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Active angiogenesis in metastatic renal cell carcinoma predicts clinical benefit to sunitinib-based therapy

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Background: Sunitinib represents a widely used therapy for metastatic renal cell carcinoma patients. Even so, there is a group of patients who show toxicity without clinical benefit. In this work, we have analysed pivotal molecular targets involved in angiogenesis (vascular endothelial growth factor (VEGF)-A, VEGF receptor 2 (KDR), phosphorylated (p)KDR and microvascular density (MVD)) to test their potential value as predictive biomarkers of clinical benefit in sunitinib-treated renal cell carcinoma patients.

Methods: Vascular endothelial growth factor-A, KDR and pKDR-Y1775 expression as well as CD31, for MVD visualisation, were determined by immunohistochemistry in 48 renal cell carcinoma patients, including 23 metastatic cases treated with sunitinib. Threshold was defined for each biomarker, and univariate and multivariate analyses for progression-free survival (PFS) and overall survival (OS) were carried out.

Results: The HistoScore mean value obtained for VEGF-A was 121.6 (range, 10–300); for KDR 258.5 (range, 150–300); for pKDR-Y1775 10.8 (range, 0–65) and the mean value of CD31-positive structures for MVD visualisation was 49 (range, 10–126). Statistical differences for PFS ($P=0.01$) and OS ($P=0.007$) were observed for pKDR-Y1775 in sunitinib-treated patients. Importantly, pKDR-Y1775 expression remained significant after multivariate Cox analysis for PFS ($P=0.01$; HR: 5.35, 95% CI, 1.49–19.13) and for OS ($P=0.02$; HR: 5.13, 95% CI, 1.25–21.05).

Conclusions: Our results suggest that the expression of phosphorylated (i.e., activated) KDR in tumour stroma might be used as predictive biomarker for the clinical outcome in renal cell carcinoma first-line sunitinib-treated patients.

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults. It accounts for ~3% of adult malignancies and 90–95% of neoplasms arising from the kidney (Kashyap *et al*, 2005). In Europe, RCC is diagnosed in around 88 400 patients and 39 300 people die each year (Ferlay *et al*, 2010). Therefore, despite recent advances in our understanding of molecular processes involved in the RCC pathogenesis, which have led to novel targeted therapies, it remains necessary to develop alternative strategies to improve patient outcomes (Castellano *et al*, 2013).

Currently, sunitinib malate or SU11248 (Sutent, Pfizer Inc., New York, NY, USA) represents a widely used therapeutic option for metastatic RCC (Scartozzi *et al*, 2013). This drug was approved by the United States Food and Drug Administration for treatment of patients with specific types of cancer

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(Kamba and McDonald, 2007). Its activity is based on the inhibition of vascular endothelial growth factor receptor 2 (KDR), platelet-derived growth factor receptor (PDGFR), c-KIT and fetal liver tyrosine kinase 3 (Mendel *et al*, 2003; Schueneman *et al*, 2003).

Vascular endothelial growth factor (VEGF) by binding with its specific receptor (KDR) promotes a variety of responses in endothelium, including hyperpermeability, endothelial cell growth, angiogenesis and enhanced glucose transport (Connolly, 1991; Senger *et al*, 1994). At the molecular level, the membrane receptor dimerises subsequently to ligand binding, and the intracellular C-terminal tyrosine residues are phosphorylated. This event activates the kinase domain initiating the signalling transduction cascade that ultimately results in increased expression of several target genes (Krause and Van Etten, 2005). The ligand–receptor interaction leads to a KDR autophosphorylation on Y1175 (Takahashi *et al*, 2001) that has been shown necessary for neovascularisation (McMahon, 2000), which produces high risk of angiogenesis, usually measured as an increase of microvascular density (MVD) (Chaudhary *et al*, 1999).

