Monitoring the Vascular Response and Resistance to Sunitinib in Renal Cell Carcinoma In Vivo with Susceptibility Contrast MRI

Simon P. Robinson, Jessica K.R. Boult, Naveen S. Vasudev, and Andrew R. Reynolds

Abstract

Antiangiogenic therapy is efficacious in metastatic renal cell carcinoma (mRCC). However, the ability of antiangiogenic drugs to delay tumor progression and extend survival is limited, due to either innate or acquired drug resistance. Furthermore, there are currently no validated biomarkers that predict which mRCC patients will benefit from antiangiogenic therapy. Here, we exploit susceptibility contrast MRI (SC-MRI) using intravascular ultra-small superparamagnetic iron oxide particles to quantify and evaluate tumor fractional blood volume (fBV) in the absence of any change in tumor volume. This response was associated with significantly lower microvessel density ($P < 0.01$) and lower uptake of the perfusion marker Hoechst 33342 ($P < 0.05$). The average pretreatment tumor fBV was negatively correlated ($R^2 = 0.92, P < 0.0001$) with sunitinib-induced changes in tumor fBV across the cohort. SC-MRI also revealed suppressed fBV in tumors that acquired resistance to sunitinib. In conclusion, SC-MRI enabled monitoring of the antiangiogenic response of 786-0 RCC xenografts to sunitinib, which revealed that pretreatment tumor fBV was found to be a predictive biomarker of subsequent reduction in tumor blood volume in response to sunitinib, and acquired resistance to sunitinib was not associated with a parallel increase in tumor blood volume.

Introduction

Antiangiogenic therapy has shown considerable efficacy in metastatic renal cell carcinoma (mRCC; ref. 1). Newly diagnosed mRCC patients are now treated with VEGF receptor tyrosine kinase inhibitors (sunitinib or pazopanib) as standard of care (2, 3). Approximately 80% of patients with mRCC achieve an initial period of disease control with these agents, whereas approximately 20% of patients derive no benefit. However, even responding patients inevitably progress due to acquired resistance that typically develops after a period of several months on treatment (4). Two modes of resistance to antiangiogenic therapy are thus currently recognized: innate resistance, whereby the tumor fails to respond to the therapy from the outset, and acquired resistance, whereby after a period of response to therapy, the tumor begins to regrow (5, 6). Both forms of resistance may arise due to the presence of alternative mechanisms of tumor vascularization, which are VEGF independent and allow the tumor to evade the effects of the targeted agent. These mechanisms are however poorly understood, and there are currently no validated biomarkers that predict which mRCC patients will benefit from antiangiogenic therapy.

Noninvasive imaging approaches that facilitate the detection of changes in tumor biology may form the basis for improved predictive biomarkers. Advances in imaging technologies provide a means of defining quantitative biomarkers to inform on biologically relevant structure–function relationships in tumors (7). Such imaging methods enable a better understanding of the behavior and heterogeneous distribution of such associations and inform on response and resistance to treatment (8). In addition to quantifying any therapy-induced volumetric change in vivo, functional imaging methods can also provide additional mechanistic insight.

Perfusion CT and dynamic contrast-enhanced (DCE) MRI using low molecular weight gadolinium chelates have been widely used to assess patients with mRCC and response to VEGF signaling inhibitors (9). However, these clinical studies suffered from marked measurement variability, particularly with DCE MRI. Alternative functional MRI techniques are thus being evaluated to provide more specific imaging biomarkers for the assessment of tumor vascular function and response in vivo. One approach, susceptibility contrast MRI, involves measuring the
uptake and distribution of intravenously administered ultrasmal superparamagnetic iron oxide (USPIO) particles, composed primarily of an iron (Fe$^{3+}$) oxide crystalline core with a biocompatible coating (10). USPIO particles create large susceptibility effects that increase the transverse MRI relaxation rate $R_2^*$, and whose long intravascular half-life enables steady-state, high-resolution measurements of $R_2^*$ (11). Quantitation of fractional blood volume ($fBV$, %), derived from measurements of the absolute increase in tumor $R_2^*$ following the administration of USPIO particles, provides a sensitive imaging biomarker of response to vascular-targeted therapies (12–14).

