The effect of VEGF targeted therapy on biomarker expression in sequential tissue from patients with metastatic clear cell renal cancer

Kevin Sharpe1*, Grant D Stewart2*, Alan Mackay5, Christophe Van Neste9, Charlotte Rofe1, Dan Berney1, Irfan Kayani3, Axel Bex4, Elaine Wan5, Fiach C O'Mahony2, Marie O'Donnell2, Simon Chowdhury6, Rukma Doshi1, Colan Ho-Yen1, Marco Gerlinger1, Dawn Baker7, Neil Smith7, Barry Davies7, Anju Sahdev1, Ekaterini Boleti10, Tim De Meyer9, Wim Van Criekinge9, Luis Beltran1, Yong-Jie Lu1, David J Harrison2,8, Andrew R Reynolds5, Tom Powles1*.

1 Barts Cancer Institute, Queen Mary University of London, UK
2 Edinburgh Urological Cancer Group, University of Edinburgh, UK
3 Experimental Cancer Medicine Centre, University College, London, UK
4 National Cancer Institute, Netherlands, UK
5 The Institute of Cancer Research, London, UK
6 Guys and St Thomas’ Hospital, London, UK
7 Astra Zeneca, Manchester, UK
8 School of Medicine, University of St Andrews, UK
9 University of Ghent, Belgium
10 The Royal Free Hospital London, UK

Running title: Biomarker change and treatment outcome in renal cancer

Key words: Renal cancer, Resistance, Targeted therapy, Heterogeneity, Angiogenesis

*Corresponding Author:
Professor Tom Powles MRCP, MD
Head of Translational Oncology,
Experimental Cancer Medicine Centre
Barts Cancer Institute.
Queen Mary University of London
London EC1A7BE
thomas.powles@bartshealth.nhs.uk
Fax 0207 6018522
Tel 0207 6018522

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Abstract:

Purpose: To investigate how biologically relevant markers change in response to anti-angiogenic therapy in metastatic renal cell carcinoma (mRCC) and correlate these changes with outcome.

Experimental Design: The study utilised sequential tumor tissue and functional imaging (taken at baseline and 12-16 weeks) obtained from 3 similar phase II studies. All three studies investigated the role of VEGF tyrosine kinase inhibitors (TKIs) prior to planned nephrectomy in untreated mRCC (n=85). The effect of targeted therapy on 10 biomarkers was measured from sequential tissue. CGH array and DNA methylation profiling (MethylCap-seq) was performed in matched frozen pairs. Biomarker expression was correlated with early progression (progression as best response) and delayed progression (between 12-16 weeks).

Results: VEGF TKI treatment caused a significant reduction in vessel density [CD31], phospho-S6K expression, PDL-1 expression and FOXP3 expression (p<0.05 for each). It also caused a significant increase in cytoplasmic FGF-2, MET receptor expression in vessels, Fuhrman tumor grade and Ki67 (p<0.05 for each). Higher levels of Ki67 and CD31 were associated with delayed progression (p<0.05). Multiple samples (n=5) from the same tumor showed marked heterogeneity of tumor grade, which increased significantly with treatment. Array CGH showed extensive intrapatient variability, which did not occur in DNA methylation analysis.

Conclusion: TKI treatment is associated with dynamic changes relevant biomarkers, despite significant heterogeneity in chromosomal and protein, but not epigenetic expression. Changes to Ki67 and grade indicate that treatment is associated with an increase in the aggressive phenotype of the tumor.
Translational relevance: The mechanisms of acquired resistance to VEGF-targeted therapy have not been fully elucidated. By investigating renal tumor tissue taken before and after treatment, this study demonstrates that consistent changes to tumor biomarkers occur with therapy. These dynamic changes occur in clinically relevant biomarkers such as PD-1, MET and FGF-2. Candidate drugs targeting these 3 proteins are currently under investigation in renal cancer. Our data shows that CD31 (a vascular marker) falls with treatment. Failure to achieve a sustained reduction in CD31 expression is associated with poor prognosis, suggesting that continued vascular suppression is important to maximize treatment response. Treatment is associated with an increase in tumor grade and Ki-67 expression, which suggests the development of a more aggressive tumor phenotype.
**Introduction**

Anti-angiogenic tyrosine kinase inhibitors (TKIs) are established as first line therapy for metastatic clear cell renal cancer (mRCC) (1, 2). However, a marked variability of response to therapy occurs. A minority of tumors are inherently resistant to therapy (early progression) while a larger proportion are initially sensitive and subsequently develop resistance to therapy (delayed progression) (3). There are currently no clinically validated tumor biomarkers for renal cancer which predict the onset of treatment resistance (4). Most studies to date have focused on tumor samples taken prior to therapy, however, preclinical data show that treatment with anti-angiogenic agents induces dynamic changes in tumor biology (5, 6). We hypothesise that identification of these dynamic changes is required to elucidate the mechanisms of resistance to anti-angiogenic agents.