Sunitinib is recommended by international guidelines for the first-line treatment of RCC on the basis of significant reported advantages in comparison with other therapeutic options such as interferon alpha. In a phase III trial, progression-free survival (PFS) was longer and response rates were higher in patients with metastatic RCC who received sunitinib compared with those who received interferon alpha (Motzer *et al*, 2007; Zama *et al*, 2010). Furthermore, its efficacy in patients with RCC refractory to cytokine-based therapy was also demonstrated in two phase II trials (Motzer *et al*, 2006a, b). Despite sunitinib activity and its clear benefit, there is a group of patients who show toxicity without clinical benefit (Motzer *et al*, 2007, 2009). Similar outcomes are showed in the work of Tannir *et al* (2012), a phase II clinical trial in patients with advanced non-clear cell RCC treated with sunitinib. They proposed a therapeutically relevant biological heterogeneity in this kind of patients (Tannir *et al*, 2012). Therefore, there is a rising interest in identifying biomarkers that could be useful to determine the profile of candidate patients who will benefit from sunitinib treatment and, in contrast, discard those patients who could undergo toxicities, poor outcomes or being refractory to this therapy.

In this work, we have analysed several molecular targets involved in the VEGF pathway in a cohort of metastatic RCC patients treated with sunitinib. Interestingly, results obtained from this preliminary study show evidence that phosphorylated (i.e., activated) KDR-Y1775 has a potential value as predictive biomarker of clinical benefit in RCC patients treated with sunitinib-based therapies and may also contribute to the future design of more personalised therapies that improve the poor outcomes observed in patients with RCC.

MATERIALS AND METHODS

Patients. The study involved 23 biopsies from consecutive cases of clear cell metastatic RCC treated with sunitinib in first line between 2008 and 2013 obtained from the Biobank of Fundación Jiménez Díaz Hospital (Spain). To compare biomarkers' expression with baseline data, we included a control group consisting of biopsies from non-metastatic RCC patients without treatment ($n = 25$). All patients gave written informed consent and sample collection was made with the approval of the Institutional Scientific and Ethical Committee.

Clinical–pathological data were obtained from the patient medical records and included sex, age, Eastern Cooperative Oncology group (ECOG) performance status, previous nephrectomy, site of metastases, number of disease sites and Memorial Sloan-Kettering Cancer Center risk classification (MSKCC risk factors), which stratifies patients with metastatic RCC into risk categories based on the number of adverse clinical and laboratory parameters present such as levels of serum haemoglobin, serum calcium and serum lactate dehydrogenase, ECOG performance status and time between diagnosis and treatment (Motzer *et al*, 2002).

Immunohistochemistry. Consecutive 4 μm tissue sections were obtained from formalin-fixed paraffin-embedded samples. Antigen retrieval was performed in PT-Link (Dako, Glostrup, Denmark) for 20 min at 95 °C in high pH buffered solution (Dako). Endogenous peroxidase was blocked by immersing the sections in 0.03% hydrogen peroxide for 5 min. Slides were washed for 5 min with Tris-buffered saline solution containing Tween 20 at pH 7.6 and incubated with the primary antibodies (VEGF-A (Clone VG1 M7273, Dako) specific labels VEGF-A121, VEGF-A165 and VEGF-A189 isoforms), VEGF receptor 2 (Ref. 2479, Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated-VEGF receptor 2 at Tyr1175 (Ref. 2478, Cell Signaling Technology, Inc.) and CD31 (Clone JC70A, Dako) for 20 min at room temperature, followed by incubation with the appropriate anti-Ig horseradish peroxidase-conjugated polymer (EnVision, Dako) to detect antigen–antibody. Sections were then visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with haematoxylin. All immunohistochemical stainings were performed in a Dako Autostainer and the same sections incubated with non-immunized serum were used as negative

Table 1. Baseline demographic and clinical characteristics

Variable	Sunitinib ($N = 23$)
Median age, years (range)	62 (34–81)
Sex, *n* (%)	
Male	11 (48)
Female	12 (52)
ECOG performance status, *n* (%)[a]	
0	7 (30)
1	14 (61)
2	2 (9)
Previous nephrectomy, *n* (%)	20 (87)
Site of metastases, *n* (%)	
Brain	1 (4)
Lung	8 (35)
Liver	3 (13)
Bone	5 (22)
Lymph nodes	6 (26)
No. of disease sites, *n* (%)	
1	11 (48)
2	8 (35)
⩾3	4 (17)
MSKCC risk factors, *n* (%)[b]	
0 (favourable)	14 (61)
1–2 intermediate (intermediate)	9 (39)

[a]ECOG denotes Eastern Cooperative Oncology Group.
[b]Risk factors associated with shorter survival according to the Memorial Sloan-Kettering Cancer Center (MSKCC) risk classification are a low serum haemoglobin level, an elevated corrected serum calcium level, an elevated serum lactate dehydrogenase level, a poor ECOG performance status and an interval of <1 year between diagnosis and treatment.

controls. As positive control, sections of a renal human tumour with known expression of the markers were stained.

