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Sunitinib-induced changes in circulating endothelial cell-related proteins in patients with metastatic renal cell cancer

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Vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors are effective agents in the treatment of metastatic renal cell cancer (mRCC). We here investigated whether inhibition of VEGFR signalin by sunitinib causes changes in plasma proteins associated with tumor endothelium. Forty-three patients with mRCC received sunitinib 50 mg/day in a 4-weeks on 2-weeks off schedule. Sequential plasma samples were obtained before treatment (C1D1), on C1D14, on C1D28, and on C2D1 before start of cycle 2. Plasma levels were assessed for VEGF, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular cell adhesion molecule-1 (sICAM-1), von Willebrand factor (vWF), circulating angiopoietin-2 (Ang-2) and soluble Tie-2 (sTie-2). Total tumor burden was calculated at baseline and at first evaluation. Progression-free survival (PFS) and overall survival (OS) were determined. Tumor burden was positively associated with baseline circulating Ang-2 [Spearman's rho (ρ) = 0.378, p = 0.028] and vWF (ρ = 0.417, p = 0.008). During sunitinib treatment, circulating Ang-2 and sTie-2 significantly decreased ($p < 0.001$ for both), plasma levels of sVCAM-1 and VEGF significantly increased (p = 0.022 and $p < 0.001$), whereas those of sICAM-1 and vWF remained stable. These protein changes had recovered on C2D1. The reduction in circulating Ang-2 levels on C1D28 was positively correlated with the percentage decrease in tumor burden (ρ = 0.605; p = 0.002). Baseline protein levels and subsequent changes were not associated with PFS or OS. In conclusion, sunitinib-induced changes in Ang-2, sTie-2, sVCAM-1 and VEGF are related to the administration schedule, while reduction in Ang-2 is also associated with decrease in tumor burden.

The development of antiangiogenic agents has significantly improved the perspectives of patients with metastatic renal cell cancer (mRCC), known as a disease resistant against standard chemotherapy.[1] Currently, sunitinib is most widely prescribed for first-line treatment of mRCC.[2] Sunitinib is an oral tyrosine kinase inhibitor (TKI) which targets several receptors including vascular endothelial growth factor receptor (VEGFR)-1, -2 and -3, platelet-derived growth factor receptors-α and -β, c-KIT and FLT3.[3] Among these receptors, VEGFR on tumor associated-endothelium is considered to be a relevant target for sunitinib in RCC.[4] In the majority of RCC tumors, high levels of VEGF are being produced as a result from a defective function of the *von Hippel-Lindau* tumor suppressor gene.[5]

VEGF is a potent angiogenic factor which plays a key role in tumor vascularization[6] and is involved in proliferation, migration and tube formation of endothelial cells as well as endothelial cell survival.[7] In response to VEGF, the endothelium is activated and several proteins are expressed by endothelial cells including vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), von Willebrand factor (vWF), angiopoietin-2 (Ang-2) and Tie-2 (Tie-2).[8–11] VCAM-1 and ICAM-1 are adhesion molecules and their soluble ectodomains (sVCAM-1 and sICAM-1) can be proteolytically released from the endothelial cell surface into the circulation.[12–14] Next to upregulation of VCAM-1 and ICAM-1, VEGF is also known to activate exocytosis of intracellular secretory granules in endothelial cells, the so-called Weibel–Palade bodies, thereby releasing vasoactive substances, including von Willebrand factor (vWF) and Ang-2.[8,9] The glycoprotein vWF is produced uniquely by endothelial cells upon endothelial cell activation and is essential for platelet adhesion to the subendothelial matrix after vascular injury. Ang-2 acts as a natural antagonist of its receptor Tie-2.[15] Endothelial shedding of soluble Tie-2 (sTie-2) is stimulated by VEGF as well.[10]

Inhibition of VEGF(R) signaling may affect endothelial cell activation and function, consequently leading to an

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altered release of endothelial cell-related proteins. Measurement of these proteins in blood might be useful to monitor the effect of anti-VEGF therapy in cancer patients. Because sunitinib inhibits VEGFR signaling, we selected plasma VEGF, sVCAM-1, sICAM-1, vWF, circulating Ang-2 and sTie-2 in patients with mRCC to determine their value as potential biomarkers. We not only assessed the effect of sunitinib on these circulating proteins but also explored whether alterations in plasma protein levels were associated with the change in tumor burden as well as treatment outcome.

Patients and Methods

Patients, treatment and evaluation

A total of 43 consecutive mRCC patients treated with sunitinib in two Dutch medical centers were included in this study. Each patient signed a protocol-specific informed consent. Collection of data was part of three protocols,[16–19] which were approved by the medical ethics review boards of the institutes.

Sunitinib was administered orally at a dose of 50-mg daily, consisting of 4 weeks on treatment followed by a 2-weeks rest period in cycles of 6 weeks. Dose reductions of sunitinib were allowed depending on the type and severity of adverse events.

