

Clinical response to therapy targeted at vascular endothelial growth factor in metastatic renal cell carcinoma: impact of patient characteristics and Von Hippel-Lindau gene status

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OBJECTIVE

To describe the relationship among patient characteristics, Von Hippel-Lindau (VHL) gene status and clinical outcome in metastatic renal cell carcinoma (RCC) in patients receiving vascular endothelial growth factor (VEGF)-targeted therapy.

PATIENTS AND METHODS

All patients with metastatic RCC who received therapy with interferon- α plus bevacizumab, SU11248 or AG013736 at the authors' institution were considered. Clinical features were collected and activation status of the VHL gene (*VHL*) was determined from baseline paraffin-embedded tumour samples. Tumour response, time to tumour progression (TTP) and overall survival were recorded.

RESULTS

Forty-three patients were evaluable for determination of *VHL* status and clinical response. There was an objective response in 18 patients (43%; 95% confidence interval 28–59%). The median TTP for the entire cohort was 8.1 months. There was an improved clinical outcome in patients with the following clinical features: male gender, lack of hepatic metastases, no previous radiation therapy and higher baseline haemoglobin level. Twenty-six patients (60%) had evidence of *VHL* mutation or promoter methylation; such patients had an objective response rate of 48%, vs 35% in patients with no *VHL* mutation or methylation. Patients with *VHL* methylation or a mutation predicted to truncate or shift the *VHL* reading frame had a median TTP of 13.3 months, vs 7.4 months

in patients with none of these features ($P=0.06$).

CONCLUSION

VEGF-targeted therapy is active in metastatic RCC and the response can be associated with certain clinical features. The TTP with VEGF-targeted therapy might be prolonged in patients with *VHL* methylation or mutations that truncate or shift the *VHL* reading frame. Further investigation of VHL pathway components is needed to understand the biology of the response to VEGF-targeted agents in metastatic RCC.

KEYWORDS

renal cell carcinoma, VHL, mutation, methylation, VEGF-targeted therapy

INTRODUCTION

The biology underlying RCC has been investigated in the search for therapeutic targets in this historically treatment-refractory disease. The von Hippel-Lindau (VHL) tumour-suppressor gene (*VHL*) is disrupted, through mutation or promoter methylation, in most sporadic clear cell RCC tumours [1–8]. The resulting loss of VHL protein function is thought to stabilize hypoxia-inducible factor α (HIF α), leading to induction of hypoxia-regulated genes, including vascular endothelial growth factor (VEGF), a potent pro-angiogenic protein [9–15].

Many therapeutic approaches to VEGF blockade have been investigated in patients

with metastatic RCC. Small-molecule tyrosine kinase inhibitors of VEGF receptor-2 (VEGFR-2) and platelet-derived growth factor receptor-B (PDGFR-B), including SU11248 (sunitinib, Sutent[®], Pfizer Inc., USA) and AG013736 (axitinib, Pfizer) have reportedly had substantial objective response rates in patients with cytokine-refractory RCC [16,17]. In addition, a randomized trial of the anti-VEGF antibody, bevacizumab (Avastin[®], Genentech, South San Francisco, CA, USA), in advanced, cytokine-refractory RCC showed a significant improvement in time to disease progression in patients treated with bevacizumab vs placebo [18]. These results prompted a phase III trial in untreated patients with metastatic RCC of interferon- α monotherapy vs interferon- α plus bevacizumab [19].

Taken together, these clinical results with VEGF-targeted therapy in metastatic RCC suggest substantial anti-tumour effects. Further investigation into patient and tumour characteristics that might predict the response to these agents could improve the clinical outcome and possibly provide a greater understanding of the biology of the response to this therapy. Relevant to the RCC tumour biology described above, a hypothesis can be proposed that *VHL* mutation or methylation would render a tumour more VEGF-dependent and thus more susceptible to VEGF-targeted therapy. Thus we examined the clinical characteristics, *VHL* status and clinical outcome of patients with metastatic RCC receiving VEGF-targeted therapy.