Here we prospectively collected sequential tissue from three trials that were all designed to investigate the role of anti-angiogenic tyrosine kinase inhibitors (TKIs) prior to planned nephrectomy in treatment naïve mRCC patients. The expression of relevant biomarkers before and after 12-16 weeks of TKI therapy was measured from the primary renal tumor. The absolute levels and dynamic changes to biomarkers were assessed and correlated with outcome in 85 patients. Patients with early or delayed disease progression were studied separately and compared with non-progressors. The markers examined were selected because they fulfilled one or more of the following criteria: (a) markers linked mechanistically with response and / or resistance to anti-angiogenic therapy, (b) markers of pathways that are currently under investigation as a therapeutic option in RCC, and (c) markers of immunological significance. Markers examined included: vessel density (CD31), pericyte coverage, Ki67, FGF2, MET receptor, PDL-1, phosphorylated S6K, CD45, CD3 or FOXP3 (7-15). Multiple sampling of the same tumor was performed to further explore the importance of heterogeneity in RCC. This included histological, chromosomal and DNA methylation analysis.
The hypothesis was that VEGF therapy resulted in dynamic changes to specific biomarkers which correlated with outcome, despite the presence of tumor heterogeneity. Our biomarker work was performed on tissue from the renal cancer and not metastatic sites, which are responsible for mortality. Therefore the final part of our work examined the relationship between the primary renal tumor and metastatic sites. This was performed by comparing metabolic response to VEGF therapy in these sites using FDG-PET.

**Patients, Materials and Methods**

**Clinical trials**

Patients included in this analysis originated from 3 independent prospective single arm phase II studies, two using sunitinib and one pazopanib (16-18). All 3 studies investigated VEGF TKI therapy prior to planned nephrectomy in biopsy proven untreated metastatic renal clear cell carcinoma. The trials were institutional review board approved [EudraCT 2006-004511-21, 2006-006491-38 and 2009-016675-29]. The analysis of biomarkers from the sequential tissue taken was a secondary endpoint of all of these prospective studies. Patients signed written informed consent for this research.

The 3 studies had very similar inclusion criteria: Two of the studies investigated sunitinib (50mg: 4/2 weeks) for 12-16 weeks prior to nephrectomy while the final study investigated pazopanib (800mg OD) for 12-14 weeks prior to planned nephrectomy. Both sunitinib studies are complete and published while the pazopanib study has reached interim analysis which is published. The 3 studies all included a translational component where key proteins were measured from excess tissue from the renal biopsy and nephrectomy. Paraffin embedded samples were treated in an identical manner. Sequential fresh frozen tissue was also available from one study (n=15) (2006-006491-38). VEGF TKI therapy was stopped 2-14 days (median 3 days) before the nephrectomy. All patients restarted the same VEGF TKI therapy after the nephrectomy irrespective of progression during the treatment break.
Not all patients had tissue available. Tissue was available in 56 patients at baseline and in 61 patients after 12-16 weeks of therapy. The commonest reason for tissue not being available at baseline was inadequate amounts of excess material being present after histological analysis. The commonest reason for sequential tissue not being available was treatment cessation prior to the 12-16 week time point.

Patients were separated into 3 groups 1] Early Progression: Those with progression of disease as their best response to therapy. 2] Delayed progression: Those patients with progression directly after the subsequent tissue collection at 12-18 weeks (after initial stable disease). 3] Non-progression: The remaining population. Characteristics of these patients are shown in Table 1. Patients with early and delayed progression have a significantly worse outcome than the non-progressors justifying the investigation of the 3 groups.

**Histological, CGH array and DNA methylation analysis**

Sequential samples were assessed for Fuhrman Grade, Ki67 expression and presence of necrosis by 2 independent pathologists. Subsequent histological analysis investigated five separate samples from the same tumor for heterogeneity in study NCT 2006-006491-38 (n=23). Patients were separated into low grade (Fuhrman 1 and 2), high grade (Fuhrman 3 and 4) or mixed grade (presence of both low and high grade). Those patients with mixed grade were defined as having histological heterogeneity. An indirect comparison was made with multiple biopsies taken from untreated nephrectomy samples (sourced from treatment naïve mRCC patients from the same research group) (n=22). Discrepancies between histopathologists were resolved by central review where required. Five different areas were selected from each tumor and one slide per area was assessed.

