Genomics

Molecular Cancer Research

RNA-seq Reveals Aurora Kinase–Driven mTOR Pathway Activation in Patients with Sarcomatoid Metastatic Renal Cell Carcinoma

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Abstract

Sarcomatoid metastatic renal cell carcinoma (mRCC) is associated with a poor prognosis, and the biology of the disease has been inadequately characterized. RNA sequencing (RNA-seq) was performed on adjacent benign, clear cell, and sarcomatoid components from clinical specimens with sarcomatoid mRCC. M phase and cell-cycle pathways were enriched in sarcomatoid versus adjacent clear cell components, suggesting greater cell proliferation. The expression of aurora kinase A (AURKA) was increased as part of these pathways, and its increased expression was validated by quantitative PCR (qPCR). Immunohistochemical (IHC) analysis revealed that AURKA levels were increased in sarcomatoid tissue compared with their benign or clear cell parts. The increase in AURKA correlated with increased mTOR pathway activity, as evidenced by increased expression of phosphorylated mTOR (S2448) and ribosomal protein S6K (T389). When AURKA was stably expressed in a RCC cell line (Renca), it resulted in increased expression and activity of mTOR, suggesting that overexpression of AURKA can activate the mTOR pathway. These results warrant the analysis of a larger clinical cohort and suggest that targeting AURKA and/or mTOR in patients with sarcomatoid mRCC should be explored.

Implications: Comparative RNA-seq of adjacent sarcomatoid and clear cell histology of RCC indicates a proliferative phenotype and increased AURKA-dependent activation of mTOR signaling in sarcomatoid RCC, which could be targeted by available agents. *Mol Cancer Res; 13(1); 130–7. ©2014 AACR.*

Introduction

The landscape of therapy for metastatic renal cell carcinoma (mRCC) has evolved markedly in recent years. Acknowledging that clear cell mRCC (representing 80%–85% of cases) is driven by aberrations in the *VHL* gene and resultant increases in VEGF, agents have evolved to inhibit VEGF-mediating signaling (1). These agents include VEGF tyrosine kinase inhibitors (TKI), VEGF-directed monoclonal antibodies, and mTOR inhibitors (2). Although the introduction of these agents appears to have improved the overall outcomes for patients with mRCC, there are still subsets of patients that warrant further study, including those patients with mRCC and a sarcomatoid component. Sarcomatoid elements may coexist with any other RCC histology and are found in only a small percentage of patients, but they generally portend a poor prognosis (3, 4). Prospective studies have examined combinations of cytotoxic therapy, yielding modest results at best. For instance, in Eastern Cooperative Oncology Group (ECOG) trial 8802, examining Adriamycin and gemcitabine chemotherapy in a series of 39 patients, a median overall survival (OS) of 8.8 months was observed. Retrospective efforts have identified similarly modest benefit with VEGF-directed therapies—in our own institutional series, no difference in survival was noted among patients treated with chemotherapy or targeted agents (5, 6). In a recent retrospective analysis of patients with RCC treated with mTOR inhibitors (temsirolimus or everolimus), which included 23 patients with clear cell RCC with sarcomatoid features, Voss and colleagues found that 13% of patients had partial response as their best response, 30% had stable disease, and 57% had progressive disease, suggesting a modest benefit in a subset of these patients (7). With the lack of prospective studies, there is no consensus as to the systemic management of "sarcomatoid" mRCC.

Sarcomatoid mRCC is characterized histologically by the appearance of spindle-shaped mesenchymal cells (4). The observation that the greater percentage of sarcomatoid involvement is associated with a worse outcome and apparent rapid progression of disease suggests increased proliferative capacity

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-14-0352

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(8). Interestingly, sarcomatoid components are typically interspersed throughout RCC tumors, leading to complex histologic assessment. This tumor heterogeneity may contribute to the poor response of patients with sarcomatoid RCC to targeted therapies. Despite the high lethality of sarcomatoid RCC, little is known concerning the biology of this disease. In this study, we sought to gain a better understanding of sarcomatoid RCC using an RNA-sequencing approach to provide a direct comparison to adjacent clear cell carcinoma and histologically benign-appearing tissues.