Expression of the studied markers was assessed in a blinded fashion by two investigators (FR and SZ). Vascular endothelial growth factor-A was expressed in the cytoplasm of tumour cells. Vascular endothelial growth factor receptor 2 was detected in the membrane and cytoplasm of endothelial cells, and, occasionally, in activated fibroblast of tumour stroma and malignant cells. Only expression in endothelial cells was considered for the analysis. For pKDR and CD31, staining in endothelial cells was required for considering a tumour as positive. For VEGF-A, KDR and pKDR, a semiquantitative HistoScore (HScore) was calculated. The HScore was determined by estimation of the percentage of cells positively stained with low, medium or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: $\text{HScore} = (\text{low\%}) \times 1 + (\text{medium\%}) \times 2 + (\text{high\%}) \times 3$ and the results ranged from 0 to 300. Microvascular density was calculated by the Chalkley counting procedure (Pallares *et al*, 2006). Briefly, a 25-point Chalkley eyepiece graticule (Olympus X250, Tokyo, Japan; Chalkley grid area 0.196 mm^2) was applied to the ocular of the microscope and at medium magnification ($\times$ 200); the three most vascular areas of the tumour were quantified.

Statistical analysis. All statistical analyses were performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). Clinical and histopathologic information as well the immunohistochemical results were collected in a database.

For potential VEGF-A and KDR association with the disease outcome, patients were divided into three expression groups (tertiles: low, medium, high) on the basis of their HScores. For MVD analysis, patients were divided according to its absolute number of CD31-positive structures. To evaluate the prognostic value of VEGF-A, KDR and MVD in our cohort, survival curves were estimated using the Kaplan–Meier method with the three groups as a factor. Significant survival differences between groups were determined by the log rank test. The third tertile was established as the cut-off point, leaving low- and high-risk patient

