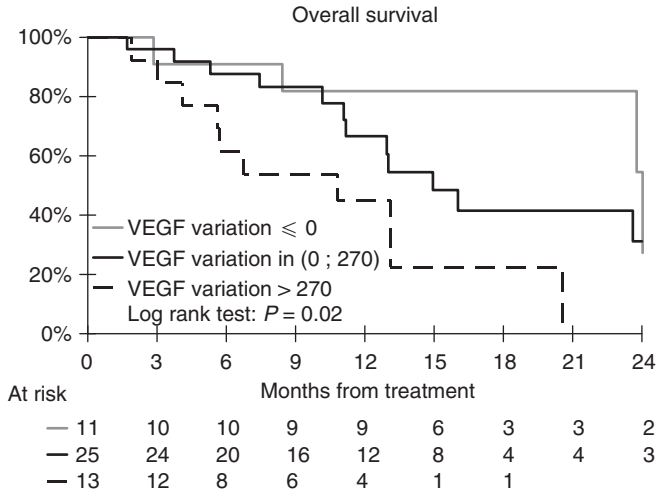

Figure 2 Overall survival according to changes in day 1–day 14 VEGF levels.

Thus, any biomarker that can predict clinical benefit would be of great value. To date, none of the expected biological markers, such as *VHL* status or VEGF plasma levels, has predicted response to targeted therapies in mRCC. In the present exploratory study, we reported the potential interest of a BMD progenitor cell subset, identified by the CD45^{dim}CD34^{+}VEGFR2^{+} phenotype in a cohort of 55 mRCC pts treated with multitargeted TKI. Interestingly, we observed a correlation between pretreatment CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell levels phenotype and both PFS and OS. Early (i.e., within the two first week of treatment) changes in this progenitor cell subset and in plasma VEGF and SDF-1α levels were also associated with PFS or OS.

Increased numbers of CEC are considered as a useful marker of vascular integrity in pts with vascular disorders (Blann *et al*, 2005) although their role in tumour neoangiogenesis is less clear. The rarity of CEC and the controversy concerning the reliability of their measurement using flow cytometry have yielded conflicting as well as limited data in cancer pts (Strijbos *et al*, 2008). In this study, no association between CEC levels and outcome was observed. Whether CEC recruitment is an intrinsic characteristic of some tumour types or a marker of drug class effect or associations, or whether our method lacks sensitivity to discriminate different patient outcomes, remains an open question.

Circulating endothelial progenitor can home to sites of neovascularisation and differentiate into endothelial cells, a process called postnatal vasculogenesis that was widely proposed as a mechanism for vascular repair, and tumour metastasis, neoangiogenesis and growth promotion (Lyden *et al*, 2001; Spring *et al*, 2005; Nolan *et al*, 2007; Gao *et al*, 2008). Initial (Lyden *et al*, 2001) and subsequent studies have indeed identified CEP incorporation into the endothelial layer of tumour neovessels (Spring *et al*, 2005; Nolan *et al*, 2007), and their critical role in promoting progression of micro- to macro-metastases (Gao *et al*, 2008). Other historical data have refuted such a contribution (Purhonen *et al*, 2008; Yoder and Ingram, 2009). Despite this debate, all of these studies agree on the existence of a robust recruitment of both endothelial and hematopoietic BMD progenitor cells into the neoangiogenic perivasculature, thus supporting the important role these cells may play in the microenvironmental molecular and cellular events necessary for tumour invasion and metastasis. In humans, the phenotypical and functional characterisation of CEP has been hampered by the extreme rarity of these cells, the lack of consensus on surface marker phenotype, the important phenotypical overlap with hematopoietic progenitors as well as the absence of standard *in vitro* or *in vivo* assays for

