Reversible Epithelial to Mesenchymal Transition and Acquired Resistance to Sunitinib in Patients with Renal Cell Carcinoma: Evidence from a Xenograft Study

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Abstract

Tyrosine kinase inhibitors (TKI) targeting angiogenesis via inhibition of the vascular endothelial growth factor pathway have changed the medical management of metastatic renal cell carcinoma. Although treatment with TKIs has shown clinical benefit, these drugs will eventually fail patients. The potential mechanisms of resistance to TKIs are poorly understood. To address this question, we obtained an excisional biopsy of a skin metastasis from a patient with clear cell renal carcinoma who initially had a response to sunitinib and eventually progressed with therapy. Tumor pieces were grafted s.c. in athymic nude mice. Established xenografts were treated with sunitinib. Tumor size, microvascular density, and pericyte coverage were determined. Plasma as well as tissue levels for sunitinib were assessed. A tumor-derived cell line was established and assessed in vitro for potential direct antitumor effects of sunitinib. To our surprise, xenografts from the patient who progressed on sunitinib regained sensitivity to the drug. At a dose of 40 mg/kg, sunitinib caused regression of the subcutaneous tumors. Histology showed a marked reduction in microvascular density and pericyte dysfunction. More interestingly, histologic examination of the original skin metastasis revealed evidence of epithelial to mesenchymal transition, whereas the xenografts showed reversion to the clear cell phenotype. In vitro studies showed no inhibitory effect on tumor cell growth at pharmacologically relevant concentrations. In conclusion, the histologic examination in this xenograft study suggests that reversible epithelial to mesenchymal transition may be associated with acquired tumor resistance to TKIs in patients with clear cell renal carcinoma.

Mol Cancer Ther; 9(6); 1525–35. ©2010 AACR.

Introduction

Following the approval of the tyrosine kinase inhibitors (TKI) sorafenib and sunitinib by the Food and Drug Administration, the treatment paradigm for metastatic renal cell carcinoma (RCC) has significantly changed (1). Until few years ago, immunotherapies such as IFNα and interleukin-2 (IL-2) were the only treatment options for metastatic RCC. Cytokine therapies are typically associated with flu-like side effects, and high-dose IL-2 administration requires inpatient admission with intensive monitoring because of the severe toxicities. The observed objective response rate for high-dose IL-2 is 15% to 20%. However, in a small number of patients, durable responses are observed (1). Receptor TKIs such as sunitinib and sorafenib have a relatively broad spectrum of targets (2). Their clinical efficacy in RCC is thought to be primarily due to inhibition of angiogenesis-related receptor kinases such as the vascular endothelial growth factor (VEGF) receptors and the platelet-derived growth factor receptor (3, 4). Clear cell RCC is known for the commonly high expression of the potent proangiogenic VEGF. This is due to the frequently deleted, mutated, or epigenetically silenced von Hippel-Lindau (VHL) gene, which leads to the constitutively high expression of hypoxia-inducible factor 1α (HIF-1α; ref. 5). HIF-1α is a transcription factor involved in cell adaption to hypoxic conditions that induce the expression of VEGF and platelet-derived growth factor and, consequently, the stimulation of new blood vessel growth (6).

In clinical studies, sorafenib and sunitinib have shown a prolongation of progression-free survival in cytokine-pretreated and untreated patients, respectively (7, 8). The final results of a large randomized phase III study comparing sunitinib with IFNα as first-line treatment has shown a significant improvement in progression-free survival, a greater overall survival, and a ~40% objective response rate (8). Despite this success, RCCs treated with
sunitinib will eventually develop resistance and progress. The mechanism of resistance remains unclear. Hypotheses include the presence of compensatory changes in blood vessel composition, the increased production of additional proangiogenic growth factors, increased pericyte coverage, accumulation of bone marrow–derived cells, and tumor cell resistance to direct cytotoxic effects (9). In the attempt of addressing this important clinical question, we have established a primary xenograft model from a patient who had initial response to sunitinib but eventually progressed and developed new metastatic lesions to the skin. We characterized this model with regard to its histology, HIF-1α expression, and baseline angiogenesis and, most importantly, to its sensitivity to TKIs.