Computed tomography (CT) or magnetic resonance imaging (MRI) was performed at baseline and every two to three cycles of treatment to assess clinical response according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 (RECIST).[20] At baseline and at first evaluation, total tumor burden was calculated, which was defined as the sum of the longest diameters of all lesions, including primary tumors.[21,22]

PFS was defined as the time between the first day of sunitinib and the date of progressive disease (PD) according to RECIST version 1.0,[20] or clear clinical evidence of PD. Overall survival (OS) was the time between the first day of sunitinib treatment and the date of death or the date at which patients were last known to be alive.

Blood samples analyses

Sequential plasma samples were obtained before treatment with sunitinib defined as cycle 1 day 1 (C1D1), on cycle 1 day 14 (C1D14), on cycle 1 day 28 (C1D28), and after 2 weeks of rest on cycle 2 day 1 (C2D1). At the time of blood sampling, the first 2-mL blood was discarded before collection of 7 mL of EDTA blood. Within 30 min after collection, samples were centrifuged at 3,000 rpm for 10 min at 4°C. Plasma was removed and stored immediately at −80°C until analysis.

Concentrations of VEGF, sVCAM-1 and sICAM-1 were assessed with Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). A commercially available ELISA for vWF antigen was obtained from American Diagnostica (Greenwich, CT). Plasma concentrations of circulating Ang-2 were measured with the human Ang-2 DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN). According to the ELISA kits the range of protein levels in normal volunteers was below 115 pg/mL for VEGF, in the range of 349–991 ng/mL for sVCAM-1, 100–307 ng/mL for sICAM, 630–1,950 mU/mL for vWF[23] and 27–287 pg/mL for circulating Ang-2.[24]

Samples from the same patient were pipetted into the same 96-wells plate. Samples were run in duplicate and the mean value was recorded. In 20 patients, sTie-2 levels (Quantikine ELISA kit; R&D Systems) were measured on C1D1 and on C1D14. These patients were selected on the basis of the highest percentage change in plasma levels of circulating Ang-2 on C1D14 when compared to that on C1D1. According to the ELISA kit, the range of sTie-2 was in the range of 17–36 ng/mL in normal volunteers.

Of all 43 patients, blood samples were available on C1D1 and on C1D14, while on C1D28 and on C2D1 blood samples were available in 33 and 35 patients, respectively. Reasons for missing blood samples were a temporary or permanent discontinuation due to sunitinib-related adverse events or evident progression of disease. In 5 of 43 patients, the amount of plasma sample was limited and only VEGF and vWF levels could be measured.

Statistics

Statistical analysis was performed using SPSS software (SPSS for Windows 16.0, SPSS, Chicago, IL). Data were expressed as median with range. The Mann–Whitney *U* test and the Kruskal–Wallis test were used to examine the associations between plasma protein levels and patient characteristics. Changes in variables were expressed as the percentage change from baseline values. The Wilcoxon Signed Ranks test was used to compare plasma levels on C1D14, C1D28 and C2D1 with those at baseline. Correlations between continuous data sets were analyzed using Spearman's correlation test. In addition, Fisher's exact test was performed to determine associations between categorical variables. A two-tailed probability value of $p < 0.05$ was considered significant.

For PFS and OS, data collection was closed on August 1, 2010. PFS and OS were calculated using the Kaplan–Meier method. All patient characteristics were tested univariately against PFS and OS using Kaplan–Meier and Cox-regression analysis, depending on the tested variables. The plasma protein levels at baseline and their changes on day 14 were dichotomized by median splitting followed by univariate testing against PFS and OS using Kaplan–Meier analysis. Log-rank test was used to test the differences between survival curves. Candidate variables with p value ≤ 0.05 were selected for the multiple Cox-regression survival analysis with PFS and OS as depending variables. Additional patient characteristics were introduced in the multivariate analyses based on univariately tested results if p value ≤ 0.05. All results from the multivariate analyses with p values < 0.05 were considered significant. The analyses were preplanned before the study started. Since this was an explorative study, no correction for multiple testing was done.

Results

Patient characteristics and treatment

Table 1 summarizes the baseline characteristics of the 43 patients. Thirty-eight (88%) patients had clear cell histology and 12 patients (28%) had a primary tumor *in situ*. Six (14%) patients had one metastatic site, 10 (23%) patients had two metastatic sites and 27 (63%) patients had at least three metastatic sites. According to the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria[25] most patients were categorized into the intermediate and poor risk group (each 44% of patients), whereas 12% of the patients was categorized into the favorable risk group.

According to RECIST, seven patients achieved a partial response (PR), 21 patients had stable disease (SD), 13 patients had PD and two patients could not be evaluated as a result of early termination due to sunitinib-related adverse events. At the time of the analysis, only three (7%) patients were still alive. Overall, the median PFS time was 7.0 months (range, 0.5–47.1 months) and the median OS time was 12.3 months (range, 0.5–41.1 months). In comparison with a previously published compassionate use cohort of sunitinib-treated mRCC patients,[26] the median PFS and OS in this study were relatively short, which can be explained by a higher number of patients in the MSKCC poor risk group as well as a lower number of patients in the favorable risk group.