PATIENTS AND METHODS

All patients with metastatic RCC who received therapy with SU11248 or AG013736 or interferon- α plus bevacizumab as part of a clinical trial at authors' institution were considered for evaluation. Clinical information for each patient was collected as specified by the treatment protocol. Data on age, gender, performance status, tumour histology, number and site of metastases, baseline laboratory values, including haemoglobin level, lactate dehydrogenase (LDH) and corrected serum calcium [total calcium $-0.8 \times (4.0 - \text{serum albumin})$] and previous local and systemic therapy were available for all patients and considered for this analysis. In addition, VEGF-targeted treatment received, best objective response, time to tumour progression (TTP) and overall survival (OS) were recorded. The clinical response to therapy (objective response and percentage of tumour shrinkage) was determined uniformly in all patients by one investigator (B.I.R.) with the Response Evaluation Criteria in Solid Tumors (RECIST) criteria, using the sum of the unidimensional measurements of the tumours [20].

All available formalin-fixed, paraffin-embedded RCC tumour material before treatment for each patient (nephrectomy, metastasectomy and/or tumour biopsy) was retrieved from the site where the relevant procedure was undertaken. An initial 5- μm section was cut from each formalin-fixed, paraffin-embedded sample, stained with haematoxylin and eosin, and reviewed by one pathologist (J.S.) unaware of the patient treatment and clinical outcome. Tumour areas that contained >90% clear cell RCC were marked for microdissection. Clear cell histology and grade were verified by the pathologist at the time of review of the haematoxylin and eosin-stained slide before microdissection.

For DNA extraction, two 10- μm tissue sections were manually microdissected as previously described [21]. Tissue was incubated in digestion buffer (500 mM KCl, 100 mM TrisHCl, 15 mM MgCl₂, 0.5% Tween 20), at 1 mm² tissue/ μL buffer. Proteinase K was added (0.4 $\mu\text{g}/\mu\text{L}$) for 3 days at 55 °C. DNA was concentrated using Microcon YM-30 columns (Amicon, New York, NY, USA) according to the manufacturer's instructions.

Exon: direction	Sequence	TABLE 1 The sequencing primers
1A: Forward	TGGTCTGGATCGCGAGGGAAT	
1A: Reverse	GACTGCGATTGCGAGAAGATGACCTGGG	
1B: Forward	GGCCCGTGTGCTGCGCTCGGTGAACT	
1B: Reverse	CCCTGCTGGGTCTGGGCCTAAGCGCCGGGCCCGT	
1B: Reverse 2	CCCCTGCGAAAATGGAC	
2: Forward	GTGGCTCTTAAACAACCTTTGC	
2: Forward 2	TCCCAAAGTGCTGGGATTAC	
2: Reverse	CCTGTACTTACCACAACAACCTTATC	
3: Forward	TTCCTTGTAAGTACTGAGACCCCTAGT	
3: Reverse	TACCATCAAAGCTGAGATGAAACAGTGAAGT	

For *VHL* sequencing, tumour DNA was quantified as previously described [22]. Two pairs of *VHL*-specific primers were used to amplify exon 1 (Table 1), while exons 2 and 3 were amplified with one pair of primers each [23]. Some samples required alternative primers for the amplification of exons 1 and 2. PCR was performed in 25 μL reaction mixtures consisting of 10 ng of template DNA, 0.2 μM of each primer pair, 4% DMSO, 1% Triton X-100, 0.1% gelatine, and 12.5 μL of AmpliTaq Gold PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA). Reaction mixtures were incubated at 95 °C for 5 min before 35 cycles of two-step PCR (15 s at 95 °C, 60 s at 62 °C), followed by 7 min at 72 °C. Amplification products were treated with ExoSAP-IT (USB, Cleveland, OH, USA) according to the manufacturer's instructions, and sequenced in forward and reverse directions using dideoxynucleotide labelling and the ABI3700 system. Normal reference DNA was also amplified and sequenced as a negative control for *VHL* mutation. RCC cell lines 769-p and 786-O served as positive controls for *VHL* mutation. Mutation analysis was undertaken using Mutation Surveyor software (SoftGenetics, State College, PA).

Patients with a normal *VHL* sequence had tumour DNA analysed for the presence of *VHL* promoter methylation. Methylation status was determined using *VHL* methylation-specific PCR primers after DNA bisulphite modification. Genomic DNA was modified using the CpGenomeTM DNA modification kit according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). The product then underwent PCR-based amplification using methylation-specific primers, and the methylation status was determined by gel electrophoresis of the PCR products, as previously described [24].