Materials and Methods

Reagents and cell culture

The human RCC cell lines RCC1.11 and RCC1.18 were kindly provided by Dr. Elisabeth Jaffe (Johns Hopkins University, Baltimore, MD). These cell lines were established from primary renal cell tumors. HS27a, an immortalized bone marrow stromal cell line, was obtained from the American Type Culture Collection. These cells were grown in RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin. The cell line IH23.1 was derived from the patient’s skin tumors and maintained in DMEM with 20% FCS, penicillin, and streptomycin. All cell lines were maintained at 37°C in a 5% CO2 and 95% air incubator.

Western blot analysis

Western blots were carried out on 4% to 15% gradient polyacrylamide gels according to methods previously described (10). The anti–HIF-1α antibody was obtained from R&D Systems.

Generation and characterization of a xenograft cell line

A tumor cell line (IH23.1) from the primary xenograft was derived by allowing tumor fragments to attach to plastic and by supporting growth in high-serum conditions. Outgrowing cells were serially passaged and stained positive with a pan-cytokeratin antibody (Sigma) for human epithelial origin (data not shown). For Western blot analysis, cell lysates of IH23.1 cells growing under normoxic conditions or exposed to 100 μmol/L CoCl2 for 6 hours to mimic hypoxia were used. HS27a cells, a human papillomavirus–immortalized fibroblast cell line, served as positive control for inducible HIF-1α.

Establishment of primary xenografts and sunitinib treatment

The patients consented to an Institutional Review Board–approved protocol for an excisional biopsy of their metastatic skin lesions. Part of the tumor tissue was fixed in formalin for H&E and immunohistochemistry. The rest was snap-frozen for protein and RNA studies or cut into 3 × 3 × 3 mm pieces for s.c. implantation in athymic nude mice (F1). The animal research protocol was approved by the Johns Hopkins University Animal Use and Care Committee and animals were maintained in accordance to the guidelines of the American Association of Laboratory Animal Care. Six-week-old male athymic nude mice were purchased from the National Cancer Institute (Frederick) and housed under pathogen-free conditions.

When the carcinomas reached a size of ~1,000 mm3, they were excised, cut into 3 × 3 × 3 mm fragments, and transplanted into a new cohort of mice for expansion and eventual drug studies. Drug studies were done on F4 and F5 generations of primary xenografts. Tumor growth was assessed twice weekly by using a caliper, and the size was expressed in cubic millimeters using the standard formula length × (width)2 × 0.52. Before starting the treatment, mice were divided into homogenous groups (8–9 per group) according to tumor burden determined by size. Treatment-related toxicity was determined by mouse weight weekly. Tumor weights were determined when mice were sacrificed at the end of the study.

Sunitinib malate (generously provided by Pfizer) was dissolved in a vehicle containing carboxymethylcellulose (0.5%), NaCl (1.8%), Tween 80 (0.4%), benzylalcohol (0.9%), and distilled water (pH adjusted to 6.0). Sunitinib malate was dispensed at a concentration of 10.4 mg/mL (8 mg/mL free base). The mixture was sonicated to achieve a stable dispersion. Animals were dosed as indicated, typically at 40 mg/kg once daily, 5 days a week, by oral gavage. In the prevention model, treatment was initiated when tumors were still small at around 30 days of implantation. In the intervention model, larger, more established tumors were allowed to form before treatment started around day 60.