When all measurable lesions were taken into account ($n = 40$), the median tumor burden was 138 mm (range, 37–321 mm). Total tumor burden could not be measured adequately in three out of 43 patients, as the disease was mainly localized in the bone. In addition, six patients could not be evaluated for the effect of sunitinib on total tumor burden due to early termination ($n = 2$), PD with massive increase in the number of metastases precluding adequate measurements ($n = 2$), and missing CT scan at first evaluation due to contraindications for administration of intravenous contrast ($n = 2$). In the remaining 34 patients, sunitinib decreased the median tumor burden from 136 mm (range, 37–321 mm) to 128 mm (range, 29–262 mm; $p = 0.192$) with a median decrease of 9% (range, −48 to +74%).

Of the clinical characteristics, the nonclear cell subtype and a higher number of metastatic sites were prognostic factors for a poor PFS (log rank = 8.328 and 11.115; $p = 0.004$ and 0.004, respectively) and poor OS (log rank = 14.160 and 7.351; $p < 0.001$ and $p = 0.025$, respectively). In addition, a higher number of MSKCC risk factors was prognostic for a poor OS (log rank = 7.840; $p = 0.020$).

Table 1. Patient characteristics

Characteristic	*N*	%
Median age (years)	60	
Range	20–84	
Sex		
Male	27	63
Female	16	37
ECOG performance status[1]		
0	18	42
1	15	35
2	6	14
3	4	9
Histological subtypes		
Clear cell	38	88
Nonclear cell	5	12
Previous treatment regimen		
None	22	51
Cytokine-based therapy	18	42
Cytokine-based therapy and antiangiogenic therapy	3	7
Previous nephrectomy		
No	12	28
Yes	31	72
Previous radiation therapy		
No	28	65
Yes	15	35
No. of metastatic sites		
1	6	14
2	10	23
≥3	27	63
MSKCC risk factors[2]		
0 (favorable)	5	12
1–2 (intermediate)	19	44
≥3 (poor)	19	44

[1]ECOG, Eastern Cooperative Oncology Group. [2]Risk groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria [based on the five risk factors: low Karnofsky performance status (<80%), high LDH (>1.5 times the upper limit of normal), low serum hemoglobin, high corrected serum calcium (>10 mg/dL), and time from initial diagnosis to treatment of less than 1 year].[25]

Plasma protein concentrations at baseline

A considerable number of mRCC patients had higher plasma levels of VEGF, sVCAM-1, sICAM-1, vWF and Ang-2 than those in healthy volunteers reported for the respective assays (see Patients and Methods section). The median level of VEGF was 97 pg/mL (range, 21–650 pg/mL). The median levels of the soluble adhesion molecules sVCAM-1 and sICAM-1 were 762 ng/mL (range, 325–3,458 ng/mL) and 295 ng/mL (range, 114–850 ng/mL), respectively. The median levels of vWF and circulating Ang-2 were 2,281 mU/mL (range, 204–4,049 mU/mL) and 1,336 pg/mL (range, 353–3,503 pg/mL), respectively. Additional measurements of the soluble receptor of Ang-2, sTie-2, were performed in 20 patients with relatively high levels of Ang-2. The sTie-2 level at baseline (median, 48 ng/mL; range, 25–114 ng/mL) was elevated as compared to that in healthy volunteers.

Table 2. Baseline plasma proteins *vs.* baseline characteristics

Characteristic	No. of cases	Circulating plasma proteins at baseline[1]				
		VEGF (pg/mL)	sVCAM-1 (ng/mL)	sICAM-1 (ng/mL)	vWF (mU/mL)	Ang-2 (pg/mL)
Sex[2]						
Male	27	102 (21–650)	782 (325–3,458)	356 (114–850)	2,233 (204–4,049)	1,246 (618–3,503)
Female	16	91 (29–331)	707 (416–1,503)	288 (179–527)	2,354 (1,019–3,756)	1,445 (353–3,466)
Histological subtypes[2]						
Clear cell	38	97 (21–363)	762 (344–3,458)	295 (179–850)	2,233 (204–3,702)	1,318 (353–3,466)
Nonclear cell	5	118 (37–191)	734 (325–1,238)	346 (114–530)	2,701 (1,898–4,049)	2,217 (1,157–3,503)
Previous nephrectomy[2]						
Yes	31	97 (29–650)	762 (344–1,365)	295 (179–850)	2,139 (204–4,049)	1,171 (353–3,135)**
No	12	98 (21–363)	714 (325–3,458)	304 (114–626)	2,749 (716–3,756)	1,781 (1,115–3,503)**
No. of metastatic sites[3]						
1	6	44 (21–99)	718 (532–1,299)	242 (187–376)	1,656 (204–2,859)	1,134 (773–3,819)
2	10	118 (75–313)	782 (501–1,365)	298 (209–527)	2,261 (1,019–3,728)	1,604 (708–3,075)
≥3	27	91 (37–650)	781 (325–3,458)	334 (114–850)	2,341 (1,146–4,049)	1,369 (353–3,503)
MSKCC risk factors[3,4]						
0 (favorable)	5	91 (29–118)	707 (636–822)	291 (187–356)	1,311 (204–2,859)	708 (353–1,372)*
1–2 (intermediate)	19	102 (21–331)	693 (344–1,503)	286 (179–441)	2,182 (716–3,756)	1,323 (430–3,466)*
≥3 (poor)	19	115 (29–650)	932 (325–3,458)	400 (114–850)	2,582 (1,583–4,049)	1,669 (739–3,503)*