Descriptive statistics were calculated to characterize the patients' features. Subsets were compared using Fisher's exact test for categorical variables (e.g. response) and the Mann-Whitney *U*-test for distributions (e.g. age). TTP was defined as the interval from the first day of protocol therapy until the criteria for RECIST-defined disease progression were met, or until a patient came off the study for any reason, whichever came first. Patients still on study were censored on the date of last disease evaluation. OS was defined as the interval from the first day of protocol therapy until death from any cause or date of last contact if alive. The Kaplan-Meier product-limit method was used to estimate the probability of TTP and OS. Subsets were compared using the log-rank test. Cox's proportional hazard model was used to identify independent predictors of TTP, using the likelihood-ratio test.

RESULTS

In all, 53 patients with metastatic RCC received therapy with SU11248, AG013736 or interferon- α plus bevacizumab between February 2003 and March 2005, on one of four protocols approved by the Institutional Review Board at the authors' institution. All patients signed an approved consent for collection of tumour tissue. All patients who received any protocol therapy were considered. Ten patients were excluded from analysis for one of several reasons: diagnosis of a malignancy other than RCC (one patient with metastatic seminoma), insufficient tissue for DNA extraction (six), poor recovery of extracted DNA (one) and unamplifiable DNA (two).

Thus, 43 patients were suitable for analysis of clinical characteristics and *VHL* status; 41 of

Characteristic	N (%) of 43 patients	TABLE 2 <i>The patients' characteristics</i>
Median (range) age, years	60 (24–79)	
Gender		
Male	29 (67)	
Female	14 (33)	
ECOG performance status		
0	27 (63)	
1	16 (37)	
Site of metastases		
Lung	32 (74)	
Lymph node	22 (51)	
Liver	12 (28)	
Adrenal gland	5 (12)	
Bone	16 (37)	
Number of metastatic sites		
1	10 (23)	
2	9 (21)	
≥3	24 (56)	
Previous therapy		
Nephrectomy	43 (100)	
Radiotherapy (any)	7 (16)	
Cytokine therapy		
High-dose IL-2	12 (28)	
Low-dose cytokines (IL-2 and/or interferon-α)	23 (53)	
No previous systemic therapy	8 (19)	
Histological subtype		
Clear cell	43 (100)	
VEGF-targeted therapy received		
SU11248	24 (56)	
AG013736	11 (25)	
Bevacizumab + interferon-α	8 (19)	
Mutated <i>VHL</i>	25 (58)	<i>ECOG, Eastern Cooperative Oncology Group; IL-2, interleukin-2.</i>
Methylated <i>VHL</i>	1 (2)	

years before the analysis of *VHL* mutation and methylation status.

The patients included in the analysis were typical of a clinical trial population of those with metastatic RCC (Table 2). All patients had the clear cell histological subtype of RCC, all had had a previous nephrectomy and 81% had received previous cytokine therapy, as required by the clinical trials of SU11248 and AG013736. All eight patients who received interferon-α plus bevacizumab therapy had had no previous systemic therapy, as required by the protocol.

Twenty-six patients (60%) had evidence of either *VHL* mutation (25) or promoter methylation (one; Table 2). Mutations occurred across all exons and introns, most commonly in exon 1 (Table 3). Of all mutations, 48% were frameshifts resulting from insertions, deletions and lost splice sites, 12% were truncating nonsense mutations, and 40% altered only one or two amino acids.

There was no difference in the frequency of *VHL* mutation or methylation with age, gender, performance status, baseline laboratory values (haemoglobin level, LDH or corrected serum calcium), number of metastatic sites or previous therapy. Patients with no bone metastases had more frequent *VHL* mutation or methylation than those with bone metastases (78% vs 31%; $P=0.004$).

In all, 42 patients were evaluable for the response; one who received AG013736 was not assessed by scans after treatment and was thus not evaluable for response. There was a RECIST-defined partial response in 18 patients (43%; 95% CI 28–59%). The median (range) time to maximum objective response (greatest reduction in tumour burden) was 10.3 (1.6–23.6) months; the median change in tumour burden for all patients was –23.5 (–99 to +141)%. Seven patients continued on therapy at a follow-up of 13.9–29.2 months; all seven had a partial response.

The patients' characteristics were examined in relation to objective response; there was no objective response among the seven patients who received previous radiotherapy, which was significantly less than those not previously treated with radiation ($P=0.01$). The baseline haemoglobin level differed between those achieving an objective response and those who did not, with higher values associated with response ($P=0.05$).