Histology and immunohistochemistry

For the CD31 staining, zinc-fixed, paraffin-embedded tissue was generated from each tumor to quantify differences in microvessel density between control and experimental groups. Sections were incubated (18 hours at 4°C) with anti-CD31 antibody (Pharmingen), a specific marker for endothelial cells after deparaffinization. Sections were incubated with a secondary biotin-conjugated rabbit anti-goat IgG antibody (1:100) for 30 minutes at 4°C. The tissue was snap-frozen for protein and RNA studies or cut into 5-μm-thick sections was followed by heat-induced antigen retrieval done with EDTA buffer (pH 9.0). Primary
antibody incubation was followed by I-View detection (Ventana Medical Systems). The reaction was developed using substrate DAB. All Slides were counterstained with hematoxylin. All antibodies were supplied as predilute by Ventana Medical Systems. Slides were scanned with the Aperio Imaging system and Tiff files were generated with the manufacturer’s software (Imagescope).

For immunostaining of E-cadherin and CAIX, paraffin sections were cut at 5 μm, placed on charged slides, and dried in a 60°C oven for 1 hour. Room-temperature slides were then deparaffinized in three changes of xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H2O2 for 10 minutes and washed with PBS/T. Antigen retrieval was done in the microwave in pH 6.0 citrate buffer for 2 × 10 minutes (E-cadherin) and for 1 × 10 minutes (CAIX), with a 15-minute cooldown followed by a PBS/T wash. Slides were then loaded on a DAKO autostainer and the following program is run: casein 0.03% [in PBS/T] was used to block for 30 minutes, blown off, and the primary antibody [E-cadherin (Novacastra), at 1/50; CAIX (Santa Cruz), 1/50] was applied to slides for 1 hour. A PBS/T wash was followed by Envision+ reagent (DAKO) for 30 minutes. PBS/T was used as a wash and the chromagen DAB+ was applied for 10 minutes (color reaction product: brown). The slides were then counterstained with hematoxylin and then dehydrated, cleared, and coverslipped. The HIF-1α (Novus Biologicals) staining procedure uses a multilevel detection system with amplification.

**Qualitative assessment of pericyte coverage**

Cardiac perfusion of the xenograft bearing animals was done as published by Dr. Donald McDonald's group (11). Briefly, mice were anesthetized with a lethal amount of ketamine/xylazine. The chest was opened rapidly and a small incision was made into the apex of the left ventricle. A blunt needle was introduced into the left

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**Figure 1. Development of skin metastases during treatment with sunitinib.**

A, the patient enrolled in the phase III randomized study received sunitinib with initial regression of lung nodules (arrows) following the first cycle and stable disease for ~10 mo. B, during the treatment, the patient developed progressive skin metastases that were excised and transplanted into athymic nude mice (circles).
ventricle, carefully advanced into the root of the aorta, and clamped into place. The right and left atria were removed by blunt force. Then, the mouse was initially perfused at around 120 mm Hg with PBS until clearance of blood and then perfused with a freshly made 1% paraformaldehyde/PBS solution for 5 minutes. The tumors were then excised, fixed for another hour at room temperature in 1% paraformaldehyde, and infiltrated with 30% sucrose for 3 to 5 days. Tissues were then embedded in optimum cutting temperature compound and cut into 30- and 100-μm sections on a Leica Cryostat. Sections were initially blocked with PBS containing 5% goat serum and 0.3% Triton X-100. Primary antibodies against CD31 (BD Pharmingen) and desmin (Millipore) were used at 1: 500 and 1:1,000 dilutions and incubated overnight in the above-mentioned buffer at 4°C. Secondary antibodies (goat; Invitrogen) labeled with Alexa 488 and Alexa 546 were used. The thick sections served for detailed confocal analysis and three-dimensional reconstruction purposes (Zeiss LSM 510). Pictures were analyzed using ImageJ software (NIH).