*$p \leq 0.05$, **$p \leq 0.01$.
[1]Data are reported as median (range). [2]Mann-Whitney *U* test was used to examine associations between plasma proteins and patient characteristics. [3]Kruskal–Wallis was used to examine associations between plasma proteins and patient characteristics. [4]Risk groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria [based on the five risk factors: low–Karnofsky performance status (<80%), high LDH (>1.5 times the upper limit of normal), low serum hemoglobin, high corrected serum calcium (>10 mg/dL), and time from initial diagnosis to treatment of less than 1 year].[25]

Baseline plasma protein concentrations and patient characteristics

Baseline plasma protein levels were assessed for a possible relationship with patient characteristics (Table 2). In this analysis, sTie-2 was not taken into account because of limited sampling. Patients with a primary tumor *in situ* had higher circulating Ang-2 levels than patients with a previous nephrectomy [median of 2,217 pg/mL (range, 1,157–3,503 pg/mL) *vs.* median of 1,318 pg/mL (range, 353–3,466 pg/mL); $p = 0.007$]. In addition, significantly higher circulating Ang-2 levels were measured in patients who were categorized into higher MSKCC risk groups ($p = 0.020$). Furthermore, patients with a primary tumor *in situ* had higher vWF levels and patients with more metastatic sites had higher VEGF levels, but these findings were not significant ($p = 0.072$ and 0.052, respectively). Higher baseline levels of sICAM-1 and vWF were also measured in patients who belonged to higher MSKCC risk groups, but differences with levels in patients in lower risk groups were not significant ($p = 0.071$ and 0.073, respectively). The levels of sVCAM-1 were not associated with patient characteristics.

In the assessment of a possible relationship between plasma proteins and the extent of disease, we hypothesized that total tumor burden may be a better reflection of disease extent than the MSKCC criteria. Indeed, larger tumor burden was associated with higher levels of circulating Ang-2 (Spearman's $\rho = 0.378$, $p = 0.028$, $n = 34$) and vWF (Spearman's $\rho = 0.417$, $p = 0.008$, $n = 39$). There were no associations between tumor burden and plasma levels of VEGF, sVCAM-1 and sICAM-1. In further analysis, we determined whether there was a relationship between circulating Ang-2 levels and other proteins. It appeared that circulating Ang-2 levels were positively associated with most proteins, being levels of VEGF (Spearman's $\rho = 0.436$, $p = 0.007$; $n = 37$), sICAM-1 (Spearman's $\rho = 0.371$, $p = 0.024$; $n = 37$), and vWF (Spearman's $\rho = 0.392$, $p = 0.018$; $n = 36$). In the separate analysis in 20 patients with relatively high levels of circulating Ang-2, these were also positively associated with sTie-2 levels (Spearman's $\rho = 0.402$, $p = 0.079$, $n = 20$).

Effect of sunitinib on circulating plasma proteins

Figure 1 shows the alterations in plasma levels of the circulating proteins during sunitinib treatment in mRCC patients. The circulating levels of sICAM-1 and vWF did not significantly change during treatment with sunitinib.

Sunitinib induced a significant rise in plasma levels of VEGF and sVCAM-1. The median VEGF levels increased from 97 pg/mL (range, 21–650 pg/mL) at baseline to 312 pg/mL (range, 57–1,722 pg/mL) on C1D14 ($p < 0.001$) and to 235 pg/mL (range, 53–1,499 pg/mL) on C1D28 ($p < 0.001$).