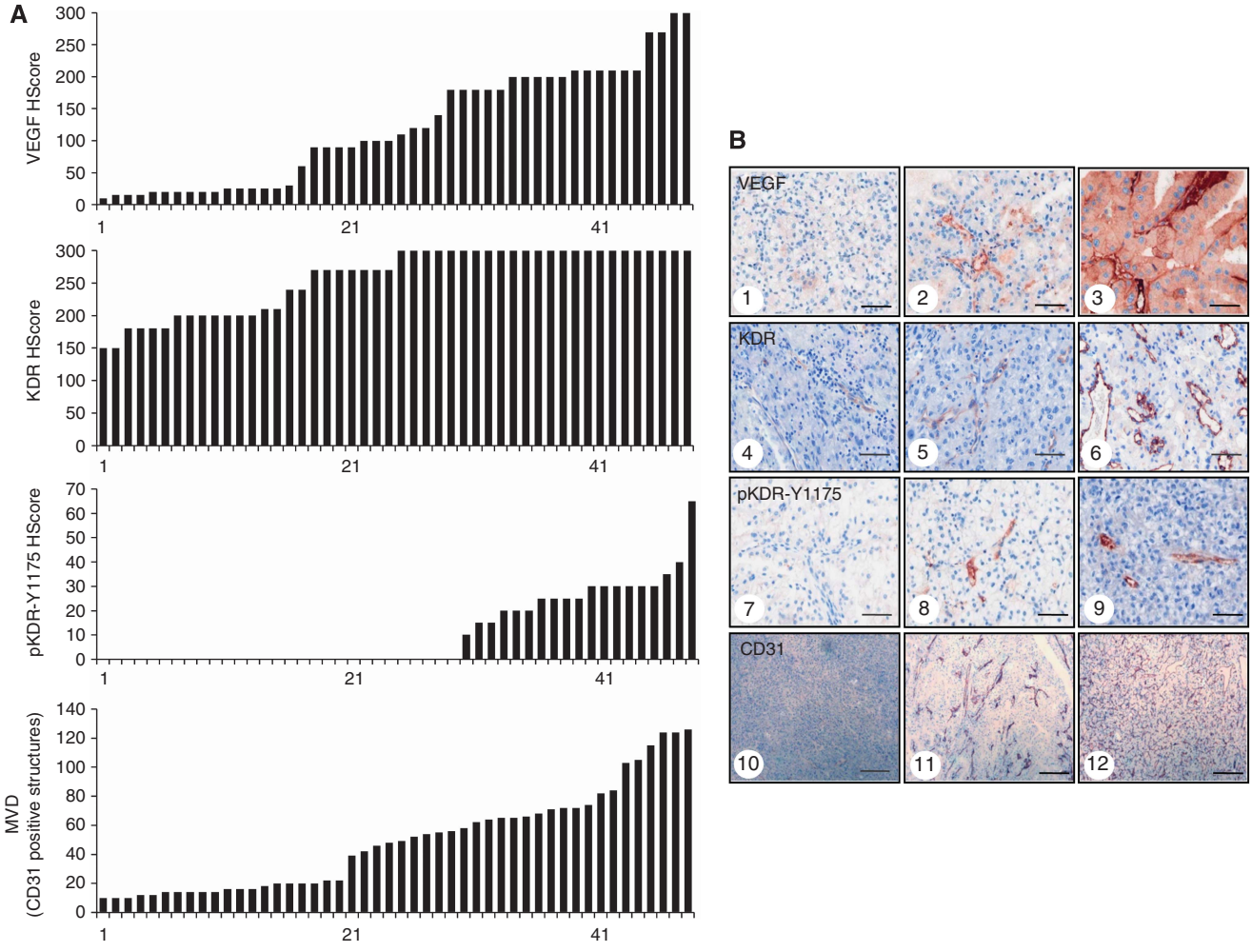

Figure 1. (**A**) Expression values for VEGF-A, KDR, pKDR-Y1775 and CD31 represented in histograms for each biomarker for the whole series of RCC patients. VEGF-A, KDR and pKDR-Y1775 are expressed as HScore in tumour and MVD as absolute number of CD31-positive vascular structures. (**B**) Representative microscopic pictures for VEGF-A, KDR, pKDR and MVD in studied metastatic RCC cases detected by immunohistochemistry. Vascular endothelial growth factor-A was detected in the cytoplasm of tumour cells with low (1), intermediate (2) and high expression (3) ($\times$ 400 magnification, scale bar: 30 μm). In addition, stromal cells showed VEGF-A staining. Expression of KDR was seen in endothelial cells, preferentially in tumour stroma. Representative images showing low (4), intermediate (5) and high expression (6) ($\times$ 400 magnification, scale bar: 30 μm). pKDR-Y1775 was exclusively detected in the endothelial cells of tumour stroma. Tumours showed low (7), intermediate (8) and high (9) levels of pKDR in vascular structures ($\times$ 400 magnification, scale bar: 30 μm). Studied cases displayed important differences in MVD, detecting sparse (10), intermediate (11) and dense (12) vascular density ($\times$ 40 magnification, scale bar: 300 μm).

groups, for MVD. The same approach was applied for VEGF-A and KDR, establishing the first tertile as the cut-off point.

For pKDR-Y1175 analysis, a cut-off point determined as positive (pKDR-Y1175 > 0) and negative expression (pKDR-Y1175 = 0) was used. Patients were divided into two groups, survival curves were estimated and differences between groups were determined by the log rank test.

Those variables that had potential prognostic suggested by univariate analysis were subjected to multivariate analysis with the Cox proportional hazards regression model. Overall survival (OS) and PFS were calculated from the date of diagnosis to the date of death or the last follow-up and to the date of sunitinib progression, respectively. A P-value <0.05 was considered as statistically significant.

RESULTS

Patient characteristics. Recruited data from patients at baseline are summarized in Table 1. The distribution of patients according to sex was similar; 52% females and 48% males. The median age for this cohort of patients was 62 years. In terms of ECOG performance status, most of patients, 61%, were clustered as equal to 1. Previous nephrectomy was carried out in 87% of the cases. Sites of metastases were diverse, including lung 35%, liver 13%, bone 22%, brain 4% and lymph nodes 26%. Number of disease sites was established as 1, 2 and $\geqslant 3$ (48%, 35% and 17%, respectively). Patients were grouped according to their MSKCC risk factor classification as favourable (61%) and intermediate (39%).