**Generation and characterization of a xenograft cell line**

A cell line of the primary xenograft was derived by allowing tumor fragments to attach to plastic and by supporting growth in high-serum conditions. Outgrowing cells were serially passaged and stained positive with a pan-cytokeratin antibody (Sigma) for human epithelial origin (data not shown). For Western blots, cell lysates of IH23.1 cells growing under normoxic conditions or exposed to 100 μmol/L CoCl₂ for 6 hours to mimic hypoxia

### Table 1. Antitumor effect of TKIs in RCC IH tumor xenografts

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor weight Mean (g)</th>
<th>Ratio</th>
<th>t test</th>
<th>Tumor volume Mean (mm³)</th>
<th>Ratio</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 9), vehicle</td>
<td>0.837</td>
<td>1.0×</td>
<td>1.000</td>
<td>873.3</td>
<td>1.0×</td>
<td>1.000</td>
</tr>
<tr>
<td>Prevention group (n = 9), 40 mg/kg sunitinib</td>
<td>0.115</td>
<td>−7.3×</td>
<td>0.006</td>
<td>134.0</td>
<td>−6.5×</td>
<td>0.003</td>
</tr>
<tr>
<td>Intervention group (n = 8), 40 mg/kg sunitinib</td>
<td>0.188</td>
<td>−4.5×</td>
<td>0.011</td>
<td>230.2</td>
<td>−3.8×</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**NOTE:** n, number of tumors measured per treatment group. Ratio, fold decrease from control in tumor weight or volume at termination of experiment.
were used. HS27a cells, a human papillomavirus–immortalized fibroblast cell line, served as positive control for inducible HIF-1α.

**Long-term exposure in vitro assay**

To assess for time-dependent and direct growth inhibitory effect of sunitinib on RCC cell lines, we used a variant of colony formation assay in which cells were continuously exposed to the drug for up to 2 weeks. All cell cultures were incubated in RPMI with 10% fetal bovine serum. Exponentially growing tumor cells were seeded at 500 per 60-mm tissue culture dish in triplicates. The cells were allowed to attach for 24 hours and subsequently exposed to sunitinb at the mentioned concentration with DMSO never exceeding 0.05%. The cells were treated for 14 days with change of medium and addition of fresh drug after 7 days. At the end of treatment, the medium was removed and the culture dishes were washed once with PBS and subsequently fix-stained with crystal violet/10% ethanol. The culture dishes were destained and pictures were acquired on a Kodak 440 imaging system. The amount of crystal violet–positive pixels for each well was determined by using the ImageJ software.

**Sunitinib blood and tumor concentrations**

Sunitinib malate was administered at a dose of 40 mg/kg p.o. to nine nude mice (three groups, n = 3). Sunitinib was extracted from plasma or tissue by acetonitrile with temazepam as the internal standard. Tissue homogenates were prepared at a concentration of 200 mg/mL in PBS and further diluted to 1:10 in human plasma before extraction. Separation of the compounds was achieved on a Waters X Terra MS C18 (50 × 2.1 mm, 3.5 mm) analytic column with a mobile phase consisting of acetonitrile containing 0.1% formic acid (65%) and ammonium acetate (10 mmol/L; 35%) using isocratic flow at 0.2 mL/min for 3 minutes. The analytes of interest were monitored by tandem mass spectrometry with electrospray positive ionization at transitions 399.0 → 283.0 and 301.2 → 355.0 for sunitinib and temazepam, respectively.

**Gene expression**

Gene expression profiles from 13 nondiseased kidney samples, 11 clear cell RCC tumor samples, and 1 skin metastasis used in this study were produced using the Affymetrix HG-U133 Plus 2.0 GeneChip platform as previously described (12). Gene expression values were preprocessed using the robust multiarray average method as
implemented in the BioConductor \textit{affy} package for the R environment (13, 14). Before data preprocessing, probe set mappings were updated (15). To predict chromosomal changes in the tumor samples, gene expression values were organized by gene mapping information, compared with the nontumor tissue, and then scanned for expression biases as implemented in the \textit{reb} package (16). Pathway analysis was performed using the parametric gene set enrichment approach as implemented in the PGSEA package (17). The VHL and hypoxia signatures were obtained from previous gene expression profiling studies (18, 19). For pathway analysis, the “up” signature component indicates the list of genes that show increased expression relative to control cells for each pathway. Likewise, the “dn” signature component indicates the set of genes that show decreased expression relative to control cells for each pathway. The \textit{VHL} gene signature was obtained from cells that contained inactivating \textit{VHL} mutations. The color in the plot indicates this pathway is inactivated.