Materials and Methods

Patient identification and ethics statement

Through a City of Hope Comprehensive Cancer Center Institutional Review Board (IRB)-approved protocol (COH 12109), we identified patients with sarcomatoid mRCC using an IRB-approved institutional database (COH 11079). The database contains clinicopathologic information, treatment-related data, and information pertaining to clinical outcome from patients who had provided written consent for information and tissues to be used for research purposes. Given the retrospective nature of the current study and the preservation of anonymity of study results as specified by protocol 12109, a waiver of consent has been granted for the current study by the City of Hope Comprehensive Cancer Center IRB. Clinicopathologic characteristics noted in the database include a Memorial Sloan-Kettering Cancer Center (MSKCC) risk score (either good, intermediate, or poor; ref. 9). Patients included in the analysis had to have paraffin-embedded tissue derived from nephrectomy. Five patients were selected for RNA-seq analysis and an additional 3 patients for subsequent assays.

RNA-sequencing

Tissue punches were procured by one of the study pathologists (H. Wu) and contained nearly 100% pure benign, clear cell, or sarcomatoid component. RNA was recovered using the FFPE-RNA kit (Qiagen). RNA sequencing was performed by the City of Hope Functional Genomics Core facility. RNA sequencing sample quality results are shown in Supplementary Table S1. Transcriptome libraries were constructed using Ribo-Zero rRNA Removal Kits (Illumina Epicenture) and TruSeq RNA Sample Preparation Kit V2 (Illumia) with minor modifications. In brief, 1 μg of total RNA from each FFPE sample was treated with 8 μL of rRNA Removal Solution (Ribo-Zero rRNA Removal Kits). The rRNA-depleted RNA was ethanol precipitated. The pellet was washed with 70% of cold ethanol and resuspended in 17 μL of Elute/Prime/Fragment Mix (Illumina). First-strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Double-stranded cDNA was further subjected to end repair, A-tailing, and adapter ligation in accordance with the manufacturer supplied protocols. Purified double-strand cDNA templates with ligated adaptor molecules on both ends were selectively enriched by 10 cycles of PCR for 10 seconds at 98°C, 30 seconds at 60°C, and 30 seconds at 72°C using Illumina PCR Primer Cocktail and Phusion DNA polymerase (Illumina). Products were cleaned using 1.0 × AmpureXP beads (Beckman Coulter). Purified libraries were validated using Bioanalyzer 2100 system with DNA High Sensitivity Chip (Agilent) and quantified with Qubit (Life Technologies). All libraries were sequenced on the Illumina Hiseq 2500 with single read 40 bp reads following the manufacturer's recommendations. The 40-bp-long single-ended sequence reads were mapped to human genome (hg19) using TopHat, and the frequency of Refseq genes was counted with customized R scripts. The raw counts were then normalized using trimmed mean of M values (TMM) method and compared using Bioconductor package "edgeR" (10). The average coverage for each gene was calculated using the normalized read counts from "edgeR." Differentially expressed genes were identified if the average coverage ≥ 1 in at least one sample, fold change ≥ 2, and $P \leq 0.05$. Gene enrichments were analyzed using DAVID (Database for Annotation, Visualization, and Integrated Discovery), which is a bioinformatic resource available online at http://david.abcc.ncifcrf.gov for functional interpretation of large lists of genes.

RT-qPCR

RNA was recovered using the FFPE-RNA Kit (Qiagen) from scrapings from slides that had been marked for pure clear cell and pure sarcomatoid components by a study pathologist (T. Tong). RNA was reverse-transcribed and qPCR was performed using a StepOne real time PCR machine (Life Technologies) using SYBR green (Invitrogen) as the detecting dye and Rox (Invitrogen) as the reference dye. Samples were tested in technical triplicate. Differences between experimental (x) and control (y) samples were normalized to RPL19 transcript levels and determined with the following calculation: $[2(C_{txgene1}/C_{tygene1})]/[2(C_{txRPL19}/C_{tyRPL19})]$. To test for differences in means, ANOVA methods were used for planned comparisons between treatment groups that were defined by linear contrast statements. For qPCR assessment of Renca cell line RNAs, samples were tested in biologic duplicates and technical triplicates. Primer sequences are shown in Supplementary Table S2.

Immunohistochemistry

The following biomarkers of interest were examined via immunohistochemical (IHC) staining: phospho-Ser2448 mTOR (pmTOR), total mTOR, phospho-Thr389 p70 S6 kinase beta (pS6K), total S6K, PTEN, AURKA, N-cadherin, and phospho-Tyr1175 VEGF receptor 2 (pVEGFR2). Tissue sections were deparaffinized, the endogenous peroxides were quenched with 3% hydrogen peroxide, and sections were treated for antigen retrieval by boiling in a pressure cooker in antigen unmasking solution (Vector Laboratories). Sections were blocked with 10% normal goat serum in TBS–0.1% Tween-20 and probed with primary antibodies: rabbit anti-PTEN (Genetex) 1:150, rabbit anti-AURKA (Biorbyt) 1:100, rabbit anti-pmTOR (Genetex) 1:150, rabbit anti-mTOR (Genetex) 1:250, rabbit anti-phospho-Tyr1175 VEGF receptor 2 (Biorbyt) 1:200, rabbit anti-phospho-Thr389 p70 S6 kinase beta (Biorbyt) 1:250, rabbit anti-S6K (Genetex) 1:250, and mouse anti-N-cadherin (DAKO) 1:50. Biotinylated goat secondary antibodies and streptavidin–peroxidase label (LabVision) were added, and DAB substrate kits were used for peroxidase detection (Vector Laboratories).