Array CGH and DNA methylation analysis (MethylCap-seq) was performed on the same sequential frozen pairs (n=14). Analysis was performed once from the biopsy and in triplicate from the nephrectomy sample for each patient.
Genomic DNA extraction was conducted using the Qiagen DNeasy Blood and Tissue (Qiagen, UK) kit as per the manufacturer’s instructions.

For array CGH, DNA was labelled with Cy3 from Nimblegen dual color labelling kit according to the manufacturer’s instructions. Similarly pooled DNA from lymphoblastoid cell lines were labelled with Cy5 and used as a reference. The sex of the cell lines was matched to that of the patient sample. Once labelled 20ug of test and reference sample were combined and added to a Nimblegen 12x135 array and hybridised, following which slides were scanned (NimbleGen MS 200 Microarray Scanner). Array CGH data were pre-processed and analysed using an in-house R script (BACE.R) in R version 2.13.0, as previously described (19, 20). Categorical aCGH states (i.e. gains, losses, and amplifications) were used for clustering, employing Wards clustering algorithm based upon Euclidean distance.

MethylCap-seq involves the in vitro capture of methylated DNA and subsequent analysis of enriched fragments by massively parallel sequencing (21). Unsupervised hierarchical clustering analysis was performed. Data analysis was performed with R 2.15.3.

Protein analysis

A tissue microrarray (TMA) was constructed from biopsy and nephrectomy tissue samples. For protein analysis, sections were pre-treated using heat-mediated antigen retrieval with sodium citrate buffer. The following antibodies were used to assess protein expression; CD31 (1:600, AstraZeneca), FGF-2 (1:100, Peprotech), MET receptor (1:200, Invitrogen), p-S6K (1:200, Cell Signalling Technology), CD3 (1:100, Abcam), CD45 (1:100, Dako), FOXP3 (1:200, Abcam), PDL-1 (1:200, Abcam). Appropriate isotype controls were employed in all cases. Results were analysed centrally by 2 histopathologists who were blinded to patient outcome data [RD, DB]. Vessel density (CD31), CD45, CD3 or FOXP3 +ve immune cells, were quantified using a computerised image analysis system (ARIOL, Applied Imaging, Genetix) using visually-trained parameters. To determine pericyte coverage, TMAs
underwent triple staining for CD31, alpha smooth muscle actin (ASMA) and DAPI and the percentage of ASMA coated vessels was scored by two observers.

**Radiological and FDG PET-CT assessment**

All patients were assessed according to RECIST v1.1. CT was performed at base line, prior to nephrectomy (week 12-14) and after nephrectomy (week 16-18). Patients then followed up with regular imaging until progression. Radiological analysis took place centrally.

FDG PET CT scans were performed at baseline and prior to nephrectomy (48 hours after cessation of VEGF TKI therapy) in 34 patients. Changes in SUV to positive lesions (SUV >2.5) with therapy was measured. A correlation between the primary renal lesion and the metastatic sites was examined. The correlation between changes in SUV and outcome in this population had been previously published (22).

**Statistical analysis**

Analysis of the data took place centrally in November 2012. Descriptive statistics were used to compare groups. Univariable and multivariable analysis was performed. Further analysis was performed to investigate if dynamic changes to biomarkers correlated with outcome. Student’s t-tests were used to compare biomarker levels in progressors and non-progressors. A p-value of <0.05 was considered to be significant. Outcome was estimated using the Kaplan-Meier method. A Fisher’s Exact test was used to compare groups expressing different histopathological features.
Results

Patient characteristics

The CONSORT diagram in Figure 1 shows the disposition of patients at baseline and their subsequent course on study. All 85 patients were diagnosed with untreated metastatic clear cell RCC. All patients started VEGF targeted therapy with the intention of having subsequent tissue taken. Table 1 compares patient’s characteristics according to their best response to therapy. Seventeen patients had early disease progression while 24 patients had delayed progression and 44 were in the non-progression group. Survival was significantly different in these three groups with early progressors having the worst outcome and those without progression having the best outcome (see Table 1). The survival for patients treated with pazopanib and sunitinib was similar (HR for pazopanib =1·16 [95% CI: 0·64-2·1]). A number of patients did not have a nephrectomy (n=19). Prior progression of disease/cessation of therapy (n=17) being the most common reason for this.

Biomarker expression and outcome from baseline tissue

To investigate biomarkers associated with early or delayed progression, baseline untreated tissue was analysed (Figure 2). None of the 10 biomarkers correlated with progression.