\textbf{Statistical analysis}

Statistical analysis was done using Student’s $t$ test and ANOVA to compare multiple groups. $P < 0.05$ was considered statistically significant.

\textbf{Results}

\textbf{Progressive disease with new skin metastases after initial response on sunitinib}

A 44-year-old gentleman underwent radical right side nephrectomy for a 3.3-cm, Fuhrman grade 4 clear cell carcinoma. Three years later, the patient was found to have a left upper lobe lesion with mediastinal lymphadenopathy on routine imaging studies. A biopsy of the lung lesion was obtained and confirmed as a recurrent conventional type clear cell carcinoma. The patient was enrolled in a phase III trial comparing the effect of sunitinib on metastatic RCC versus IFN$\alpha$. The patient was randomized to the sunitinib arm and was started on the study drug at 50 mg p.o. daily on a 4 weeks on and 2 weeks off schedule. Imaging studies after 4 weeks confirmed a partial response of his pulmonary and mediastinal lesions (Fig. 1A). The patient was continued on sunitinib and experienced stable disease after the initial partial response. Except for grade 2 diarrhea (controllable with loperamide) and hair discoloration, the patient tolerated the treatment with sunitinib relatively well and required no dose reductions. However, the patient developed progressive subcutaneous, highly vascularized skin lesions on his buttocks (Fig. 1B). These lesions were excised and the histology was consistent with RCC metastases. Subsequently, sunitinib was discontinued after approximately 10 months of treatment. The patient was considered for additional clinical trials and treatment with high-dose IL-2, but unfortunately, he developed rapidly progressive disease and bisphosphonate-resistant hypercalcemia with accelerated clinical decline.

\textbf{Sunitinib-resistant tumor regained sensitivity to TKIs when transplanted into nude mice}

To test the hypothesis whether sunitinib-resistant skin metastases maintained a resistant phenotype, we...
transplanted small pieces into athymic nude mice. After serial passages, we treated the tumor-bearing animals with sunitinib. Early treatment with sunitinib prevented tumor growth, whereas delayed administration induced tumor regression (Fig. 2; Table 1). Treatment groups showed significant changes in tumor size and vascularization as well as tumor weight when compared with control ($P < 0.05$ versus control). Similar results of tumor growth control were observed with sorafenib (data not shown).

**Treatment with sunitinib reduced microvascular density and impaired pericyte coverage**

Treatment with the TKI sunitinib led to a significant decrease in microvascular density (Fig. 3). Analysis by confocal microscopy revealed a significant difference in

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**Figure 5.** Evidence of EMT in sunitinib-resistant skin metastases and reverted phenotype to clear cell histology in the tumor xenografts. A, the primary RCC showed conventional clear cell histology (H&E), whereas the skin metastasis showed extensive fibroblast-like features and lack of clear cell features, suggesting EMT. Following transplantation of the skin metastases into athymic nude mice, the EMT phenotype reverted back to classic clear cell histology. Staining for cytokeratin and CAIX confirmed that the cells with fibroblast-like appearance are of epithelial origin. B, HIF-1α was expressed in the skin lesions but not in the original nephrectomy specimen nor in the murine xenograft. Vimentin, a classic marker of EMT, was absent in the patients primary tumor, but was strongly expressed in the skin metastases and maintained in the xenografts. E-cadherin was expressed in the normal kidney (see inset) but not in the primary tumor nor in the skin metastases. Weak expression was observed in the xenografts.
pericyte coverage. In the control group, the pericytes were closely associated with the endothelial cells and discrete dendrite-like extensions were observed covering the endothelial cells. In the tumors treated with sunitinib, the fine extensions disappeared and the pericytes were dissociated from the remaining vasculature.