Pathologic analysis and scoring

IHC staining was performed by 2 laboratory-based investigators (J.O. Jones and M. He), and stained slides were reviewed independently by the study pathologists. An estimate of the average percentage of positively staining cells (0%–100%) and their staining intensity (0–3) in the sarcomatoid and clear cell sections of each slide was recorded for each biomarker; the extent and intensity values were multiplied for a final staining score. The

Student *t* test was used to compare the staining for each biomarker between sarcomatoid and clear cell components.

Cell culture and Western blotting

Renca cells were cultured in RPMI with 10% FBS and antibiotics. Cells were transfected with an AURKA expression vector (AddGene plasmid 8510; ref. 11) using Lipofectamine transfection reagents (Life Technologies), and 2 independent clones were selected using puromycin. Cells were lysed in TBS with 0.1% Tween with protease inhibitors. Lysates were resolved by SDS-PAGE and transferred to membranes for blotting with the antibodies described above or actin (Santa Cruz, sc-1616) as a loading control.

Results

Patient characteristics

Clinicopathologic and treatment-related characteristics of the cohorts are noted in Table 1. Mean age of the patients was 60 years old, and patients were mainly male. Treatment patterns were heterogeneous among the 2 groups. Few patients received third-line therapy.

Table 1. Clinicopathologic and treatment-related characteristics of patients with sarcomatoid mRCC

Characteristic	(*n* = 8)
Age, y	
Mean	60.0
Range	(42.7–78.2)
Gender (*n*)	
Male	6
Female	2
Sites of metastasis (*n*)	
Bone	4
Lung	4
Brain	3
Liver	4
Risk group (*n*)	
Good	1
Intermediate	7
Poor	0
Treatment (*n*)	
First-line	
VEGF-TKI	4
mTOR	0
Immunotherapy	0
Chemotherapy	1
None	3
Second-line	
VEGF-TKI	1
mTOR	1
Immunotherapy	0
Chemotherapy	1
None	5
Third-line	
VEGF-TKI	1
mTOR	0
Immunotherapy	0
Chemotherapy	0
None	7

RNA-seq

RNA recovered from tissue punches from adjacent clear cell and sarcomatoid components was of high enough yield to prepare libraries and perform 40-bp reads on all samples. Exon coverage ranged from 4% to 24%, with an average coverage of 13.2% (Supplementary Table S1). Interestingly, the samples clustered by patient, not collectively by histologic subtype (benign, clear cell, or sarcomatoid), suggesting an evolution of disease from benign to clear cell to sarcomatoid that is unique to each patient (Fig. 1A). Focusing specifically on mRNAs, we found a number of differentially expressed genes (Fig. 1B; Supplementary Table S4) among groups, including many differences between sarcomatoid and surrounding clear cell disease sections. Gene ontology analysis revealed significant differences between sarcomatoid and adjacent clear cell cancers (Tables 2 and 3). The most striking differences appear to involve increased proliferation in sarcomatoid tissue, including M phase and cell-cycle pathways. To validate the RNA-seq findings, we extracted RNA from corresponding tissue samples and performed RT-qPCR for several genes found to be