Characteristic	N (%) of 25 patients	TABLE 3 <i>Characteristics of VHL mutations</i>
Location of <i>VHL</i> mutation		
Exon 1	10 (40)	
Exon 2	5 (20)	
Exon 3	7 (28)	
Intron I	2 (8)	
Intron II	1 (4)	
Type of <i>VHL</i> mutation		
Frameshift (insertion or deletion)	9 (36)	
Frameshift (loss of splice acceptor site)	3 (12)	
Nonsense	3 (12)	
Missense	7 (28)	
In-frame deletion	2 (8)	
In-frame insertion and missense	1 (4)	

the tissue specimens analysed were primary renal tumours, one was from a previous metastasectomy (soft-tissue metastasis), and one was a biopsy of an adrenal metastasis.

Tumour tissue analysed in this study had been initially procured a median (range) of 2.9 (0.08–11.4) years before starting the VEGF-targeted therapy and 3.8 (0.4–11.6)

There was no difference in response rate by baseline LDH or corrected serum calcium value. There was no difference in objective response with gender, performance status, location of metastatic sites, previous systemic therapy or age.

Of the 26 patients with *VHL* mutation or methylation, 25 were evaluable for response to therapy, excluding one who received AG013736, noted above. There was no association between the presence of a *VHL* mutation or methylation and either objective response ($P=0.53$) or overall tumour shrinkage ($P=0.99$). The small subsets precluded a statistical comparison between objective response or overall tumour shrinkage and location, type or effect of mutation on the *VHL* reading frame.

The median TTP for the entire cohort was 8.1 months; there was a difference in TTP with gender, with men having a longer TTP (median 13.3 vs 4.9 months; $P=0.02$). In addition, lack of hepatic metastases was associated with a longer TTP (median 10.8 months without liver involvement vs. 4.9 months with; $P=0.01$). Although men were less likely to have liver metastases (50% vs 17%, $P=0.04$), there was no interaction between these variables in predicting TTP. Patients with a baseline haemoglobin level of >12 g/dL had a longer TTP than those with a baseline haemoglobin of ≤ 12 g/dL (median 13.3 vs 4.9 months; $P=0.01$). There was no difference in TTP by baseline LDH or corrected serum calcium value, age (<65 vs ≥ 65 years), performance status, number of metastatic sites, nonhepatic metastatic sites or previous therapy. When the significant univariate features were considered simultaneously using Cox's proportional hazard model, a higher baseline haemoglobin and lack of hepatic metastases were significant independent predictors of a longer TTP (likelihood ratio test: haemoglobin $P=0.001$; hepatic metastases $P=0.002$).

The median TTP in patients with *VHL* mutation or methylation was 10.8 months vs 5.5 with no *VHL* mutation or methylation ($P=0.26$; Fig. 1a). Six patients with a *VHL* mutation and one with no mutation or methylation are still on study, with follow-up of 13.9–29.2 months. Patients with methylation of the *VHL* promoter or a mutation that truncated or shifted the *VHL* reading frame had a median TTP of 13.3 months vs 7.4 in patients with no methylation, truncation or shift in the *VHL* reading frame ($P=0.06$; Fig. 1b).