The effect of treatment on tumor pathology and biomarkers

Paired tumor tissue showed a significant increase in Fuhrman grade (increased = 61% of patients, vs. reduction = 29%, p<0·05), Ki67 (increased = 63%, vs. reduction=23%. p<0·05) and necrosis (increased=58%, vs. reduction=2%, p<0·05) with therapy. The proportion of samples scored Fuhrman Grade ≥ 3 increased with treatment from 31% to 78% (p<0·05).

IHC demonstrated that treatment had a significant effect on several tumor markers including CD31 [tumor vessel density] (median change -56%, p<0·05), cytoplasmic FGF2 (+90%, p<0·05) and MET receptor expression in blood vessels (p<0·05). A significant change in p-S6K (-50%: p<0·05) and changes in immune cell markers PDL-1 (-34%, p<0·05) and FOXP3 (p<0·05)
were also observed (Figure 3a). No significant changes occurred in other biomarkers examined. The comparative effect of sunitinib and pazopanib on biomarker expression was investigated. There was no difference in the effects of the 2 drugs on the expression of the majority of biomarkers in the paired samples. However, sunitinib but not pazopanib, resulted in reduced expression of the immune cell markers CD45 and CD3 [median change -84% vs. +13% (p<0·05); median change -38% vs. +117% (p<0·05) respectively].

**Biomarker expression and outcome from treated tissue**

Subsequently we examined if any of the biomarkers which significantly changed with therapy (Figure 3a) correlated with delayed progression of disease. Ki-67 and CD31 expression was significantly higher in tissue taken from patients with delayed progression compared to non-progressors (p<0·05 for both) (Figure 3b).

An increase in CD31 with therapy also correlated with reduced overall survival (p<0.05). The only other biomarker in which a change was associated with a poor outcome was Fuhrman grade, where an increase was associated with a poor outcome (p<0.05).

**The relationship between biomarker expression and overall survival**

In multivariable analysis, significant baseline prognostic factors included Heng prognostic score [HR 4·61 (1·30-16·31)], number of metastatic sites [3·37 (HR 1·16-9·78)], tumor grade [HR 6·62 (2·02-21·63)] and high pericyte coverage at baseline [HR=4·20 (1·48-11·9: p<0·05)]. The prognostic significance of a change in the biomarkers with therapy (above and below the median change) was also examined. Multivariable analysis revealed a reduction in pericyte coverage was the only factor associated with a poor outcome [HR 3·65 (1·36-9·80) p=0·01].

**Tumor heterogeneity in treated and untreated samples**

Five samples were taken from treated tumors (n=23) to examine for heterogeneity of tumor grade and biomarker expression (n= 5). The majority of treated tumors exhibited both low and high grade histological features
(mixed features). Mixed histological grade was significantly higher in the treated group compared to the untreated group (65% vs. 20%, p<0.05) (Figure 4a). Variability in biomarker expression between different regions of the same tumor was also observed (Figure 4b). GCH array analysis showed marked intra-patient variability in multiple samples (n=3) taken from the same patient. Only 2 of 15 patient's tumors clustered (Figure 4c). Further analysis showed no increase in DNA instability in the treated samples compared to untreated samples. There were no consistent chromosomal changes associated with sunitinib therapy. Analysis of the DNA methylation data showed consistent clustering of individual patient's tumors irrespective of exposure to sunitinib treatment (Figure 4d).

The relationship between the primary renal tumor and the metastatic sites
Sequential FDG-PET CT scans were performed at baseline and after 3 cycles of sunitinib (i.e. at the time of the tissue collection) in 34 patients. The relationship between the metabolic activity in the primary renal tumor and metastatic sites was examined. There was a positive correlation between baseline metabolic activity (SUV) in the primary tumor and the metastatic sites for individual patients (r=0.72: p<0.01) (Figure 5a). There was also a significant correlation in the metabolic response (ΔSUV) between response in the primary tumor and metastatic sites (r=0.46 p<0.01) (Figure 5b).
Discussion

There is currently a lack of tissue biomarkers to predict response to VEGF receptor-targeted TKIs. In this work, sequential tissue taken before and during TKI treatment was used to explore the hypothesis that dynamic changes to biomarker expression occur in resistance. We focused on the correlation of biomarker expression with progression (initial and delayed) rather than response. This is because initial response does not appear to correlate with outcome (unlike progression) and usually occurs early in the treatment process before acquired resistance appears (23). Correlation between radiological response (RECIST v1.1) and biomarker was performed but did not identify prognostic changes (data not shown).