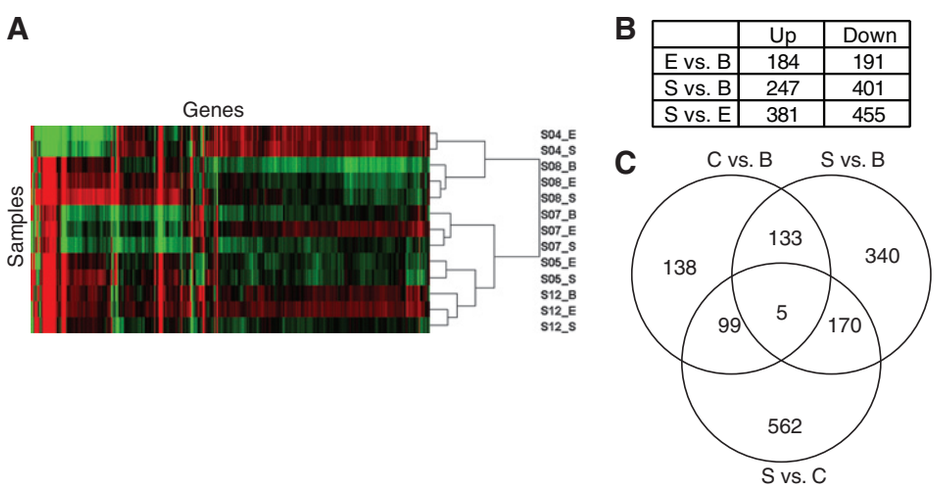

Figure 1.
RNA-seq results. RNA-seq was performed on benign, clear cell, and sarcomatoid tissue punches from 5 patient samples. A, hierarchical clustering demonstrates a strong dependence on the patient from which the sample was obtained, not on histologic disease designation. B, a Venn diagram of significant differentially regulated genes among sarcomatoid (S), clear cell (C), and benign (B) tissue sections.

Table 2. Gene Ontology pathways increased in sarcomatoid versus clear cell

Term	Count	Fold enrichment	*P*
M phase	53	8.55	1.36E−33
Cell-cycle phase	57	7.30	1.57E−32
Mitosis	42	10.13	2.00E−29
Nuclear division	42	10.13	2.00E−29
M phase of mitotic cell cycle	42	9.95	4.24E−29
Organelle fission	42	9.73	1.06E−28
Mitotic cell cycle	50	7.17	5.52E−28
Cell-cycle process	59	5.54	3.13E−27
Cell cycle	66	4.51	1.55E−25
Cell division	39	7.01	2.44E−21

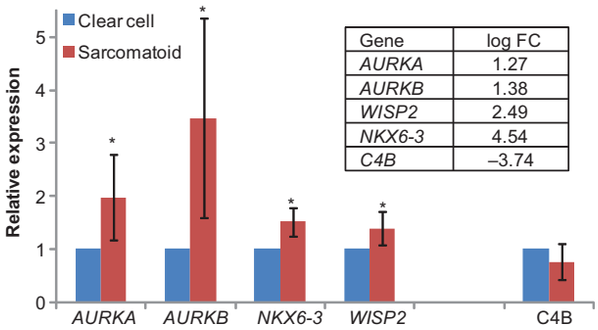

Figure 2.
RT-qPCR confirmation of RNA-seq findings. RNA was extracted from 4 of the 5 patient samples from the RNA-seq analysis, plus an additional 3 patient samples with adjacent sarcomatoid and clear cell RCC. qPCR demonstrates a pattern of expression similar to that observed in the RNA-seq analysis (inset) for all genes examined.

significantly regulated by RNA-seq. We were able to extract RNA from 4 of the 5 patient samples from the RNA-seq analysis, plus an additional 3 patients who had archived tissue containing adjacent sarcomatoid and clear cell RCC. We chose to examine the expression of several of the most highly altered genes, as determined by RNA-seq, as well as the expression of Aurora kinases A and B, as they are known to be involved in increased mitosis and cell-cycle progression (12, 13). Although the magnitude of change as determined by qPCR was not identical to that detected by RNA-seq, all of the genes we examined were altered in the same direction (Fig. 2).

Aurora kinase and mTOR pathway activation in sarcomatoid RCC

We next used IHC to determine whether AURKA was increased at the protein level as well as at the transcript level. In 6 of the 7 patient samples, AURKA expression was elevated in sarcomatoid areas compared with clear cell areas, although all clear cell tissue had some staining (Fig. 3). Aurora kinases have been shown to interact with mTOR to regulate cell-cycle progression and even the transformation of cells (14, 15). As mTOR signaling has shown to be a valid target in clear cell RCC (16) and is more significantly altered in high-grade tumors and tumors with poor prognostic features (17), we examined the correlation between AURKA overexpression and mTOR pathway activation. Expression of phosphorylated (active) mTOR and S6K was used to assess the status of the mTOR signaling pathway. Autophosphorylation of mTOR at serine 2448 is thought to indicate an active complex (18), and phosphorylation of S6K at Thr389 is a widely accepted marker of mTOR pathway activation and most closely correlates with p70 kinase activity *in vivo* (19). We observed a greater extent and intensity of pmTOR and pS6K expression in sarcomatoid versus clear cell components of tumor samples, although both were expressed to some extent in all clear cell areas (Fig. 3). In the 7 samples that included both sarcomatoid and clear cell carcinoma components on the same slide, pmTOR ($P = 0.006$), pS6K ($P = 0.001$), and AURKA ($P = 0.05$) stainings were significantly elevated in sarcomatoid areas compared with clear cell areas (Fig. 3).