The mechanisms of resistance to antiangiogenic therapy can be investigated using preclinical cancer models. We have previously established that subcutaneous xenografts of the 786-O renal cancer cell line can demonstrate resistance to sunitinib treatment (15). The aims of this study were to (i) evaluate $fBV$ derived from susceptibility contrast MRI as a noninvasive predictive imaging biomarker of 786-O xenograft response in vivo, and (ii) interrogate the vascular phenotype of 786-O xenografts exhibiting acquired resistance to sunitinib.

Materials and Methods

Cell culture, tumor propagation, drug formulation, and treatment

Human 786-O RCC cells (ATCC, LCG Standards; purchased 2011) were cultured in RPMI1640 medium supplemented with 10% FBS (Invitrogen) and maintained at 37°C in a humidified incubator with an atmosphere of 95% air, 5% CO2. Resuscitated 10% FBS (Invitrogen) and maintained at 37°C. Treatment Cell culture, tumor propagation, drug formulation, and Materials and Methods

The parameters of tumor $fBV$ (%) were subsequently calculated using the USPIO-induced change in $R_2^*$ approach (19). Parametric maps of tumor $fBV$ (%) were subsequently calculated using the USPIO-induced change in $R_2^*$ maps were calculated from the MGRE images acquired prior to and following administration of USPIO particles by fitting a single exponential to the signal intensity echo time curve on a voxel-by-voxel basis using a Bayesian maximum a posteriori approach (19).

IH and fluorescence microscopy

IH detection of endomucin was used to assess tumor microvessel density (MVD; ref. 15). Formalin-fixed paraffin-embedded sections were incubated with rat monoclonal anti-endomucin antibodies (#SC65495, Santa Cruz Biotechnology), and immunoreactivity was detected with biotinylated anti-rat IgG secondary antibodies and a DAB Substrate Kit (Vector). Slides were counterstained with hematoxylin prior to mounting in DEPEX, and visualized on a BX51 microscope (Olympus Optical). Endomucin-positive vessels were counted in 5 randomly selected high-power fields ($\times 100$) for each tumor and the number converted into vessels/mm$^2$.

Separate cohorts of mice bearing 786-O xenografts treated for 2 weeks with 40 mg/kg/day sunitinib or vehicle control were injected via a lateral tail with the perfusion marker Hoechst 33342 (Sigma-Aldrich; ref. 21). After 1 minute, tumors were rapidly excised and snap frozen over liquid nitrogen. Fluorescence signals from Hoechst 33342 were subsequently detected above a constant threshold at 365 nm from 10-μm thick frozen whole tumor sections (3 per tumor) using a motorized scanning stage (Prior Scientific Instruments) attached to the BXX1.
microscope, driven by image analysis software (CellP, Soft Imaging System). The area of each tumor section with Hoechst 33342 fluorescence was determined and expressed as a percentage of the whole tumor area, as described previously (12, 22).

Statistical analysis
All statistical analyses were performed using GraphPad Prism version 6.07. Results are presented in the form mean ± 1 SEM. Following application of a Shapiro–Wilk normality test to confirm the Gaussian distribution of the data, significance testing employed Student two-tailed t test, assuming unequal variances with a 5% level of significance. Significant correlations were determined using linear regression analysis.

Results
Susceptibility contrast MRI with USPIO particles measures fractional tumor blood volume of 786-0 xenografts
Susceptibility contrast MRI incorporating the use of USPIO particles was used to assess the perfused vasculature of subcutaneous 786-0 xenografts. The schematic data in Supplementary Fig. S1 shows a T2-weighted anatomic image (Supplementary Fig. S1A), gradient-recalled echo (GRE) images pre- and postadministration of USPIO particles (Supplementary Fig. S1B and S1C), and the calculated parametric fBV map (Supplementary Fig. S1D) obtained from a representative 786-0 xenograft. These data show that successful injection and delivery of USPIO particles into the tumor intravascular compartment resulted in a clear reduction in GRE image intensity in perfused tumor areas, allowing calculation of a parametric fBV map. Furthermore, administration of USPIO particles resulted in no noticeable adverse effects to the mice or tumor growth, and no significant difference in tumor baseline $R_2^*$ measured pre- and posttreatment, indicating no sequestration of USPIO particles that could influence subsequent fBV measurements.