The IH23 tumor cell line derived from the xenograft tumor showed no sensitivity to sunitinib treatment

To test whether the regained sensitivity of the xenograft to sunitinib was due to a direct inhibitory effect of the TKI on the tumor cells, we isolated the IH23.1 cell line from the primary xenograft. These cells expressed inducible HIF-1α and stained positive for the pan-cytokeratin marker (data not shown). We exposed the xenograft-derived cell line IH23.1 to pharmacologically relevant doses of sunitinib (≤500 nmol/L in 10% serum). Even under prolonged exposure, no direct cytotoxic effect was observed in a long-term exposure assay (Fig. 4). In comparison, in other two human RCC cell lines, RCC1.18 and RCC1.11, sunitinib treatment induced a dose-dependent inhibitory effect.

Evidence of epithelial-mesenchymal transition in sunitinib-resistant skin metastases and reverted phenotype to clear cell histology in the tumor xenografts

The patient’s primary RCC at the time of diagnosis was consistent with conventional clear cell histology with no sarcomatoid features. The skin lesions that progressed on sunitinib treatment showed pure sarcomatoid RCC without evidence of clear cell morphology (Fig. 5A). The sarcomatoid carcinoma was composed of highly vascularized nodular structures characterized by cytologically atypical spindle-shaped cells. Remarkably, this histologic feature was completely lost after transplantation in the mice and the original, conventional clear cell histology was restored in the tumor. Immunohistochemical analysis confirmed the nature of the spindle-shaped cells of the sarcomatoid carcinoma in the skin lesion as evidenced by positive staining for pan-cytokeratin (AE1/AE3) and CAIX (5A). At the same time, the mesenchymal markers vimentin and HIF-1α were expressed, suggesting an epithelial-mesenchymal transition (EMT), which, by definition, is a potentially transient/reversible phenotype of epithelial carcinoma (27). Whereas the patient’s skin metastasis used in this study were compared with gene expression profiles derived from nondiseased kidney tumor tissue (see Materials and Methods). As shown in Fig. 6, gene expression analysis of the skin metastasis strongly indicated it is of clear cell RCC origin. Its molecular profiles showed close resemblance to that of clear cell RCC, having the typical chromosomal signature of the loss of 3p and gain of 5q (Fig. 6A). In addition, pathway analysis also showed strong VHL inactivation and hypoxia signature (Fig. 6B).

Sunitinib plasma and tissue levels

To test whether the regained tumor sensitivity to sunitinib in mice was due to abnormal drug levels, we determined the peak plasma and tissue concentrations of sunitinib. Peak-range sunitinib plasma levels were determined after 2 hours of oral dosing on the 5th day of the treatment cycle at the end of the treatment study. Mean peak plasma sunitinib level was 402 ng/mL with the 40 mg/kg daily dosing, and this result was comparable to published data (20). Synchronous tissue concentrations of the tumor and skin were determined. The mean concentration of sunitinib in the tumor and skin was 4,333 and 2,963 ng/g, respectively.

Discussion

Angiogenesis inhibition as a cancer treatment modality has been validated across a spectrum of solid tumors. In clear cell RCC, clinically effective single-agent activity with the TKI sunitinib has been shown, and this drug is currently recommended for first-line treatment (1). Despite the excitement of recent successes, eventually patients will progress, stressing the importance to study the potential mechanisms of resistance. In this study, we report on the unique opportunity of establishing human tumor xenografts from a patient with metastatic clear cell RCC who initially responded to treatment with sunitinib but subsequently developed progressive disease with new metastatic lesions on treatment. To our surprise, the resistance to sunitinib in the patient did not predict its subsequent response as a xenograft. The regressed tumors showed the characteristic pharmacodynamic effects of sunitinib (i.e., decrease in microvascular density and pericyte dysfunction) whereas no direct antitumor effects of sunitinib on a xenograft-derived cell line at pharmacologically relevant doses were observed.