To explore the dependence of mTOR activation on AURKA overexpression, we also examined the expression of PTEN, which when lost is known to activate mTOR signaling (20), and of phosphorylated VEGFR2, as a marker of the VEGF signaling (21), which has also been shown to activate mTOR signaling (22). We found no difference in PTEN expression between sarcomatoid and clear cell components ($P = 0.18$) and no obvious overlap with pmTOR or pS6K staining (Fig. 3). Likewise, there was no difference in pVEGFR2 staining between sarcomatoid and clear cell components ($P = 0.25$). This suggests that activation of mTOR signaling in our samples was not likely dependent on VEGFR2 signaling or PTEN loss, especially in sarcomatoid mRCC.

Table 3. Gene Ontology pathways decreased in sarcomatoid versus clear cell

Term	Count	Fold enrichment	*P*
Coenzyme metabolic process	17	5.06	2.38E−07
Oxidation reduction	36	2.57	5.19E−07
Response to drug	19	4.01	1.26E−06
Cofactor metabolic process	18	4.20	1.34E−06
Fatty acid metabolic process	17	3.91	7.34E−06
Amine biosynthetic process	11	6.19	1.03E−05

AURKA overexpression drives mTOR activation in clear cell RCC cells

As the correlation between AURKA overexpression and mTOR pathway activation was strong, we wanted to determine whether AURKA overexpression could cause mTOR pathway activation. We chose to use the Renca cell line as a model as it has a low level of AURKA and mTOR expression and recapitulates several features of sarcomatoid RCC in mice (e.g., distant metastases including liver). Two independent clones were selected with a varying degree of AURKA expression (Fig. 4). Overexpression of AURKA caused an increase in total and phosphorylated mTOR, as well as total and phosphorylated S6K, demonstrating that AURKA overexpression can drive mTOR expression and signaling in RCC cells. The increase in mTOR protein levels does not appear to be caused by increased transcription, as RT-qPCR demonstrates a lack of increased mTOR transcript (Fig. 4). Likewise, the increased expression of AURKA has no effect on the transcription of aurora kinase B. However, a known AURKA target, ccnb1 (23), was found to be increased by RT-qPCR and in the RNA-seq comparison of sarcomatoid to clear cell tissue (Fig. 4; Supplementary Table S3).

Sarcomatoid RCC has been proposed as an example of epithelial-to-mesenchymal (EMT), as evidenced by increased expression of N-cadherin and Snail among other markers (24–26). We were able to show in our samples that the expression of N-cadherin was higher in sarcomatoid tissue than in surrounding clear cell tissue ($P = 0.05$; Fig. 5). We also observed increased N-cadherin