Tumor fBV maps obtained from a representative mouse bearing a 786-0 xenograft prior to and 2 weeks after daily treatment with 40 mg/kg sunitinib are shown in Fig. 1A and B. A marked reduction in fBV was consistently observed post-treatment, which was primarily associated with the tumor core. The fBV cumulative frequency curves for the same 786-0 xenograft are shown in Fig. 1C. The mean pre- and posttreatment fBV values for this tumor were 9.6% and 2.3%.

Figure 1.
The antiangiogenic activity of sunitinib in 786-0 RCC xenografts can be assessed in vivo using susceptibility contrast MRI. Parametric fBV maps calculated from a 786-0 xenograft prior to (A) and following 2 weeks of daily treatment with 40 mg/kg sunitinib orally (B). C, Cumulative frequency curves of fBV obtained from the same 786-0 xenograft. The mean pre- and posttreatment fBV values for this tumor were 9.6% and 2.3%. D, Line series of fBV determined for each 786-0 xenograft imaged pre- and posttreatment.
xenograft pre- and posttreatment revealed a marked left shift in distribution toward smaller values, with a substantial increase in the proportion of voxels with fBV below 5% following treatment (Fig. 1C). Susceptibility contrast MRI revealed a reduction in fBV in 8 of 9 treated tumors (Fig. 1D), resulting in a significant (P < 0.01) 71% reduction in the cohort mean fBV in the absence of any significant change in cohort mean tumor volume (Table 1). This response was associated with a significant (P < 0.01) reduction in MVD, as assessed by IHC detection of endomucin-positive vessels (Fig. 2A), and significantly (P < 0.05) reduced perfusion as evidenced by lower Hoechst 33342 uptake (Fig. 2B), in the sunitinib-treated cohort relative to control. Positive endomucin staining and Hoechst 33342 fluorescence was seen predominantly at the periphery of tumors in the sunitinib-treated mice.

Table 1. Summary of the quantitative volumetric and fBV data acquired from 786-O xenografts (n = 9) prior to and 2 weeks after daily treatment with sunitinib, and from 786-O-R xenografts exhibiting acquired resistance to sunitinib (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>786-O</th>
<th>786-O-R</th>
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<tbody>
<tr>
<td><strong>Pretreatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mm³)</td>
<td>15.2 ± 1.7</td>
<td>148 ± 24</td>
</tr>
<tr>
<td>fBV (%)</td>
<td>8.2 ± 2</td>
<td>2.4 ± 0.5</td>
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<tr>
<td><strong>Posttreatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mm³)</td>
<td>14.8 ± 24</td>
<td>49.4 ± 4.4</td>
</tr>
<tr>
<td>fBV (%)</td>
<td>2.4 ± 0.5</td>
<td>2.2 ± 0.5</td>
</tr>
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*NOTE: Data are mean ± 1 SEM.

*P < 0.01, paired t test.

Pretreatment fractional tumor blood volume is predictive of the antiangiogenic response to sunitinib in 786-0 xenografts

The data in Fig. 1D suggest that tumors exhibiting a relatively high pretreatment fBV subsequently showed the greatest reduction in fBV after 2 weeks of daily treatment with sunitinib. By simply plotting the average baseline fBV against the treatment-induced change in fBV after 2 weeks, a correlation would be expected even when there may be no relationship (23). To overcome this, and test whether the baseline fBV was indeed predictive for the subsequent reduction in fBV in response to sunitinib, the average of the final and mean baseline fBV was plotted against the change in fBV (ΔfBV) measured after 2-week treatment for each tumor (Fig. 3A). A highly significant and strong negative correlation was obtained (R² = 0.92, P < 0.0001), greater than the correlation of 0.7 that would be expected by chance (23). There was no significant relationship between sunitinib-induced reduction in fBV with change in tumor volume (Fig. 3B), pretreatment tumor fBV with pretreatment tumor volume or sunitinib-induced reduction in fBV with pretreatment tumor volume (data not shown).