Efficacy of VEGF receptor-targeted TKIs is thought to stem primarily from suppression of tumor angiogenesis. Here, TKI treatment significantly reduced tumor vessel density, supporting recent findings obtained in sequential tissue from mRCC patients (12). Importantly, we show also that failure to achieve a reduction in vessel density is associated with progression of disease. Continued suppression of the vasculature is therefore important for continued clinical response, supporting the results of a recent randomised phase III trial (24). The inhibitory effect of TKIs on the PI3K pathway (pS6K) demonstrated the broad effect of these agents and suggests possible overlapping mechanism of action with the mTOR inhibitors which also target the PI3K pathway. This may be a potential explanation for cross resistance that is speculated to occur between these 2 classes of agents in mRCC.

Preclinical studies suggest that VEGF-targeted therapy may increase tumor aggressiveness, including accelerated tumor growth (25), increased tumor invasion (26) and increased metastasis (25, 26). If manifested in patients, these effects would limit therapeutic efficacy, however, there is little or no compelling clinical evidence that VEGF-targeted therapy leads to increased tumor aggressiveness (27). Here we show that tumor grade and tumor cell proliferation were both increased in mRCC patients after treatment with VEGF.
TKIs. Also, increased proliferation (Ki-67) was associated with a poor prognosis. Therefore, we present the first evidence for a phenotype of increased tumor aggressiveness in response to VEGF-targeted therapy in human mRCC. It is possible that some of these effects, especially on tumor grade, are related to the treatment stress of VEGF therapy. This effect occurs in prostate cancer, where LHRH agonist result in a more aggressive phenotype due to treatment related factors (28).

FGF-2 may mediate resistance to VEGF-targeted therapy by providing an alternative pro-angiogenic signal for endothelial cells (6, 9). Increased circulating concentrations of FGF-2 have been reported in patients progressing on VEGF-targeted therapy (29). However, the relevance of this circulating pool is not clear, because FGF2 exerts a paracrine effect to promote angiogenesis and tumor cell proliferation (30). Here we confirm, for the first time, that in situ tumor expression of FGF-2 is increased as a result of TKI treatment. Combined, these data support a strategy to target both VEGF and FGF receptor signalling in order to achieve sustained suppression of mRCC tumor growth (9). Dovitinib targets both VEGF and FGF receptor signalling and is currently under investigation in the TKI refractory setting.

Preclinical studies show that VEGF-targeted therapy results in up-regulated expression of MET receptor in tumor cells, facilitating increased tumor invasion and metastasis (31, 32). Here we found no evidence for up-regulated MET receptor in the tumor cell compartment. Instead, we found significant up-regulation of MET receptor in the vascular compartment in response to TKI. Of interest, the MET receptor ligand, hepatocyte growth factor (HGF), may also mediate resistance to VEGF-targeted therapy by providing an alternative pro-angiogenic signal for endothelial cells (8). Conceivably, up regulated expression of MET in the vascular compartment could help facilitate this mechanism of acquired resistance.

Pericyte coverage of vessels in human tumors is variable, with conflicting reports regarding the prognostic significance (32, 33). Our results show that
high baseline levels of pericyte coverage correlate with a poor outcome, supporting previous work in RCC (33). Conflicting data also exist regarding the effects of anti-angiogenic therapy on pericyte coverage (32, 34, 35). Here we demonstrate that therapy can exert opposing effects on pericyte coverage (increase in 13%, decrease in 26% and no change in 61%). We also observed that a reduction in pericyte coverage adversely effected survival in multivariable analysis. This may be related to the fact that pericyte loss can compromise tumor vessel integrity, promoting metastasis (32, 35). Since loss of pericytes was not associated with progression in our study, but did strongly associate with overall survival, there may be a tumor growth-independent effect of pericyte loss on outcome.

Immune therapy has been used in renal cancer and is currently under evaluation in the form of PD1/PDL-1 inhibitors (36). Our work support in vivo experiments which show that VEGF TKIs have specific effects on immune parameters, including regulatory T cell expression. The suppression of PDL-1 required particular attention as the biomarker is thought to predict response to PD-1 inhibitors (37). This alteration questions the use of archived untreated tissue to estimate VEGF resistant biomarker expression.

Recent exome analysis revealed the existence of significant regional heterogeneity from one patient with mRCC (38). Our histology, protein and chromosomal analysis shows marked intra-tumoral heterogeneity across a cohort of patients. However DNA methylation results showed all samples from individual patients clustered. This suggests the variability seen in protein expression and histology results more from genetic rather than epigenetic instability. Consistent chromosomal changes were not seen with sunitinib treatment. This may in part be due to the marked heterogeneity.