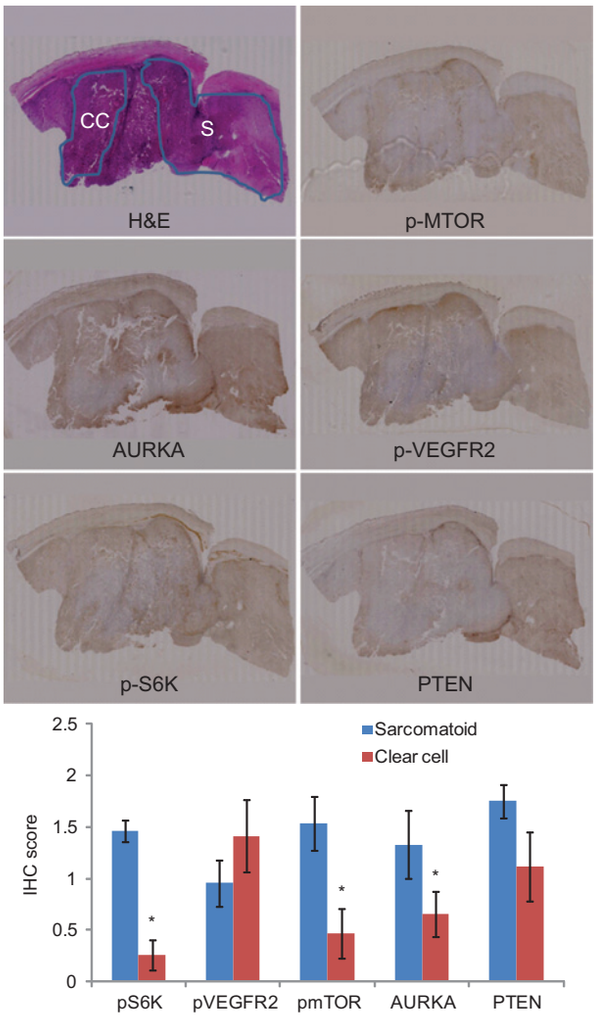

Figure 3.
Expression of AURKA and mTOR pathway proteins in sarcomatoid RCC. IHC was performed for the indicated proteins on samples from 7 patients who had both sarcomatoid and clear cell components on the same slide. Top, representative whole slide imaging for 1 patient sample is shown. Bottom, extent × intensity scoring was averaged from 7 samples who had both sarcomatoid and clear cell carcinoma components. AURKA, pmTOR, and pS6K, but not pVEGFR2 or PTEN, expression was significantly different between cancer components. Bars represent SE. The Student *t* test was used to determine significance (*, $P < 0.05$).

transcript levels in 1 of the 2 AURKA-overexpressing Renca cell lines by RT-qPCR (Fig. 5). No changes in the expression of several other EMT markers in cells overexpressing AURKA (Fig. 5) were seen, nor did we observe any morphologic changes that might be indicative of EMT (data not shown). Thus, our results do not rule out EMT in the evolution of sarcomatoid disease but do suggest that overexpression of AURKA does not contribute to the EMT process, only to the activation of mTOR signaling.

Discussion

The molecular characterization of sarcomatoid RCC has been limited, but several groups have shown that sarcomatoid disease may be an example of EMT, as evidenced by increased expression of N-cadherin and Snail among other markers (24–26). We also observed increased expression of N-cadherin in sarcomatoid sections, but interestingly, EMT pathways were not identified in the gene ontology analysis of the RNA-seq data. Other groups have shown that sarcomatoid RCC from tumors with clear cell pathology maintained the high expression of HIF pathway proteins found in the surrounding clear cell tissue (27). Other small studies have suggested greater expression of FAS ligand (28) or c-KIT (29), although the c-KIT finding has been refuted (30). Our unbiased RNA-seq exploration of sarcomatoid tissue from clear cell RCC suggested an increased expression of AURKA, which was confirmed at the transcript and protein levels. AURKA is known to be involved in increased mitosis and cell-cycle progression (12, 13) and is often overexpressed in cancer, thus several agents inhibiting AURKA activity are in clinical trials (31). Two studies have shown that AURKA expression is increased in clear cell RCC compared with normal kidney tissue and that higher expression levels correlate with advanced tumor stage and poor patient survival (23, 32). We, too, saw higher AURKA transcript levels in clear cell components than in benign kidney tissue in the RNA-seq data, but the sarcomatoid tissue had even greater expression than the clear cell tissue. Neither previous study indicated whether patients from whom samples were taken had sarcomatoid involvement, but greater sarcomatoid involvement is also known to correlate with advanced disease and poorer outcome (8), so the fact that AURKA levels correlated with more advanced disease and poorer patient survival might suggest that AURKA levels also correlated with sarcomatoid involvement.

Our observations also suggest an increase in the activity of the mTOR signaling pathway, as measured by phosphorylation of mTOR and S6K, in patients with sarcomatoid mRCC. mTOR is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription (18). mTOR forms 2 distinct complexes, mTORC1 and mTORC2. mTORC1 controls protein synthesis, in part, by regulating the phosphorylation status of p70 s6 kinase 1 (S6K), and the phosphorylation of S6K is often used to assess the activity of mTOR (33). Our results clearly demonstrate that mTOR signaling is active in the clear cell component of the RCC but is further elevated in the sarcomatoid tissue. In support of our findings, one other study also identified increased pS6K expression in patients with sarcomatoid RCC (34). The mTOR pathway has been examined in clear cell RCC to a greater extent, with different studies suggesting activation of mTOR in 60% to 85% of clear cell RCC (17, 35). Interestingly, rare mutations have been observed in the mTOR pathway in clear cell RCC, including mutations in the mTOR gene itself (36, 37). In a cohort of 77 fresh-frozen clear cell samples and matched benign tissue, Kucejova and colleagues identified additional mutations in the mTOR pathway, including 3 mutations in TSC1, 1 in PTEN, and 1 in REDD1, which is a negative regulator of mTOR (38). REDD1 is a HIF1 target gene, and its expression increases upon loss of VHL and concomitant HIF1 activation, which occurs in approximately 90% of sporadic tumors (39, 40). REDD1 expression should inhibit mTOR signaling, yet mTOR signaling appears to be active in the majority of clear cell RCC. It appears that somatic mutations in the mTOR pathway can overcome VHL/HIF1/REDD1-mediated mTOR suppression in rare cases, but other mechanisms must exist to account for the majority of mTOR activation in clear cell RCC. We were unable to sequence genes in the mTOR pathway