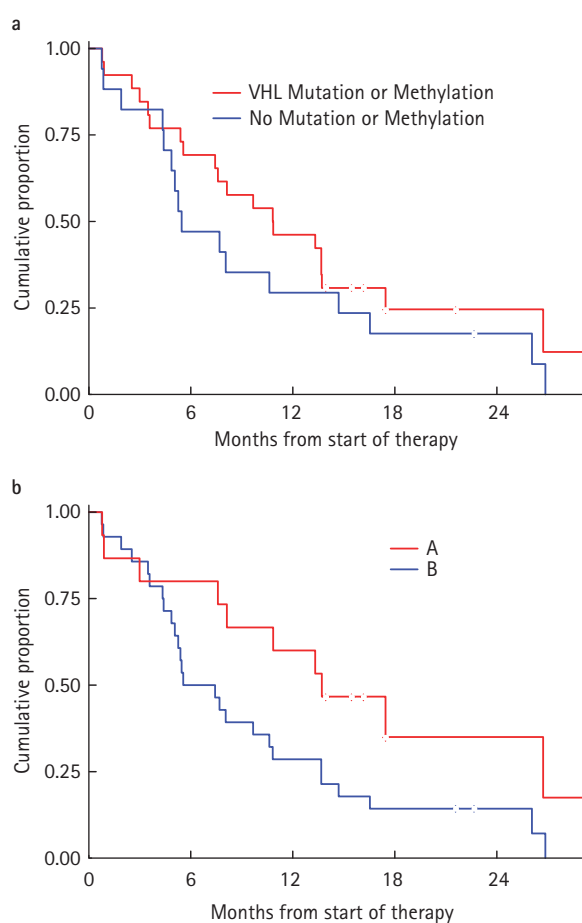


FIG. 1. The median Kaplan–Meier time to disease progression in patients with: **a**, *VHL* mutation or methylation was 10.8 months, vs 5.5 months in those with no *VHL* mutation or methylation ($P=0.26$); and **b**, with methylation, truncation or a shift in reading frame of *VHL* was 13.3 months (red) vs 7.4 months in those with no methylation, truncation, or shift in reading frame of *VHL* (blue; $P=0.06$).

The median OS was 18.0 months, with no difference with *VHL* status ($P=0.97$). Among the 23 patients who died, 22 were evaluable for response. Four achieved a partial response, 13 had stable disease (with seven having a decrease in tumour burden), and five had a best response of progressive disease. Among those who died, death was at a median of 5.2 (0–18.0) months after disease progression.

DISCUSSION

Clear cell RCC is characterized by *VHL* inactivation in most patients. The physiological consequences of this genetic event include the production of VEGF and PDGF by tumour cells, which act through receptors on nearby endothelial cells to promote angiogenesis and tumour growth. Recently, therapies directed against the VEGF protein or the VEGFR and PDGFR showed substantial clinical activity in RCC. Identifying clinical and/or tissue-based predictors of response might help to treat more patients

and further the understanding of the biology underlying RCC and response to VEGF-targeted agents. In the present analysis we sought to correlate patient clinical and tumour characteristics, specifically the presence of *VHL* mutation or methylation, and the anti-tumour effect of VEGF-targeted therapy in metastatic RCC.

The analysis showed that patients with a higher baseline haemoglobin level had a better outcome for both objective response rate and TTP, while other characteristics were associated with either an improved objective response (lack of previous radiotherapy) or improved TTP (male gender, lack of hepatic metastases). *VHL* status did not affect objective response/tumour shrinkage, but patients with *VHL* methylation or mutations that truncated or shifted the *VHL* reading frame appeared to have a longer TTP than those with no methylation, truncation or shift in the reading frame. This latter finding is hypothesis-generating and must be interpreted with caution, given the small, retrospective subset analysis, heterogeneity of

treatment received and the imprecision of delineating the impact of *VHL* status on downstream events such as VHL protein production and ultimately VEGF expression.

No previous studies specifically examined the predictive value of *VHL* status after VEGF-targeted therapy. Previous reports examined the prognostic value of *VHL* mutations in RCC tumours of all stages, without considering the systemic therapy received [25–27]. One of these analysis [26] showed that the presence of a *VHL* mutation was associated with better cancer-specific survival in a multivariate Cox proportional hazard analysis ($P = 0.023$). Another series failed to associate *VHL* mutations with a better cancer-specific survival in a subset of patients with RCC and metastatic disease [27]. Thus, there are limited and inconsistent data on the prognostic value of *VHL* mutations in RCC, and no previous predictive data. Further investigation of more uniformly treated patients is needed to define the predictive and prognostic value of *VHL* status in RCC patients.

Many of the clinical variables identified as associated with the outcome of VEGF-targeted therapy in this analysis (baseline haemoglobin level, previous radiation and hepatic metastases) were previously identified as prognostic in RCC and/or included in prognostic schemes [28–31]. The present study cohort could not be fully characterized according to these schemes, given lack of Karnofsky performance status data and a variable number of previous systemic treatment regimens. The better clinical outcome in patients with, e.g. a higher baseline haemoglobin level, might simply reflect the better prognosis of such patients regardless of therapy. However, alternative hypotheses can be proposed to explain why these specific characteristics might have predictive and prognostic value. It is possible that elevated baseline haemoglobin levels are a reflection of *VHL*-mediated production of erythropoietin and general activation of this pathway, rendering these patients more susceptible to VEGF-targeted therapies. However, erythropoietin levels were not measured in these patients, and thus this hypothesis requires prospective investigation. For hepatic metastases, enhanced sensitivity to VEGF-targeted agents might be expected, based on mouse models with conditional inactivation of *VHL* that develop a highly vascular phenotype, characterized by hepatic tumours [32,33]. Anecdotally, hepatic RCC