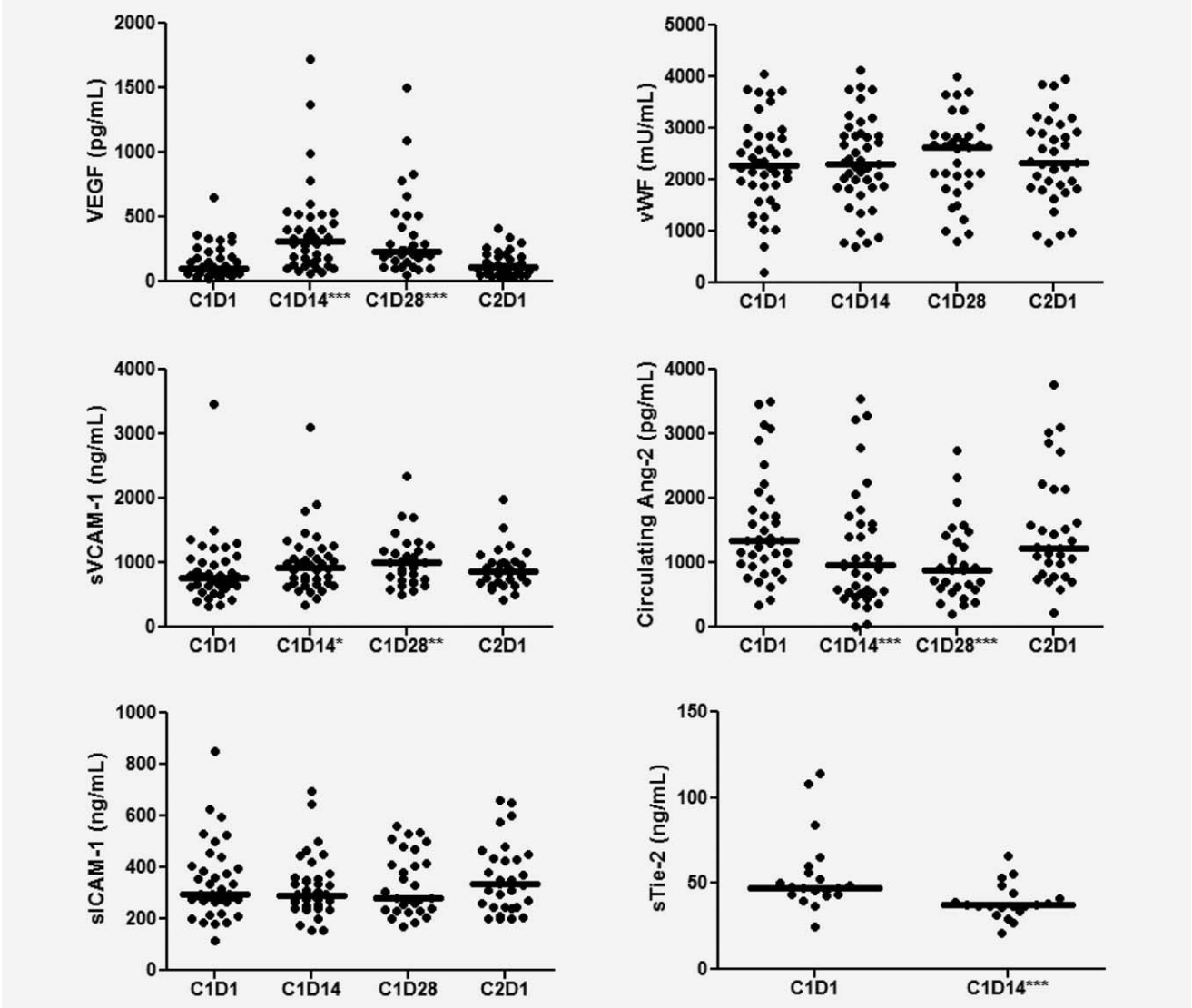

Figure 1. Plasma levels of circulating markers at baseline (C1D1), defined as cycle 1 day 1 (C1D1), on C1D14, C1D28, and on C2D1 in patients with mRCC treated with sunitinib 50 mg/day for 4 weeks in a 6-week cycle. Horizontal bars represent median values. *$p \leq 0.05$, compared to baseline value. **$p \leq 0.01$, compared to baseline value. ***$p \leq 0.001$, compared to baseline value.

The median sVCAM-1 levels increased from 762 ng/mL (range, 325–3,458 ng/mL) at baseline to 931 ng/mL (range, 335–3,095 ng/mL) on C1D14 ($p = 0.022$) and to 994 ng/mL (range, 507–2,336 ng/mL) on C1D28 ($p = 0.002$). After the 2-weeks rest period (C2D1), both VEGF levels and sVCAM-1 levels grossly returned to baseline values, being 114 pg/mL (range, 34–412 pg/mL; $p = 0.964$) and 870 ng/mL (range, 417–1,988 ng/mL; $p = 0.196$), respectively.

Sunitinib induced a significant decline in levels of circulating Ang-2 and sTie-2. Two weeks after the start of treatment, the median level of circulating Ang-2 had significantly decreased from 1,336 pg/mL (range, 353–3,503 pg/mL) to 958 pg/mL (0–3,534 pg/mL; $p < 0.001$) and that of sTie-2 from 48 ng/mL (range, 25–114 ng/mL) to 37 ng/mL (range, 21–66 ng/mL; $p < 0.001$). On C1D28, the change in Ang-2 persisted (median, 884 pg/mL; range, 206–2,746 pg/mL; $p < 0.001$), but on C2D1 the levels of circulating Ang-2 had recovered and had grossly returned to baseline values (median, 1,232 pg/mL; range, 229–3,771 pg/mL; $p = 0.178$).

We next explored whether the significant changes in the concentration of one protein in the 4 weeks of sunitinib treatment were associated with that of another among patients. On C1D14, the percentage change in levels of circulating Ang-2 was negatively associated with the percentage change in the levels of VEGF (Spearman's $\rho = -0.457$, $p = 0.005$, $n = 36$). Although changes in sTie-2 were also negatively associated with changes in VEGF (Spearman's $\rho = -0.456$, $p = 0.043$, $n = 20$), there was no association between the percentage change in the levels of circulating Ang-2 and that of the other proteins, in particular not