The control group comprised of 25 biopsies from non-metastatic RCC patients without treatment. The median age of this group was 67 years, and the distribution of patients according to sex was 60% males and 40% females.

Vascular endothelial growth factor-A, KDR, pKDR-Y1775 and MVD in RCC. To evaluate the expression of the selected proteins, immunohistochemistry assays were performed in patients treated with SU11248. A control group was included to establish a reference value for each marker. Vascular endothelial growth factor-A expression was diffusely detected in the cytoplasm of tumour cells, as well as in the stromal, including fibroblasts, and endothelial cells. Most of the cases showed stronger staining in the tumour than stroma. Expression of KDR was seen in endothelial cells, preferentially in tumour stroma. In addition, KDR was also detected in isolated fibroblasts and malignant cells. Expression of pKDR-Y1775 was only observed in the endothelial cells of vascular structures in the tumour. Conversely, endothelial cells of vessels in adjacent non-tumoral renal tissue did not express pKDR-Y1775. Finally, CD31 expression was present in all vascular structures, both in tumour and non-tumoral renal tissue (Figure 1).

HScore values of all patients for VEGF-A, KDR and pKDR-Y1775 as well as absolute number of CD31-positive structures for MVD visualisation are represented in histograms (Figure 1). The HScore mean value obtained for VEGF-A staining was 121.6 (range, 10–300);

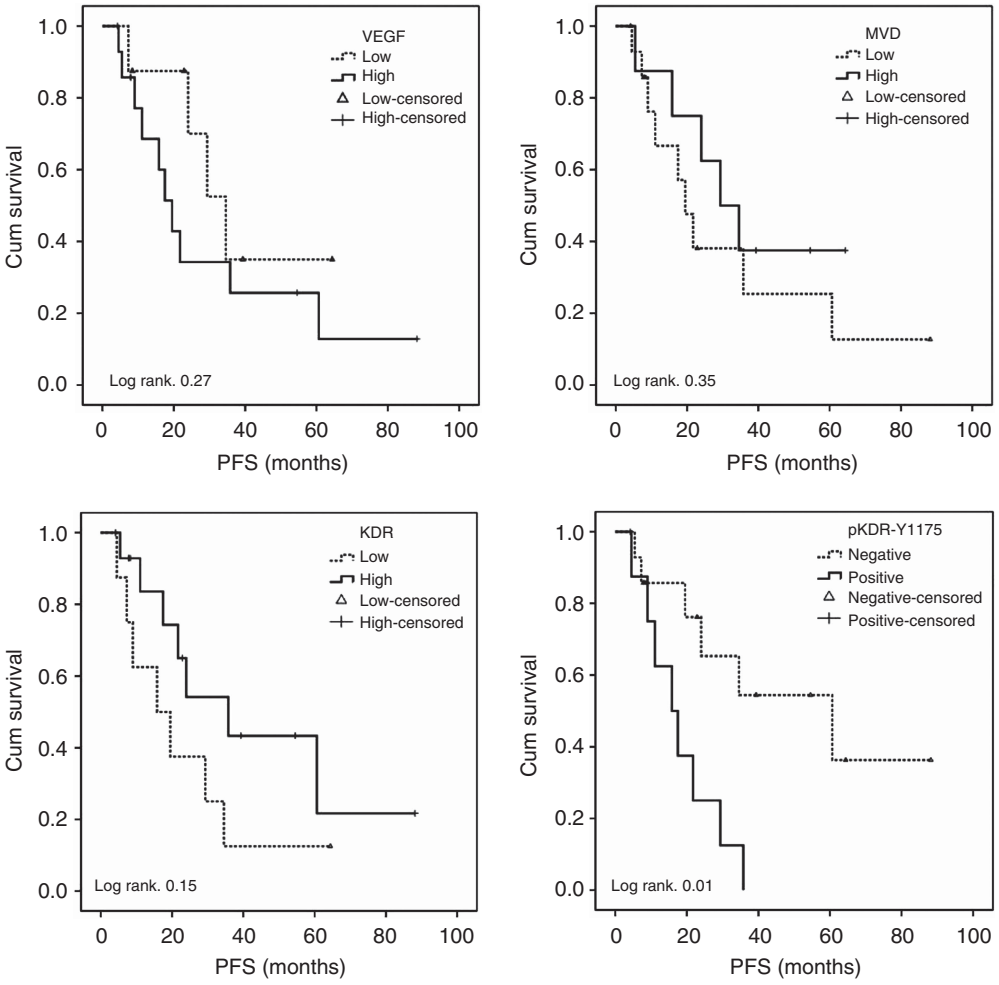

Figure 2. Kaplan–Meier analysis for PFS based on VEGF-A, KDR, pKDR-Y1775 and MVD levels in sunitinib-treated metastatic RCC patients.