metastases in patients treated with VEGF-targeted therapy have shown remarkable necrosis and liquefaction, despite no overall change in tumour diameter. The finding of a worse outcome in patients with hepatic metastases in this study highlights that the biology of hepatic metastases and interaction with VEGF-targeted therapy in this subset of patients requires further investigation. Other characteristics, such as male gender and lack of previous radiotherapy, cannot at present be clearly linked to RCC tumour biology affecting the susceptibility to therapy. This study is limited by the few patients and the restricted set of clinical and laboratory features considered. Additional, prospective investigation of a more comprehensive set of clinical and laboratory characteristics in patients with metastatic RCC treated with VEGF-targeted therapy is needed.

The present analysis showed *VHL* mutation or methylation in 60% of renal tumours, consistent with previous reports. We did not analyse the loss of heterozygosity of *VHL*, after which somatic mutation of the remaining allele would render the gene inactive. However, the vast majority of RCC tumours with somatic *VHL* mutations have loss of heterozygosity at the short arm of chromosome three (3p) [1,2,26,34,35]. Patients in this series were not treated uniformly, although analysis of small subsets by VEGF-targeted treatment received showed no differences in clinical outcome by *VHL* mutation status (data not shown). Last, most of the patients received cytokine therapy after initial tissue procurement; the effect of this therapy on tumour biology and response to subsequent VEGF-targeted therapy is unknown.

The biology of RCC and the *VHL* pathway is complex. Several factors could explain the lack of a clear relationship between *VHL* status and clinical response to VEGF-targeted therapy. Lack of a greater clinical response in patients with a *VHL* mutation might be explained by one of the proposed non-angiogenic functions of the *VHL* protein, including cell-cycle control [36], chemokine receptor expression [37] and extracellular matrix assembly [38]. One or more of these functions might drive the biology in a given tumour, thus rendering the tumour less susceptible to VEGF-targeted therapy. In addition, the impact of a given *VHL* mutation on subsequent *VHL* protein structure/function, and thus on VEGF expression, is not

well-characterized. Indeed, further analysis of the present data showed that certain types of *VHL* mutations might be associated with a better outcome. Additional modelling of mutated *VHL* protein structure and function is required for further insight.

The clinical responses in tumours with no *VHL* mutation might be due to *VHL*-independent activation of HIF (e.g. through PI3K/AKT/mTOR or other downstream pathways [39]) or through the many other regulators of VEGF expression, including cytokines [40,41], growth factors [42–46], hormones [47], hypoxia [48–50] and alternative tumour-suppressor genes [51–53]. Additional study of HIF activation status and *VHL*-mediated gene and protein expression in these tumours is needed to test this hypothesis.

VEGF-targeted therapy has clear and substantial anti-tumour effects and is now a standard therapy in advanced RCC. Analysis of predictive clinical and molecular features of targeted therapeutics is critical in RCC and all cancers. Such analyses further the understanding of the biology of both the tumour and the tumour/drug interaction that leads to response. This insight provides an understanding of the mechanism of response and resistance, which can expand the benefit of existing agents and identify additional therapeutic targets. The present analysis is an initial step towards this in RCC. Additional prospective clinical and tissue analysis is required for a greater understanding of the biology of response to VEGF-targeted agents in metastatic RCC.

FOOTNOTE

Full details of specific *VHL* mutations for each individual patient are available at <http://cc.ucsf.edu/people/waldman/rini/>

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CONFLICT OF INTEREST

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Abbreviations: VEGF(R), vascular endothelial growth factor (receptor); VHL, von Hippel-Lindau; VHL, tumour-suppressor gene; HIF, hypoxia-inducible factor; platelet-derived, growth factor (receptor); LDH, lactate dehydrogenase; TTP, time to tumour progression; OS, overall survival; RECIST, Response Evaluation Criteria in Solid Tumors.