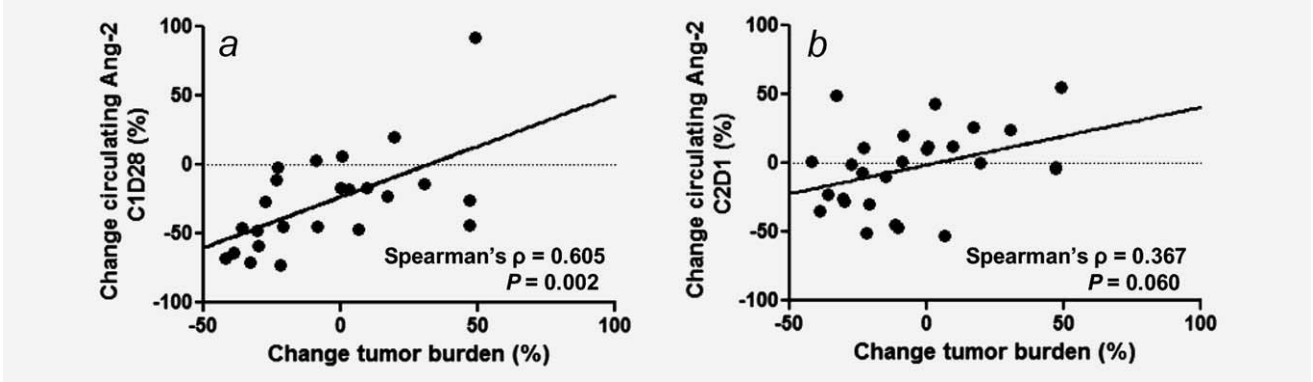

Figure 2. Association between percentage change in total tumor burden at first evaluation and percentage change in circulating Ang-2 levels on C1D28 (*a*) and C2D1 (*b*) in patients with mRCC treated with sunitinib 50 mg/day for 4 weeks in a 6-weeks cycle.

between circulating Ang-2 and sTie-2 (C1D14, Spearman's $\rho = 0.080$, $p = 0.738$, $n = 20$).

Associations between changes in plasma proteins and tumor burden

We assessed the effect of sunitinib on total tumor burden at first evaluation in relation to changes in plasma proteins. There were no associations between changes in tumor burden and the increase in plasma levels of VEGF or sVCAM-1. Of interest, the percentage decrease in circulating Ang-2 on C1D28 was positively associated with the percentage decrease in tumor burden (Spearman's $\rho = 0.605$, $p = 0.002$; $n = 24$; Fig. 2). When the outlyer with 92% increase in circulating Ang-2 was excluded, the association was still positive (Spearman's $\rho = 0.551$, $p = 0.006$; $n = 23$). In addition, there was a trend on C1D14 (Spearman's $\rho = 0.330$, $p = 0.087$, $n = 28$) and on C2D1 (Spearman's $\rho = 0.367$, $p = 0.060$, $n = 27$). In patients with a $> 30\%$ decrease in total tumor burden ($n = 6$) the circulating Ang-2 levels on C2D1 had not recovered to baseline values. On C2D1, the median absolute decrease in circulating Ang-2 in these patients was −363 pg/mL (range, −668 to 903 pg/mL), while that in patients without clinically relevant tumor reduction ($n = 21$) was +7 pg/mL (range, −1,293 to 414 pg/mL; Mann–Whitney *U* test, $p = 0.064$). In addition, patients with PR as best response according to RECIST had a significantly larger decrease in circulating Ang-2 on C1D28 than that in the other patients (Mann–Whitney *U* test, $p = 0.041$). Remarkably, one patient showed an absolute decrease of circulating Ang-2 of 1,295 pg/mL on C1D28, but an absolute increase of 903 pg/mL on C2D1. Clinically, this patient experienced a rapid tumor rebound during the rest period of the first treatment cycle. Consequently, in the subsequent cycles the sunitinib dose was changed to 37.5 mg per day in a continuous schedule and the patient showed a 33% reduction in total tumor burden at first evaluation. For the purpose of illustration, Figure 3 shows examples of CT images in a responding and a nonresponding patient.

Plasma proteins and clinical outcome

The associations between circulating protein levels and clinical outcome were explored. Baseline protein levels or sunitinib-induced changes in protein levels were not associated with PFS. In univariate analysis, only pretreatment sVCAM-1 plasma levels above the median value (762 ng/mL) were associated with poor OS (log rank = 5.219; $p = 0.022$). Baseline characteristics entered into the multivariate Cox model for OS analysis included histological subtype, the number of metastatic sites, and MSKCC risk factors. In multivariate analyses, pretreatment plasma levels of sVCAM-1 were no longer predictive of OS (hazard ratio: 1.835; 95% CI, 0.825–4.079; $p = 0.136$).

Discussion

In this study, we explored the effect of sunitinib on levels of circulating proteins involved in VEGF-regulated endothelial cell activation and function including VEGF, sVCAM-1, sICAM-1, vWF, Ang-2 and sTie2. At baseline, mRCC patients showed elevated plasma levels of all proteins as compared with those reported in healthy volunteers. Most of these protein levels were related to factors associated with the extent of the disease. At baseline, total tumor burden was positively associated with vWF and circulating Ang-2. In addition, levels of circulating Ang-2 were positively associated with VEGF, sICAM-1, vWF and sTie-2. Treatment with sunitinib induced an increase in VEGF and sVCAM-1, a decrease in circulating Ang-2 and sTie-2, while levels of sICAM-1 and vWF were not affected. After the 2-weeks rest period, changes in circulating proteins had grossly recovered. The change in circulating Ang-2 levels was positively associated with the percentage change in total tumor burden. Sunitinib-induced changes in plasma protein levels were not significantly associated with PFS or OS.