for KDR in endothelial cells 258.5 (range, 150–300); for pKDR-Y1775 10.8 (range, 0–65); and the mean value of CD31-positive vascular structures for MVD staining was 49 (range, 10–126).

To determine the predictive potential of these proteins in metastatic RCC patients treated with sunitinib in first line, we estimated a cut-off point of 60 for VEGF-A, of 200 for KDR, of 0 for pKDR-Y1775 and of 48 for MVD.

pKDR-Y1775 in tumour stroma predicts clinical outcome. On the basis of these cut-off points, Kaplan–Meier analysis for categorical values of each marker was performed to assess the correlation between the expression levels and prognosis status in patients treated with sunitinib in terms of PFS and OS.

Vascular endothelial growth factor-A , KDR and MVD did not show statistical difference in terms of PFS and OS (Figures 2 and 3) (Tables 2 and 3).

In relation to pKDR-Y1775, log rank test showed statistical differences for this biomarker in terms of both PFS (log rank 0.01) and OS (log rank 0.007). The median survival time for the patients without the expression of pKDR (negative) was 23.4 months (range, 5–88) for PFS and 27.6 months (range, 8–88) for OS, whereas those cases with positive pKDR-Y1775 expression were associated with worse outcome, with a median survival time of 15.8 for PFS (range, 4–36) and 25.9 months (range, 4–51) for OS. Univariate analysis showed statistical differences for both PFS ($P=0.017$, HR: 4.02, 95% CI, 1.28–12.63) (Figure 2 and Table 2) and OS ($P=0.015$ HR: 5.34, 95% CI, 1.39–20.5) (Figure 3 and Table 3)

After multivariate Cox proportional hazards regression analysis, pKDR-Y1775 expression remained significant for both PFS ($P=0.01$; HR: 5.35, 95% CI, 1.49–19.13) and OS ($P=0.02$; HR: 5.13, 95% CI, 1.25–21.05) suggesting that phosphorylation of KDR in Y1175 could be an independent predictive factor of sunitinib response in patients with clear cell metastatic RCC (Tables 2 and 3).

DISCUSSION

This study evaluates the role of VEGF-A, KDR, pKDR-Y1175 and MVD in metastatic RCC after receiving sunitinib and their potential to predict significant clinical benefit in terms of statistically longer PFS and OS.

Vascular endothelial growth factor pathway has been largely characterised in RCC as a key mechanism in the angiogenesis development (Takahashi *et al*, 1994; Nakagawa *et al*, 1997; Tomisawa *et al*, 1999), and as a result, a relevant therapeutic target (Rini, 2009). Sunitinib is a multitargeted receptor tyrosine kinase inhibitor of VEGF receptors, among others, which interacts with the ATP binding pocket of these kinases and acts as competitive inhibitor with ATP. Its efficacy in patients with RCC refractory to cytokine-based therapy was demonstrated in two phase II trials (Motzer *et al*, 2006a, b) as well as in previously untreated patients in a phase III trial (Motzer *et al*, 2007).