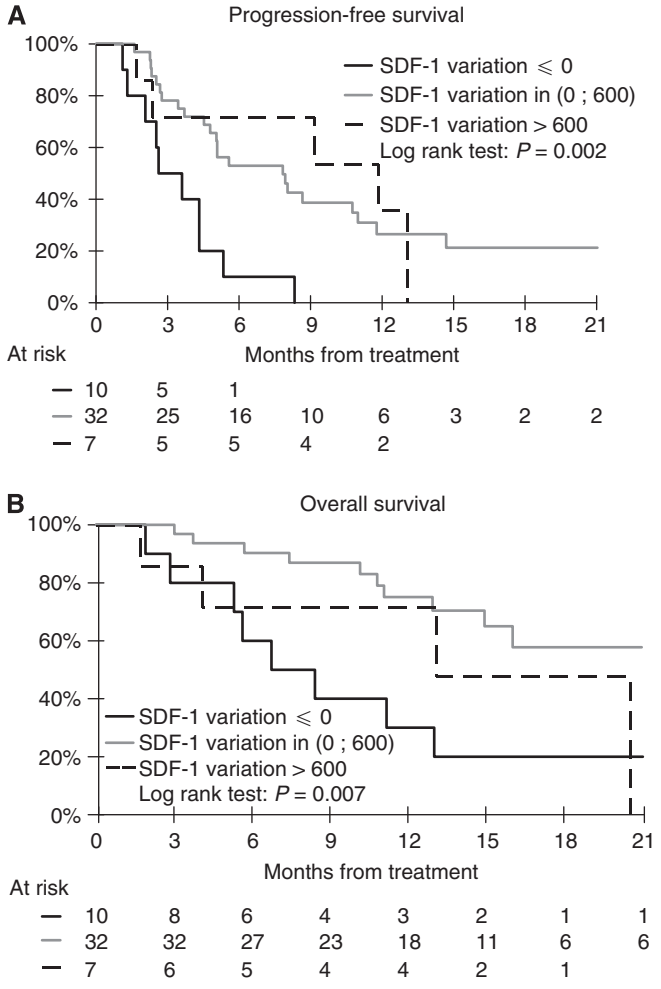

Figure 3 Progression-free survival and OS according to changes in day 1–day 14 SDF-1α levels. (**A**) Progression-free survival according to changes in day 1–day 14 SDF-1α levels. (**B**) Overall survival according to changes in day 1–day 14 SDF-1α.

functional characterisation (Yoder and Ingram, 2009). In light of this, we preferred to refer to the specific sub-population investigated herein as CD45^{dim}CD34^{+}VEGFR2^{+} cells (rather than to CEP). We used a rigorous four-color FCM assay to detect the circulating CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} cell subset in 10 ml of progenitor-enriched whole blood, which characteristics included (i) sampling of an important volume of blood, (ii) a pre-enrichment step, (iii) use of a viability marker (7AAD) and (iv) a multiple gating strategy (Farace *et al*, 2007; Taylor *et al*, 2009). In contrast to a recent report in pts with non-small cell lung cancer, we did not detect any sub-population with a CD45^{-}CD34^{+}VEGFR2^{+}CD133^{-} phenotype harbouring size and structural characteristics of viable cells in our FCM analyses (Vroling *et al*, 2010). We observed that a high level of CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells (>2%) was the single baseline marker associated with poor PFS ($P=0.01$) and OS ($P=0.006$) thus suggesting that baseline CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell levels could allow to discriminate mRCC with poor outcome. Furthermore, we observed that pts with stable or increased levels of CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells between baseline and day 14 had a lower risk of tumour progression ($P=0.03$). However, the association between day 1 and day 14 changes in CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell levels and OS did not reach statistical significance ($P=0.07$). The present exploratory study was conducted in a small cohort of 55 pts with mRCC and the

absence of a control group (i.e., non-TKI-treated pts) did not allow to determine whether $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels were prognostic or predictive. Furthermore, several cell subpopulations and plasma markers were evaluated, thus introducing potential biases of multiple testing. When the analysis was repeated in the 43 pts with metastatic clear cell carcinoma and in the 34 pts with this single histologic subtype receiving a single modality of treatment (i.e., sunitinib), these associations between levels of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells and clinical outcome remains significant. Biomarkers for RCC would be best developed in specific histologic subtypes, that is: clear cell RCC or non-clear cell RCC, especially because the biology of these tumour types is different and may differently influence the tumour microenvironment and have different biomarkers relevant to their predictive and prognostic value. Future studies in a cohort of mRCC pts with a single tumour histology (i.e., clear cell carcinoma) receiving a single antiangiogenic treatment (i.e., sunitinib) are needed to confirm our results and to determine the eventual prognostic or predictive value of $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels in this tumour subtype.