The presence of heterogeneity is challenging as it may mask the detection of biologically significant changes due to sampling bias. However, despite this heterogeneity, we still observed significant changes in several key biomarkers, including CD31, Ki67, S6K, PDL-1, MET receptor and FGF2. In our opinion, the ability to detect a significant change in these biomarkers...
across a sample that is intrinsically heterogeneous, only acts to further reinforce the biological significance of these changes.

Finally, a positive correlation between a metabolic response in the primary tumor and the metastatic sites occurred, suggesting that primary and metastatic sites may respond in a similar fashion to therapy. However, it does not necessarily mean that dynamic changes to biomarkers in the metastatic sites match those seen in the primary tumor. Collection of sequential tissue from specific metastatic sites is extremely challenging. Therefore, further exploration of this relationship may not be possible.

There are several shortcomings in this work. Specifically, not all patients were able to have sequential tissue taken due to lack of excess tissue for sampling and patients coming off study. Also, the tissue originated from three rather than one study (although they were almost identical in design). Despite being the largest series to report in this setting the number of samples remains modest. Moreover multiple testing occurred due to the number of biomarkers selected. In addition, our analysis of sequential biomarker expression may be influenced by the natural course of disease rather than treatment. Finally, this work was a post hoc analysis of tissue from 3 studies and was exploratory in nature. Therefore these results are hypothesis generating and require further exploration in clinical and laboratory bases models. While potential biomarkers from sequential RCC tissue (such as CD31) appear feasible, there are major challenges before this becomes clinical practice. The 2 major hurdles are the feasibility of taking sequential tissue in large randomised trials and the chromosomal and protein variability effecting biomarker expression.
References


Table 1: Patient characteristics prior to study entry

<table>
<thead>
<tr>
<th></th>
<th>All patients*</th>
<th>Early PD</th>
<th>delayed PD</th>
<th>Patients without early or delayed PD</th>
</tr>
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<tbody>
<tr>
<td>Number of patients</td>
<td>85</td>
<td>17</td>
<td>24</td>
<td>44</td>
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<tr>
<td>Age</td>
<td>61 (range: 38-82)</td>
<td>62 (range: 42-82)</td>
<td>60 (range: 41-78)</td>
<td>60 (range: 38-75)</td>
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<tr>
<td>Gender</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>male</td>
<td>65 (76%)</td>
<td>13 (76%)</td>
<td>16 (67%)</td>
<td>36 (82%)</td>
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<tr>
<td>female</td>
<td>20 (24%)</td>
<td>4 (24%)</td>
<td>8 (33%)</td>
<td>8 (18%)</td>
</tr>
<tr>
<td>MSKCC group risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>intermediate</td>
<td>63 (74%)</td>
<td>10 (59%)</td>
<td>17 (71%)</td>
<td>36 (82%)</td>
</tr>
<tr>
<td>poor</td>
<td>22 (26%)</td>
<td>7 (41%)</td>
<td>7 (29%)</td>
<td>8 (18%)</td>
</tr>
<tr>
<td>Number of metastatic sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28 (33%)</td>
<td>1 (6%)</td>
<td>7 (29%)</td>
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</tr>
<tr>
<td>2</td>
<td>33 (39%)</td>
<td>10 (59%)</td>
<td>9 (38%)</td>
<td>14 (32%)</td>
</tr>
<tr>
<td>3+</td>
<td>24 (28%)</td>
<td>6 (35%)</td>
<td>8 (33%)</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Pazopanib</td>
<td>27 (32%)</td>
<td>3 (18%)</td>
<td>10 (42%)</td>
<td>14 (32%)</td>
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<tr>
<td>Sunitinib</td>
<td>58 (68%)</td>
<td>14 (82%)</td>
<td>14 (58%)</td>
<td>30 (68%)</td>
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<tr>
<td>Dose reduction prior to progression</td>
<td>27 (32%)</td>
<td>1 (6%)</td>
<td>9 (28%)</td>
<td>17 (39%)</td>
</tr>
<tr>
<td>Platelet count</td>
<td>300 (76-857)</td>
<td>294 (76-857)</td>
<td>311 (112-627)</td>
<td>309 (98-722)</td>
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<td>Neutrophil count</td>
<td>5·8 (2·3-15·5)</td>
<td>5·35 (2·3-13·3)</td>
<td>6·2 (2·3-15·5)</td>
<td>5·8 (2·4-15·2)</td>
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<td>Best response to treatment</td>
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<td></td>
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<tr>
<td>PD</td>
<td>17 (20%)</td>
<td>17 (100%)</td>
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<tr>
<td>SD</td>
<td>54 (64%)</td>
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<td>14 (17%)</td>
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<td>Site of disease progression</td>
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<td>Lung</td>
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<td>10 (59%)</td>
<td>11 (46%)</td>
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<tr>
<td>Liver</td>
<td>6 (7%)</td>
<td>3 (18%)</td>
<td>3 (13%)</td>
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<tr>
<td>Bone</td>
<td>6 (7%)</td>
<td>1 (6%)</td>
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<td>Brain</td>
<td>5 (6%)</td>
<td>2 (12%)</td>
<td>3 (13%)</td>
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<td>Lymph nodes</td>
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<td>4 (17%)</td>
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<td>Reason for no nephrectomy</td>
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<tr>
<td>Progressive disease</td>
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<tr>
<td>Patient choice</td>
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<td></td>
</tr>
<tr>
<td>Other</td>
<td>2 (2%)</td>
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<td></td>
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<tr>
<td>Total</td>
<td>24 (28%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose reduction within prior to planned sequential biopsy</td>
<td>23 (26%)</td>
<td>5 (31%)</td>
<td>8 (33%)</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Access to further targeted therapy</td>
<td>22 (26%)</td>
<td>4 (25%)</td>
<td>7 (29%)</td>
<td>11 (25%)</td>
</tr>
<tr>
<td>Overall survival (months)</td>
<td>18</td>
<td>4·1</td>
<td>14·7</td>
<td>25·3</td>
</tr>
<tr>
<td></td>
<td>14·7-23</td>
<td>2·11·2</td>
<td>11·0-20·4</td>
<td>22·5-NA</td>
</tr>
</tbody>
</table>