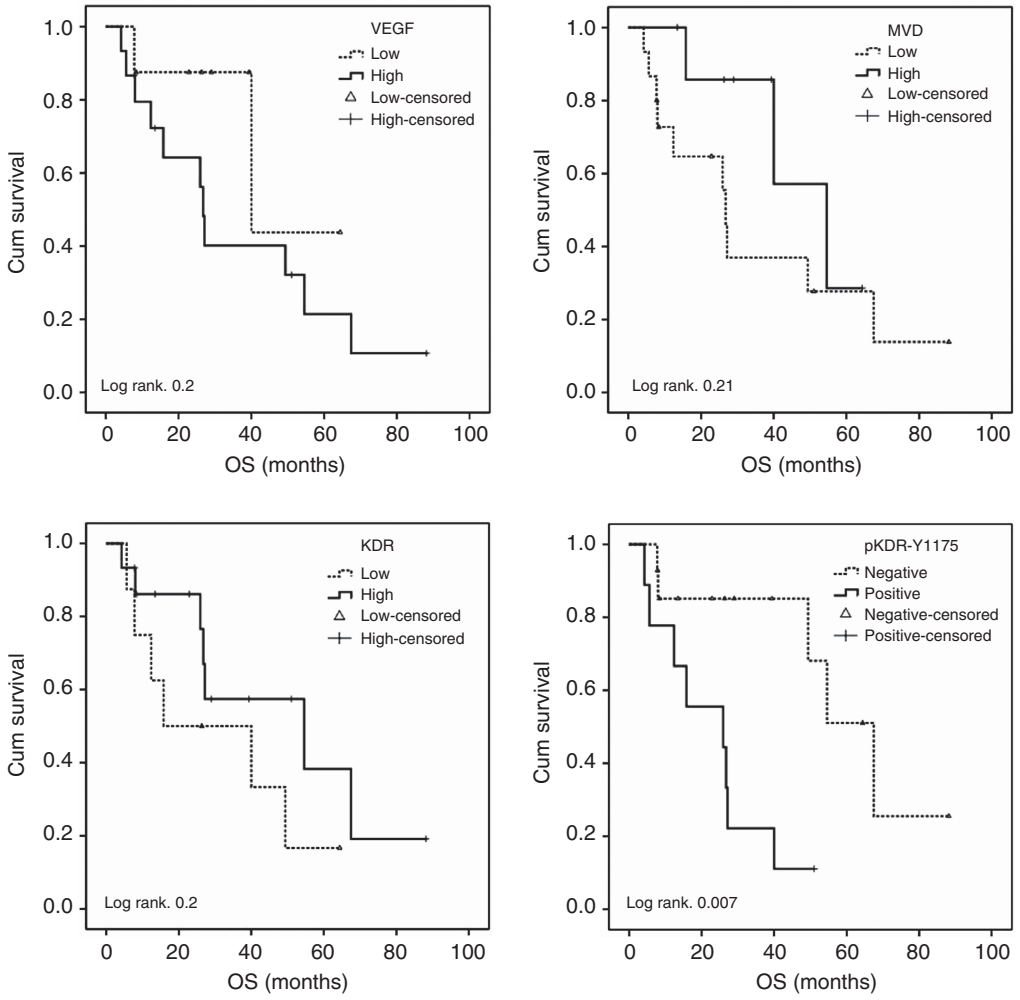

Figure 3. Kaplan–Meier analysis for OS based on VEGF-A, KDR, pKDR-Y1775 and MVD levels in sunitinib-treated metastatic RCC patients.

Table 2. PFS: uni- and multivariate analyses in RCC patients

	Univariate PFS analysis				Multivariate PFS analysis			
		95% CI				95% CI		
	HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
Age	0.98	0.94	1.02	0.36				
Gender				0.18				0.13
Male	1.00				1.00			
Female	2.07	0.70	6.09		2.66	0.75	9.48	
ECOG				0.26				
0	1.00							
1–2	2.08	0.58	7.51					
No. of disease sites				0.71				
1	1.00							
⩾2	1.22	0.42	3.55					
MSKCC risk factors				0.45				0.66
Favourable	1.00				1.00			
Intermediate	1.52	0.5	4.62		1.32	0.37	4.67	
VEGF				0.28				
Low	1.00							
High	1.9	0.59	6.12					
KDR				0.16				
Low	1.00							
High	0.47	0.16	1.35					
pKDR-Y1775				0.017[a]				0.01[a]
Negative	1.00				1.00			
Positive	4.02	1.28	12.63		5.35	1.49	19.13	
MVD				0.35				
Low	1.00							
High	0.59	0.19	1.80					

Abbreviations: CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; HR = hazard ratio; KDR = vascular endothelial growth factor receptor 2; MVD = microvascular density; MSKCC = Memorial Sloan-Kettering Cancer Centre; PFS = progression-free survival; pKDR = phosphorylated KDR; VEGF = vascular endothelial growth factor.
[a]Denotes statistical differences ($P \leqslant 0.05$).