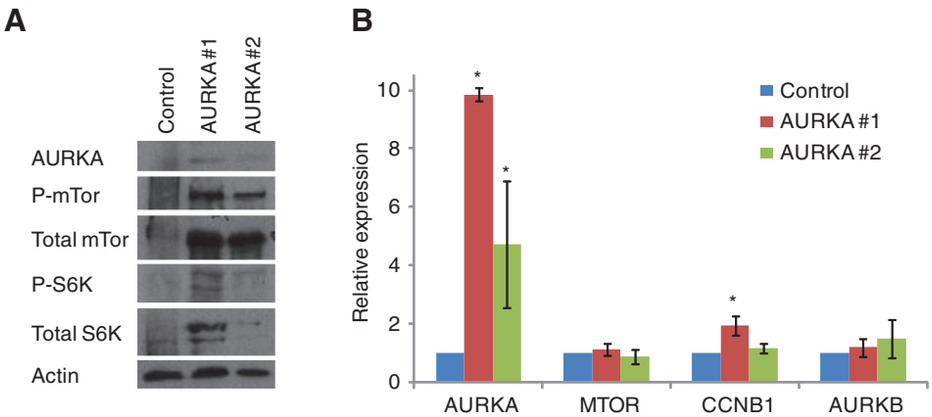

Figure 4.
AURKA overexpression drives mTOR activation in Renca cells. Renca cell lines were transfected with an AURKA expression vector and 2 independent cell lines were selected. After several weeks of selection, lysates were harvested from both AURKA cell lines and parental Renca cells, separated by SDS-PAGE, and immunoblotting was performed for the indicated proteins (A), or RNA was harvested for RT-qPCR (B). Overexpression of AURKA caused increased expression of mTOR and S6K, as well as phosphorylation of both proteins. The increased expression of mTOR was not reflected at the transcript level, although the AURKA target ccnb1 was increased. Bars represent SE. ANOVA methods were used to derive *P* values (*, $P < 0.05$).

due to the fragmented nature of the DNA in the FFPE samples, but our data would suggest that pathogenic overexpression of AURKA can activate mTOR signaling, thus overcoming REDD1-mediated suppression. In fact, it has been shown that AURKA is a transcriptional target of HIF1α and that AURKA expression increases with loss of VHL, which helps explain the elevated levels of AURKA observed in clear cell RCC (41–43). Our results suggest a model where loss of VHL causes increased HIF1α expression, which in turn causes increased AURKA expression, which activates mTOR signaling. This is likely a general model for clear cell RCC that may be exacerbated in sarcomatoid tissue. Further studies will be needed to confirm the activity status of the mTOR pathway in ccRCC and, more importantly, if such markers correlate with response to mTOR-targeted therapies, but our studies suggest that mTOR pathway activation is highly relevant in patients with sarcomatoid RCC. Although 2 FDA-approved mTOR inhibitors are in clinical use for mRCC, the activity of these compounds in sarcomatoid mRCC is limited to 1 retrospective study in 23 patients (7) and 2 other case reports (44, 45). To our knowledge, there are no current trials investigating aurora kinase inhibitors in RCC, but perhaps dual targeting of mTOR and aurora kinases in patients with sarcomatoid RCC would be an effective strategy. Our findings in cultured RCC cells that overexpression of AURKA can drive mTOR pathway signaling support this hypothesis. Developing a mouse model of sarcomatoid RCC would also be helpful in preclinical testing of such novel combinations.

We did not observe a significant difference in expression of pVEGFR2 or PTEN when comparing tissues with adjacent sarcomatoid and clear cell components, suggesting that neither PTEN loss nor VEGF signaling was responsible for mTOR activation. VEGF signaling is sensitive to numerous targeted agents that are thought to act primarily through VEGFR2. The lack of significant differences in pVEGFR2 staining between sarcomatoid and clear cell components does not completely rule out differences in VEGF–VEGFR signaling in these patients that could be targeted by current therapies. It is possible that VEGFR2, although thought to be the primary mediator of VEGF signaling, is not the most important receptor for VEGF in sarcomatoid tissue. It would be beneficial to examine the expression of other VEGFRs and whether they are active (phosphorylated) or not. It would also be