The histologic data and tumor growth experiments suggest that the tumor microenvironment contributed to the acquired resistance to sunitinib in this patient. Although the exact mechanism of resistance remains unclear, the association of sunitinib-resistant skin tumors with an EMT phenotype is intriguing. EMT has been associated with metastasis, drug resistance, and angiogenesis (21–25). A growing number of interdependent pathways have been linked to the induction of EMT, which, by definition, is a potentially transient/reversible phenotype of epithelial cancers (23, 25–27). Whereas the patient’s skin metastasis...
showed homogenous fibroblast-like spindle cell histology with cytokeratin and vimentin immunoreactivity, the primary xenograft reverted back to conventional clear cell carcinoma histology. Interestingly, the staining for vimentin, a marker for EMT, remained positive in the xenograft. Our observation is supported by other clinical data. Sarcomatoid differentiation is a growth pattern characterized by spindle-shaped histology (i.e., fibroblast-like appearance; ref. 28). Although not a histologic subtype per se, a sarcomatoid phenotype is observed across all subtypes of RCC, typically portends a poor prognosis, and recently has been associated with an increased resistance to VEGF pathway inhibitors (29). Interestingly, similar to our findings, the fibroblast-like cancer cells with sarcomatoid features express epithelial and mesenchymal markers (cytokeratin and vimentin)—a phenotype consistent with the definition of EMT.

Our observation does not rule out the possibility that by passaging the human tumor into nude mice, we may have selected a clear cell carcinoma population that may be responsible for the reverted phenotype. However, several points suggest that this is not the case. The strong vimentin positive staining in the skin metastasis and the xenograft tumors but not in the original nephrectomy speaks against the argument of selection. The two original skin metastases were propagated simultaneously in several immunocompromised mice and the reverted phenotype and regained sensitivity to sunitinib were observed in multiple isolates. The histologic examination by our pathologist also did not reveal any clear cell in the original skin metastasis. The chromosomal signature, VHL inactivation, and hypoxia signature in the skin metastasis indicated the clear cell origin. The comparison of chromosomal aberrations between the skin metastasis and the xenograft revealed significant homology, suggesting similar tumor cell populations (data not shown). All these considerations suggest that our observation was not merely due to a positive selection mechanism but rather to a true phenotypic change.

In our experimental model, we measured the sunitinib levels to rule out the possibility that the regained tumor sensitivity in mice was not due to higher drug exposure as compared with humans. Although the effective, target modulatory plasma concentration is comparable between mouse and humans (i.e., 50–100 ng/mL; refs. 20, 30), there is a significant difference in sunitinib half-life in the two species. In humans, the half-life typically exceeds 40 hours (41–80), allowing convenient once-daily dosing. Median peak level at 50 mg daily is 72 ng/mL with high median trough levels of 44 ng/mL, suggesting a continuous 24-hour target modulatory effect (30). In mice, however, a higher daily dose was required to observe a meaningful antitumor effect. At 40 mg/kg, peak levels of ∼1,000 ng/mL have been reported with a trough level of ∼2 ng/mL, suggesting a much shorter half-life as compared with humans (20). Preclinical data suggest that meaningful antitumor effects occur only when a durable target inhibition of VEGF receptor 2 (at least 12 hours) is
achieved with plasma concentrations \( \sim 50 \) to \( 100 \) ng/mL (20). Higher \( C_{\text{max}} \) levels did not correlate with greater tumor activity in preclinical models (20). Therefore, even if in our experiments we achieved higher peak levels in mice with the \( 40 \) mg/kg dosage as compared with humans, the exposure to sunitinib within 12 to 24 hours was very similar to that one achieved with \( 50 \) mg in humans. However, our observation does not completely rule out the possibility that a dose escalation strategy of sunitinib therapy may still overcome resistance.