Acquired resistance to sunitinib is not associated with a parallel increase in tumor fBV in 786-0 xenografts

Notably, in 6 tumor-bearing mice, we observed a period of growth control during the early phase of sunitinib treatment, which was followed by tumor regrowth while still on...
The initial response and subsequent relapse of mRCC patients treated with VEGF receptor tyrosine kinase inhibitors, such as sunitinib, is well documented (4). Currently, there are no validated biomarkers that accurately predict which mRCC patients will benefit from antiangiogenic therapy, and the mechanisms associated with the innate and acquired resistance are poorly understood. In this preclinical study, quantitation of tumor fBV using susceptibility contrast MRI was evaluated (i) for its potential as a noninvasive predictive imaging biomarker of 786-0 xenograft response to sunitinib, and (ii) to quantify the degree of functional vascularization of 786-0 xenografts exhibiting acquired resistance to chronic treatment with sunitinib, in vivo (15).

Susceptibility contrast MRI yielded a pretreatment mean fBV of approximately 8% in untreated 786-0 xenografts, consistent with similar measurements reported across a range of subcutaneous rodent tumor models using different USPIO preparations (12, 14, 24, 25). Daily treatment with sunitinib for 2 weeks induced a marked reduction in the fBV of 786-0 xenografts in vivo, with tumor uptake of USPIO particles, and therefore patent vasculature, restricted to the tumor periphery posttreatment. Importantly, this response was associated with histologically confirmed reduction in MVD and perfused vessels, providing strong validation of fBV as a quantitative imaging biomarker of functional tumor vasculature, and its response to sunitinib, in this model of RCC (7). Similar reductions in tumor fBV, measured by susceptibility contrast MRI, have been reported following treatment with other antivascular therapies (12, 14, 24, 26–28). The data provide further support for the clinical development and application of USPIO particles for the assessment of human tumor vasculature and its response to treatment. Recent studies have highlighted the efficacy and safety of the USPIO particle preparation ferumoxytrol for MRI investigations in both adults and children (29–31), and in imaging-embedded oncology clinical trials (32).

RCC 786-0 xenografts exhibiting a relatively larger fBV subsequently showed the greatest reduction in fBV in response to sunitinib. Furthermore, a strong and highly significant negative correlation between baseline tumor fBV and its subsequent response to daily treatment with sunitinib over 2 weeks was obtained, suggesting that baseline fBV has prognostic value for subsequent tumor vascular response to sunitinib and is a predictor of the magnitude of the reduction in fBV following treatment. The absence of any correlation of sunitinib-induced change in fBV with changes in tumor volume reiterates the shortcomings of the RECIST criteria to correctly assess human tumor response to antiangiogenic therapies, and the need for robust noninvasive vascular imaging readouts (33).

In the clinic, perfusion CT and DCE MRI, and the quantitative biomarkers they provide [Hounsfield unit (HU) of density and the volume transfer constant Ktrans, respectively], have been predominantly used to assess patients with mRCC and response to VEGF signaling inhibitors (9). Several imaging-embedded investigations reported that highly vascular renal tumors had a beneficial outcome following treatment (34–37) and that early reductions in HU and Ktrans related to subsequent beneficial survival (34, 38, 39). Marked measurement variability, particularly in Ktrans, was apparent in these clinical studies, likely a consequence of different pharmacokinetic modeling approaches.
used to analyze the CT and DCE MRI data. The data herein strongly suggest that quantitation of FBV using susceptibility contrast MRI may provide a simpler, more sensitive and specific imaging biomarker for predicting and assessing the vascular response of mRCC in the clinic. In this regard, the potential of arterial spin-labelling MRI, which is wholly noninvasive and yields absolute quantitation of tissue blood flow (mls/100 g/minute), has also been highlighted (40, 41).