* Two patients were not included due to early cessation of treatment.

** Significantly longer OS compared to patients with early and late progression (p<0.05).
Figure legends

Figure 1 is a CONSORT diagram showing the disposition of patients at baseline and their subsequent course on study.

Figure 2 compares expression in biomarker patients with and without disease progression. The tissue to investigate biomarker expression was taken at baseline, prior to any therapy. It separated patients in 3 groups (patients with early progression, those with delayed progression and those without progression). A Student’s t-test was used to compare progressors and non-progressors and p-values < 0.05 were considered significant. Patients with delayed progression do not have significantly different biomarker expression compared to non-progressors in tissue taken prior to treatment. Furthermore early progression was not associated with a significantly different expression of any of the markers investigated.

Figure 3 (a) investigates the effect of treatment on biomarker expression. Analysis of biomarkers occurred only where paired samples (untreated and treated in the left and right column respectively) where available. Pre-treatment samples were taken before the onset of therapy and post-treatment samples were taken 12-16 weeks after the treatment onset. A proportion of patients did not have post treatment samples taken (n=17). The untreated samples act as a control to identify the effects of treatment on tissue. (b) investigates the association of delayed progression with those biomarkers that changed significantly with treatment. Tissue was taken after 12-16 weeks of therapy. Patients were separated into 2 groups (those with delayed progression and those without progression). Patients with delayed progression have higher levels of Ki67 and CD31 (a Student’s t-test was used and p-values < 0.05 were considered significant).

Figure 4 (a) The stacked bar chart demonstrates increased morphological heterogeneity in sunitinib treated patient samples compared with sunitinib
naive patients (P<0·01, Fisher’s Exact Test). Numbers in the bar charts indicate the number of patients in each category. Tissue sample examples show sections classified as low grade (Furman 1 or 2) or high grade (Furman grade 3 or 4) by 2 histopathologists (DH and MO). (b) Examples of IHC intrapatient heterogeneity in treated samples. Scores for CD31, Ki67 and CD45 are shown from patients with at least five cores available from different tumor regions taken after treatment. (c): Analysis was performed on 15 matched pairs of untreated (biopsy) and treated (nephrectomy) tissue. Categorical aCGH states (i.e. gains, losses, and amplifications) were used for clustering, employing Wards clustering algorithm based upon Euclidean distance. (d) Hierarchical clustering of DNA methylation data. Analysis was performed on 14 matched pairs of untreated (biopsy) and treated (nephrectomy tissue). The 1000 loci featured by the largest variance (after quantile normalization and log transformation) were used for clustering, employing complete clustering based upon Euclidean distance.