Early and more recent studies, including the pivotal phase III trial of sorafenib in advanced mRCC (Jacobsen *et al*, 2000; Escudier *et al*, 2009), have indicated that baseline VEGF levels correlate with disease activity and prognosis. However, other studies have produced inconclusive results. It currently remains uncertain whether VEGF status alone is an adequate predictive marker for efficacy of VEGF-targeted therapy in mRCC. Herein, baseline VEGF and other factor levels did not correlate with PFS or OS. Unlike a previous study reporting larger changes in VEGF levels in pts demonstrating objective tumour responses during sunitinib treatment (Deprimo *et al*, 2007), we observed that the magnitude of increase in VEGF levels under treatment was associated with OS ($P=0.02$). Surprisingly, a decrease in SDF-1α levels was also found to be strongly associated with both PFS ($P=0.002$) and OS ($P=0.007$). Plasma factor changes, that is, increased levels in circulating VEGF and concomitant decreased levels in sVEGFR2 have been reported as class effects of TKI in pts. Recently, the observation of increased levels of multiple proangiogenic factors in sunitinib-treated tumour-free mice has implied a systemic multi-organ endocrine response to VEGF and PDGF inhibition in normal tissues (Ebos *et al*, 2007). Elucidating underlying mechanisms with respect to the multiple targets directly or collaterally affected by such agents may prove difficult. VEGF and SDF-1α are critical regulators governing the mobilisation and recruitment of a heterogeneous population of BMD proangiogenic vascular and hematopoietic progenitor cells to tumours. Initial studies have shown that VEGF-targeted therapy may inhibit the mobilisation of CEP (Willett *et al*, 2004) and induce the increase in SDF-1α blood levels during tumour escape (Batchelor *et al*, 2007). Our results do not support these findings although the dynamics of these processes remain to be determined under continued therapy. Overall, our results suggest that inadvertent changes induced by TKI treatment, that is, upregulation of VEGF, decrease in SDF-1α, and subsequent changes in BMD $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels are associated with poor outcome in mRCC pts treated with TKI.

None of the six angiogenesis/endothelial markers evaluated correlated with tumour response as documented by RECIST. As angiogenesis/endothelial markers are mainly host-derived, their changes during antiangiogenic treatment are expected to reflect the angiogenic–antiangiogenic balance resulting from a complex interplay between the biological actors of angiogenesis and the therapeutic agent. These markers may not be as informative for tumour shrinkage as could be tumour-derived factors. Clinical results of VEGF-targeted therapy have shown little evidence of tumour shrinkage and have rather suggested a cytostatic effect, therefore tumour response criteria by RECIST may not be a good indicator of clinical benefit of antiangiogenic agents. An important issue in identifying biomarkers is the endpoint of the actual response in pts. Progression-free survival or OS may be more accurate clinical read-outs for host angiogenesis marker evaluation.

Escape from antiangiogenic therapy has been demonstrated in both pre-clinical and clinical settings (Bergers and Hanahan, 2008). Emerging data have implicated evasive resistance mechanisms where host adaptive responses circumvent the specific angiogenic blockade. Low oxygen tension, HIF-1α accumulation and subsequent SDF-1α and VEGF effectors were reported to promote angiogenesis and tumour growth via the recruitment of various proangiogenic BMD sub-populations (Bergers and Hanahan, 2008). Evidence supporting the link between therapy-induced hypoxia and BMD proangiogenic cell populations stemmed from a study demonstrating that vascular disrupting agents (VDA) induced vasculogenic 'rebounds' that homed to the vasculature of treated tumours thereby promoting tumour neovascularisation and subsequent re-growth (Shaked *et al*, 2006). In a previous clinical study, we observed the presence of $CD45^{dim}$ $CD34^{+}$ $VEGFR2^{+}$ progenitor cell mobilisation in a small series of cancer pts included in phase I trial combining a VDA (AVE8062, Sanofi-Aventis, Antony, France) with chemotherapy (Farace *et al*, 2007). Further studies are needed to determine whether subpopulations of BMD progenitors may be biomarkers of response or resistance to antiangiogenic therapies.

In conclusion, our study shows for the first time an association between baseline levels of a BMD $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell subset and outcome in mRCC pts treated with TKI. Also, we present novel data on therapy-induced changes of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell, VEGF and SDF-1α levels that were also associated with PFS or OS. Large prospective studies in a homogeneous cohort of mRCC pts are warranted to confirm our results as it will allow necessary multivariate analysis to assess the eventual prognostic or predictive value of these markers in mRCC pts treated by TKI.

ACKNOWLEDGEMENTS

The authors acknowledge Pfizer, France for supporting the study.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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