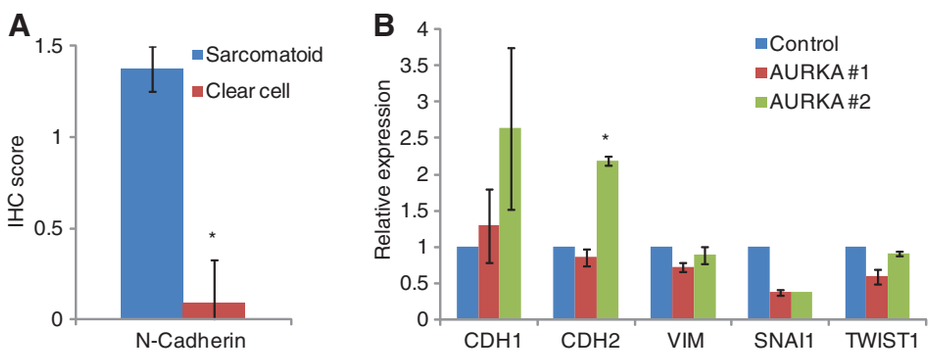

Figure 5.
AURKA overexpression does not cause EMT in Renca cells. A, extent × intensity scoring was averaged from 7 samples who had both sarcomatoid and clear cell carcinoma components. N-cadherin expression was significantly different between cancer components. Bars represent SE. The Student *t* test was used to determine significance (*, $P < 0.05$). B, RT-qPCR was performed on parental and AURKA-overexpressing Renca cells as in Fig. 4. Bars represent SE. ANOVA methods were used to derive *P* values (*, $P < 0.05$).

beneficial to examine downstream markers of VEGF–VEGFR signaling to determine whether this pathway is active in sarcomatoid and nonsarcomatoid mRCC. The lack of overlap between VEGF and mTOR signaling in sarcomatoid and clear cell components in patients harboring both cancers suggests that there may be rationale to treat these patients with the combination of VEGF- and mTOR-directed approaches. Although recent randomized phase II and III studies exploring these combinations have shown little improvement in efficacy and increased toxicity as compared with existing single-agent regimens, these studies explored a generalized population of patients with mRCC and did not particularly include an analysis of patients with sarcomatoid component (46–49). A more specific evaluation of these combinations in a subgroup of patients with sarcomatoid mRCC may be warranted.

There are several limitations to our study, primarily the small sample size and the use of FFPE tissue for deep-sequencing approaches. Patient samples with clearly documented sarcomatoid components are rare and often overlooked, but this RNA-seq assessment of 5 patients provides important insights into the biology of sarcomatoid disease that can now be explored in larger cohorts. Our RNA-seq methodology produced only an average of 13.2% exon coverage, which is on par with other studies that have used FFPE tissue for RNA-seq; however, the RNA quality and exon coverage would likely be improved using frozen samples instead. Furthermore, because of nucleic acid fragmentation, we were unable to perform reliable exome sequencing on most of the samples, even following several methods of library preparation. Such data would be useful to identify chromosomal gains and losses as well as the accumulation of somatic mutations as clear cell carcinoma evolves into sarcomatoid disease. Fragmented nucleic acid also limited the confidence in findings from 100-bp paired-end RNA-seq reads. We identified 2 fusion transcripts from this approach but could not independently confirm either (Supplementary Table S3). Despite our admittedly small sample size, our study provides important biologic insights and the data presented herein should be used to inform larger translational studies investigating sarcomatoid mRCC. These findings could also suggest previously untested therapeutic avenues for the disease, such as combinations of aurora kinase and mTOR inhibitors. Until such efforts are undertaken, the prognosis of sarcomatoid mRCC will likely remain dismal.

Disclosure of Potential Conflicts of Interest

T.K. Choueiri has received other commercial research support from Pfizer and is a consultant/advisory board member of Bayer, GSK, NCCN, Up-to-Date, Novartis, and ASCO. J.A. Karam is a consultant/advisory board member of Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acknowledgments

The authors thank Sofia Loera and the Pathology Core Facility for assistance with slide preparation. Tissue samples were provided by the City of Hope Biospecimen Repository, which is funded in part by the National Cancer Institute. Other investigators may have received specimens from the same patients. The authors also thank the generous contributions of Frank Di Bella and Paul Musco.

Grant Support

This work was supported by NIH K12 2K12CA001727-16A1 (S.K. Pal).

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Received June 18, 2014; revised July 25, 2014; accepted August 5, 2014; published OnlineFirst September 2, 2014.

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