At baseline, plasma levels of all selected proteins were increased in patients with mRCC as compared with those in healthy volunteers. Elevated plasma levels of VEGF, sVCAM-1, sICAM-1 and sTie2 were grossly comparable with those

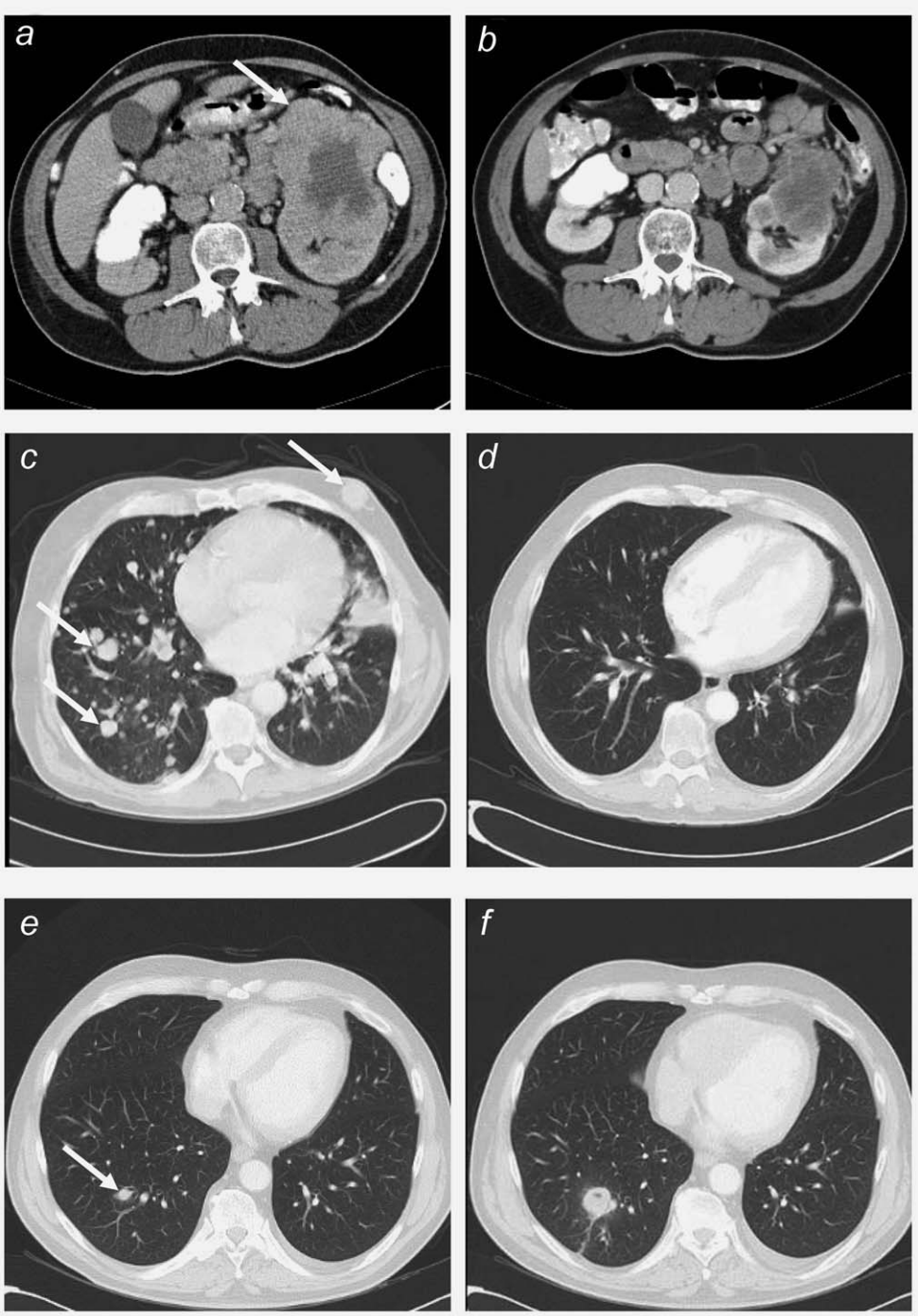

Figure 3. Examples of CT images in two patients with mRCC treated with sunitinib. (*a–d*) A responding patient with a primary tumor *in situ* (*a*, arrow), multiple lung and subcutaneous metastases (*c*, arrows) at baseline and at first evaluation during sunitinib (*b*, *d*). The patient showed a 71% decrease in circulating Ang-2 on C1D28 and a 33% decrease in total tumor burden at first evaluation. (*e*, *f*) A nonresponding patient with lung metastases at baseline (*e*, arrow) and at first evaluation during sunitinib (*f*). The patient showed a 92% increase in circulating Ang-2 on C1D28 and a 49% increase in total tumor burden at first evaluation.