Although anti-angiogenic therapy has resulted in a complete revolution in the treatment of metastatic RCC patients, the response varies widely from patient to patient in terms of PFS and OS, no apparent explanation is found in most cases (Motzer *et al*, 2007, 2009). Certainly, it is the differential grade in the outcome that justifies the need to identify biomarkers that can predict the clinical benefit of sunitinib.

In addition to clinical and laboratory-based factors used as prognostic criteria, being MSKCC the most known (Motzer *et al*, 1999), several molecules have been explored as potential biologic indicators in terms of response to SU11248. Some of these studies have showed association between levels of VEGF-A soluble isoforms and PFS (Paule *et al*, 2010; Porta *et al*, 2010). Other recent studies have found an association between several proteins involved in hypoxia and SU11248 efficacy as well as low VEGFR3 expression associated with worse outcome (Garcia-Donas *et al*, 2013). Circulating endothelial cells as well as circulating bone marrow-derived progenitor cells have also been explored as valuable biomarkers (Gruenwald *et al*, 2010; Farace *et al*, 2011). Even at genetic level, novel studies have revealed a differential outcome based on the presence of polymorphisms in *VEGF* and *VEGFR* genes (Scartozzi *et al*, 2013) or based on miRNA expression profiles (Gamez-Pozo *et al*, 2012). Terakawa *et al* (2013) suggested that it would be useful to consider the expression levels of KDR to identify the metastatic RCC patients likely to be benefited from treatment with sunitinib; although several biomarkers were studied, only VEGFR2 expression appeared to be independently related to PFS as well as OS on multivariate analysis. In the analysis carried out in our panel of patients, we described for the first time the correlation of pKDR-Y1175 expression with PFS and OS in patients with metastatic RCC in terms of clinical benefit of sunitinib-based therapy.

Presently, little is known about the predictive role of pKDR-Y1175 in response to treatment. The phosphorylation profile and the intracellular location of KDR were investigated in both normal and neoplasic kidneys (Fox *et al*, 2004). Although the phosphorylated epitopes were different from our marker (Y1059 and Y1214), this study showed that pKDR is present in a wide variety of renal tumours, suggesting that anti-VEGFR therapy might have direct effects on tumour cells. Furthermore, pKDR-Y1775 has been associated with poor prognosis in endometrial carcinomas (Giatromanolaki *et al*, 2006).

Angiogenesis and its signalling proteins have been largely studied in several tumour types and their importance in tumour progression is widely accepted. However, their role in the modulation of response to anti-angiogenic therapies in cancer is still under debate. Some evidences recently showed correlations between angiogenesis and response to tyrosine kinase inhibitors that target receptors of angiogenesis (Rosa *et al*, 2013), including sunitinib. Supporting this research, our analysis provides novel

Table 3. OS: uni- and multivariate analysis in RCC patients

	Univariate OS analysis				Multivariate OS analysis			
		95% CI				95% CI		
	HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
Age	1.00	0.95	1.04	0.98				
Gender				0.08				0.15
Male Female	1.00 2.87	0.87	9.44		1.00 2.6	0.7	9.64	
ECOG				0.15				
0 1–2	1.00 4.5	0.57	35.01					
No. of disease sites				0.2				
1 ⩾2	1.00 2.17	0.65	7.23					
MSKCC risk factors				0.06				0.19
Favourable Intermediate	1.00 2.93	0.96	8.95		1.00 2.24	0.65	7.73	
VEGF				0.22				
Low High	1.00 2.59	0.56	11.95					
KDR				0.21				
Low High	1.00 0.48	0.15	1.51					
pKDR-Y1775				0.015[a]				0.02[a]
Negative Positive	1.00 5.34	1.39	20.5		1.00 5.13	1.25	21.05	
MVD				0.22				
Low High	1.00 0.44	0.12	1.64					

Abbreviations: CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; HR = hazard ratio; KDR = vascular endothelial growth factor receptor 2; MVD = microvascular density; MSKCC = Memorial Sloan-Kettering Cancer Centre; OS = overall survival; pKDR = phosphorylated KDR; VEGF = vascular endothelial growth factor.
[a]Denotes statistical differences ($P \leqslant 0.05$).

data of the role of active angiogenesis in RCC patients to predict the benefit of sunitinib. These findings require further validation in additional clinical series to confirm the potential impact in terms of outcome prediction.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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