To confirm that the effects observed in our study were not simply due to higher peak levels, we exposed a cell line derived from the primary xenograft in vitro to increasing drug concentrations. When compared with other RCC cell lines, we observed no inhibitory effect with sunitinib concentrations up to \( 500 \) ng/mL and continuous exposure for 2 weeks. It is important to notice that sunitinib is highly protein bound (90–95%; ref. 20), and because only 10% of serum was used, the effective free drug concentration is probably much higher, potentially 10-fold or more, as compared with human serum concentration. The same observation holds true for the increased tumor and skin drug levels as compared with plasma. Sunitinib accumulates in tissues (and probably in tumors) by binding to proteins and extracellular matrix. Yellow discoloration of the skin is clinically observed and it explains the high distribution volume (in the excess of \( 2,000 \) liters; ref. 20). Our observation does not rule out the possibility that sunitinib may still exert a direct antitumor effect, and additional mechanisms of resistance may be involved in patients with clear cell carcinoma treated with TKIs.

In this report, we describe the de novo onset of an EMT-like phenotype in a patient with conventional clear cell RCC on sunitinib treatment, the associated acquired resistance to the treatment with sunitinib, and the reversion to an epithelial histology in a primary xenograft model, which again is responsive to the treatment with sunitinib. Different mechanisms of resistance such as the expression of additional proangiogenic growth factors, the recruitment of bone marrow–derived cells, increased pericyte coverage, as well as angiogenesis-independent growth patterns have been described (9). We believe that EMT can be added to the list of potential resistance mechanisms. Treatment-associated tumor hypoxia has been reported to induce an EMT in several tumor models (31). Interestingly, we observed an induction of HIF-1α staining in the skin metastasis as compared with the nephrectomy sample that resolved in the xenografts. How frequently EMT as a mechanism of acquired resistance occurs is unknown and deserves further investigation. We recognize that our results represent hypothesis-generating data that need to be confirmed by future studies involving larger series of patients. Furthermore, our findings should caution about the interpretation of data obtained with angiogenesis inhibitors in primary xenograft models. Resistance mechanisms in the tumor microenvironment may be transient in nature and could be lost as the human tumors are populated with mouse stromal elements.

The reverted histologic phenotype observed in the xenografts also suggests that the escape mechanisms against anti-VEGF therapies may be transient. According to this hypothesis, patients who have initially received clinical benefit from treatment with TKIs and then developed resistant disease may respond again to TKIs following a break from anti-VEGF therapies. The “holiday” period from anti-VEGF therapies may lead to “reset” the tumor microenvironment and reestablish a primarily VEGF-driven tumor growth. This hypothesis is supported by anecdotic reports of patients who were treated with sunitinib with initial response and subsequent progression who responded again to sunitinib following different targeted therapies such as mTOR inhibitors. The apparent transient/reversible mechanism of resistance to anti-VEGF therapies may also explain why clinical benefit has been reported by sequencing different anti-VEGF therapies despite the fact that these agents target the same VEGF pathway. Sufficient time elapsed between treatments could reset the tumor microenvironment.

The ongoing molecular profiling of tumor/stromal cells to anti-VEGF therapies in both preclinical and clinical settings will continue to shed light on the mechanisms of resistance and will guide clinicians to offer the optimal sequence/combination of targeted agents to patients with advanced clear cell renal carcinoma.

Disclosure of Potential Conflicts of Interest

R. Pilip: grant support and paid consultant, Pfizer. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank Mary Vaughan and the pathology core facility at Roswell Park Cancer Institute for their technical assistance with the immuno/histochemistry studies.

Grant Support

The National Comprehensive Cancer Network, Pfizer and a generous gift from Dr. Richard Turner and Deidre Turner. H.J. Hammers was supported by a DOD Prostate Postdoctoral Training Grant.

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Received 12/10/2009; revised 03/12/2010; accepted 03/23/2010; published OnlineFirst 05/25/2010.

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Molecular Cancer Therapeutics

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