Until recently, remarkably few preclinical studies have exploited relapsing and/or acquired resistant tumor models to study mechanisms of resistance to targeted therapies. One reason for this is the inherent longevity, and hence practical implications, associated with developing a resistant phenotype in xenografts in vivo. However, the radiology and quantitative imaging biomarkers in such models are likely to provide more accurate preclinical platforms for evaluating both novel therapeutics and drug resistance. We recently described the development of a 786-0 RCC xenograft model of acquired resistance to daily dosing with sunitinib, with tumors exhibiting late resistance approximately 2 to 3 months after treatment initiation (15). In the current study, susceptibility contrast MRI clearly revealed impaired FBV in progressing 786-0-R xenografts in vivo (see also Supplementary Fig. S2).

A rebound in tumor angiogenesis, mediated by VEGF-independent mechanisms, has been suggested as one mechanism by which tumors may evade antiangiogenic therapy (5, 6). However, this has been predicated on numerous studies that have relied on histopathologic determination of tumor vessel density and have not incorporated any direct measure of perfused/functional tumor vasculature in vivo. Our noninvasive susceptibility contrast MRI data obtained in 786-0 xenografts demonstrate that acquired resistance to sunitinib is not associated with functional revascularization in situ and suggest that tumors can gain acquired resistance to antiangiogenic therapy without the need to induce rebound angiogenesis.

How do we explain why acquired resistance to antiangiogenic therapy can be observed without an accompanying rebound revascularization? Tumor adaptation to treatment with VEGF signaling inhibitors may involve a metabolic adaptation in cancer cells, which permits cancer cells to survive despite a treatment-induced reduction in tumor vasculature and the associated hypoxic environment (42, 43). Intriguingly, we recently demonstrated that sunitinib-resistant 786-0 tumor xenografts are more hypoxic than parental 786-0 xenografts in vivo (44). Furthermore, metabolic symbiosis between tumor cells distal and proximal to surviving vessels has also been recently implicated in acquired resistance to sunitinib in RCC (45). Therefore, it appears possible that the 786-0-R xenografts analyzed in the current study can acquire resistance to antiangiogenic therapy, without recourse to rebound revascularization, because there is a shift in tumor metabolism that compensates for the reduced vascular supply.

In trying to elucidate the complex mechanisms responsible for resistance to antiangiogenic therapy, our study also highlights the important contribution from using vascular imaging strategies that correctly inform on the extent and distribution of functional tumor vasculature. Furthermore, longitudinal monitoring of tumor FBV with susceptibility contrast MRI could facilitate expedient switching of VEGF receptor tyrosine kinase inhibitors as part of sequential therapeutic strategies designed to overcome acquired resistance, and which appear to be beneficial in the treatment of patients with mRCC (46, 47).

In conclusion, we have shown that quantitation of FBV using susceptibility contrast MRI provides a sensitive imaging...
biomarker for both predicting and assessing the response of 786-0 RCC xenografts to treatment with sunitinib. Clinical MRI investigations incorporating USPIO preparations are increasingly being performed, and determination of BV may thus positively impact on mRCC patient stratification with antiangiogenic therapy. In addition, we provide strong evidence that the phenotype of 786-0 xenografts exhibiting acquired resistance to sunitinib is not associated with functional revascularization in vivo.

Disclosure of Potential Conflicts of Interest

N.S. Vasudev has received speakers bureau honoraria from Novartis and is a consultant/advisory board member for Pfizer, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S.P. Robinson, A.R. Reynolds
Development of methodology: S.P. Robinson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.P. Robinson, J.K.R. Boult, N.S. Vasudev
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.P. Robinson, J.K.R. Boult, A.R. Reynolds

References


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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.P. Robinson
Study supervision: S.P. Robinson

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