Figure 5: Sequential FDG-PET CT scans performed. The metabolic activity (SUVmax) (figure 5a) and response (Δ SUV) (figure 5b) in a metastatic target lesion (SUV max) and the primary renal tumor were compared. The baseline scan was performed 48 hours prior to sunitinib. The subsequent scan occurred prior to the nephrectomy (patients had been off therapy for 48 hours).
Figure 1. CONSORT diagram

(a)

Reasons for unavailable tissue
• Inadequate material after biopsy (n=26)
• Tissue missing (n=3)

Reasons for unavailable tissue
• Early disease progression (n=17)
• Other health issues (n=2)

Tissue available at baseline (n=56)

Tissue available after therapy (n=66)

Continued response (n=42)
Delayed progression (n=24)

(b)

<table>
<thead>
<tr>
<th>Study (NCT)</th>
<th>Agent</th>
<th>Number</th>
<th>Tissue available at baseline*</th>
<th>Patients with primary progression</th>
<th>Tissue** available at 12-14 wks</th>
<th>Patients with delayed Progression</th>
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</thead>
<tbody>
<tr>
<td>2006-006491-38</td>
<td>sunitinib</td>
<td>N=36</td>
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<td>10</td>
<td>26</td>
<td>10</td>
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<tr>
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<td>7</td>
<td>4</td>
<td>17</td>
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<tr>
<td>2009-016675-29</td>
<td>pazopanib</td>
<td>N=27</td>
<td>23</td>
<td>3</td>
<td>23</td>
<td>9</td>
</tr>
</tbody>
</table>

Total N=85

* Tissue was not available at baseline due to inadequate viable tissue after diagnosis of clear cell renal cancer was made
** Tissue was not available at surgery due to prior progression of disease or other health issues
Figure 2: The relationship between biomarker expression and disease progression from untreated tumor samples

- CD31 score
- FGF-2 cytoplasm
- FGF-2 nuclear
- Met tumour score
- Met +ve vessels (%)
- Pericyte coverage (%)
- CD45 score
- CD3 score
- PD-L1 score
- s6K score

*p = 0.48
*p = 0.19
*p = 0.99
*p = 0.48
*p = 0.91
*p = 0.15
*p = 0.15
*p = 0.98
*p = 0.09
*p = 0.29
*p = 0.16
*p = 0.78
*p = 0.91
*p = 0.97
*p = 0.66
Figure 3: Molecular markers before and after 12-16 weeks of VEGF TKI therapy in matched pairs:

(a)  
- CD31: score (%)  
  - Pre-treatment: 1  
  - Post-treatment: 2  
  - p < 0.01

- Cytoplasmic FGF-2  
  - Pre-treatment: 300  
  - Post-treatment: 400  
  - p < 0.01

- Nuclear FGF-2  
  - Pre-treatment: 100  
  - Post-treatment: 200  
  - p = 0.59

- Met - tumour cells  
  - Pre-treatment: 400  
  - Post-treatment: 400  
  - p = 0.84

- Met +ve vessels (%)  
  - Pre-treatment: 0  
  - Post-treatment: 0  
  - p = 0.04

- Pericapsule coverage (%)  
  - Pre-treatment: 0  
  - Post-treatment: 0  
  - p = 0.17

- CD8+ T-cells (%)  
  - Pre-treatment: 0  
  - Post-treatment: 0  
  - p = 0.20

- CD3+ T-cells (%)  
  - Pre-treatment: 0  
  - Post-treatment: 0  
  - p = 0.29

(b)  
- KDR score  
  - delayed progression: 100  
  - no progression: 80  
  - p = 0.02

- CD3+ score (%)  
  - delayed progression: 3  
  - no progression: 4  
  - p = 0.04

- FGF-2 cytoplasm  
  - delayed progression: 100  
  - no progression: 200  
  - p = 0.39

- c-met +ve vessels  
  - delayed progression: 0  
  - no progression: 5  
  - p = 0.36

- sK score  
  - delayed progression: 400  
  - no progression: 300  
  - p = 0.95

- PD-L1 score  
  - delayed progression: 300  
  - no progression: 400  
  - p = 0.21

- FOXP3 +ve cells (%)  
  - delayed progression: 0  
  - no progression: 0  
  - p = 0.41
Figure 4  
(a) A comparison of histology heterogeneity in VEGF treated (n=23) and untreated samples (n=22).

(b) Heterogeneity of biomarker expression from the same tumor.

(c) Array CGH analysis performed on sequential tissue before and after sunitinib: multiple samples were taken from the treated tissue.

(d) DNA methylation analysis performed on sequential tissue before and after sunitinib: multiple samples were taken from the treated tissue.
Figure 5 (a) The correlation between SUVmax in the metastatic sites and SUV max in the primary renal tumor with treatment for individual patients.

(b) The correlation between the change in SUV max in the metastatic sites and the change in SUV max in the primary renal tumor

\[ R = 0.72 \ p < 0.01 \]

\[ r = 0.46 \ p < 0.01 \]
The effect of VEGF targeted therapy on biomarker expression in sequential tissue from patients with metastatic clear cell renal cancer

Kevin Sharpe, Grant D Stewart, Alan Mackay, et al.

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