reported in other mRCC cohorts.[13,27–29] The high-plasma level of circulating Ang-2, however, is a new finding in mRCC. In RCC tumor tissue, expression of Ang-2 has been described predominantly on tumor-associated endothelium,[30,31] but Ang-2 expression has been observed in RCC tumor cells as well.[30] Currently, growing evidence indicates that Ang-2 plays an important role in tumor angiogenesis.[32] Ang-2 can collaborate with VEGF to induce angiogenesis in

a synergistic manner[33] and can facilitate the angiogenic process regulated by tumor-derived VEGF between existing host vessels that co-opt with tumor cells.[32] In our study, circulating Ang-2 was the strongest factor associated with the extent of disease, which was characterized by a concurrent primary tumor *in situ*, a higher risk group according to MSKCC and a larger total tumor burden. Nevertheless, levels of circulating Ang-2 or any other proteins were not independently associated with outcome in sunitinib-treated mRCC patients. In contrast to these findings, other studies have shown a correlation between high levels of circulating Ang-2 and a poorer prognosis in melanoma patients[34] and in patients with advanced colorectal cancer treated with bevacizumab.[35] Although baseline-circulating Ang-2 was not prognostic for outcome in our mRCC patients, a decrease in circulating Ang-2 appeared to be predictive for sunitinib-induced reduction in total tumor burden.

Levels of circulating Ang-2 and sTie-2 both declined after sunitinib treatment. Previous studies have shown that sunitinib and bevacizumab cause downregulation of Ang-2 mRNA in human tumor cells including glioma and rectal cancer xenografts.[36,37] These reports and this study suggest that inhibitors of VEGF(R) signaling may decrease Ang-2 expression in tumors, which subsequently results in a decline of circulating Ang-2. In addition, inhibition of VEGF(R) signaling may reduce shedding of sTie-2, which is exclusively expressed on endothelial cells as described for RCC,[30] thereby decreasing endothelial cell binding of Ang-2 to its receptor.

As conflicting data have been published on tissue localization of Ang-2 expression in malignancies,[30,35] we further explored whether the changes in circulating Ang-2 were possibly of endothelial origin. Hence, HUVECS were treated with sunitinib to determine changes in Ang-2 in supernatant. Although sunitinib had an inhibitory effect on HUVEC proliferation, secretion of Ang-2 by these cells was not affected (unpublished data). Since plasma levels of vWF, which is also stored in the Weibel–Palade bodies of endothelial cells, were not affected in our patients, these findings suggest that sunitinib mostly reduced Ang-2 production from tumor cells. In accordance, we found an association between the decrease in circulating Ang-2 levels and a reduction in total tumor burden in sunitinib-treated mRCC patients.

Apart from circulating Ang-2, sunitinib treatment resulted in changes in plasma levels of VEGF and sVCAM-1. As expected, VEGF levels increased after administration of sunitinib. A reactive rise in plasma VEGF is usually observed upon treatment with TKIs targeting VEGFR.[38] In a preclinical study, sunitinib increased plasma VEGF levels in a dose-dependent manner, which was caused by a tumor-dependent response as well as a systemic tumor-independent response.[39] Currently, the origin of the tumor-independent release of VEGF has not been identified. In this study, sunitinib treatment also induced a rise in sVCAM-1, while plasma levels of the other soluble adhesion molecule, sICAM-1, remained stable. Similarly, an increase in sVCAM-1 has been reported after bevacizumab-based therapy,[40,41] while sICAM-1 levels were not notably affected.[41] The increase of sVCAM-1 associated with inhibition of VEGF(R) has yet to be clarified. It has been described that nitric oxide (NO), which is produced as a result of VEGFR-2 signaling,[42] represses gene transcription of VCAM-1 in human saphenous vein endothelial cells.[43] This observation suggests that sunitinib may promote endothelial expression of VCAM-1 by inhibiting the repressing function of NO. In contrast, it has been suggested that the NO-dependent pathway is not involved in VEGF-induced mRNA expression of VCAM-1 in human umbilical vein endothelial cells (HUVECS).[11]

The results of this study indicate that sunitinib, a promiscuous drug against multiple tyrosine kinases, affects Ang-2 signaling, which may reflect an additional beneficial effect of sunitinib in patients with mRCC. In these patients, circulating Ang-2 levels are elevated and are associated with the extent of disease. During sunitinib treatment, the decline in circulating Ang-2 early predicts reduction in tumor burden. Presently, antiangiogenic drugs that selectively target the Ang-2 pathway are under development.[44] Recently, AMG 386, a drug that selectively inhibits the interaction of Ang-1 and Ang-2 with Tie-2, has shown promising antitumor activity in patients with advanced solid tumors.[45,46] These Ang-2 inhibitors may also be promising agents for the treatment of RCC, as mRCC patients show increased levels of circulating Ang-2.

In conclusion, sunitinib-induced changes in Ang-2, sTie-2, sVCAM-1 and VEGF are related to the administration schedule of the drug, while reduction in Ang-2 is also associated with decrease in tumor burden. Our findings warrant further studies in mRCC patients to clarify the role of the Ang-2 pathway in sunitinib treatment and efficacy.

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