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Anti-tumor response to combined anti-angiogenic and cytotoxic chemotherapy in recurrent metastatic chromophobe renal cell carcinoma: Response signatures and Proteomic Correlates

Abhishek Maiti, MD¹, Robert E. Brown, MD², Paul G. Corn, MD³, Ravi Murthy, MD⁴, Dhakshina Moorthy Ganeshan, MD⁵, Apostolia M. Tsimberidou, MD⁶, and Vivek Subbiah, MD⁶

¹Department of Internal Medicine, University of Texas Health Sciences Center at Houston

²Department of Pathology and Laboratory Medicine, University of Texas Health Sciences Center at Houston

³Department of Genitourinary Medical Oncology, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston

⁴Department of Interventional Radiology, The University of Texas MD Anderson Cancer Center, Houston

⁵Department of Diagnostic Radiology, The University of Texas MD Anderson Cancer Center, Houston

⁶Department of Investigational Cancer Therapeutics, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston

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Corresponding Author: Vivek Subbiah, MD, Department of Investigational Cancer Therapeutics, Unit 455, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, Texas 77030., vsubbiah@mdanderson.org.

Author contributions

Conception and design: VS, AM, REB, ATM

Development of methodology: VS, AM, REB, AMT

Acquisition of data: VS, AM, REB, ATM, PGC

Analysis of data: VS, AM, REB, PGC, AMT

Contributed reagents/materials/analysis tools: VS, REB, KP, ATM

Drafting, review, and revision of manuscript: AM, REB, PGC, AMT, KP, VS

Place of Work: Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, 1515 Holcombe Blvd, Houston, Texas 77030; Department of Pathology and Laboratory Medicine, University of Texas Health Sciences Center at Houston, 6431 Fannin Street, Houston, Texas 77030

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Introduction

Chromophobe renal cell carcinoma (ChRCC) is the third most common renal neoplasm, accounting for ~5% of renal cancers, with approximately 3000 new cases annually in the United States^{1,2}. Roughly 25% of RCC patients have metastatic disease at presentation³. ChRCC typically have good outcomes with 5-year overall survival (OS) ranging from 80-100%⁴⁻⁶. However, a small proportion of ChRCC behave aggressively with tendency for metastasis and/or recurrence^{4,5,7,8}. These patients characteristically are resistant to systemic therapy⁹, and have poor prognosis with median OS being around 28 months^{10,11}.

As ChRCC have a higher rate of low stage and low grade tumors discovered early, nephron sparing nephrectomy has emerged as an effective strategy¹². Consequently there is scarce data, and very limited options for metastatic ChRCC. Targeted therapy based on molecular profiling has clinically benefitted many rare cancers.¹³ However, for those cancers that have no actionable aberrations it is challenging to identify therapy options once they are refractory to standard therapy. Herein we report our experience with a patient with recurrent refractory metastatic ChRCC who initially did not respond to a combination of a multi-kinase VEGFR2 inhibitor vandetanib, and mTOR inhibitor everolimus, but later showed prolonged response to oxaliplatin, capecitabine, and bevacizumab. Next generation exome sequencing failed to reveal actionable aberrations but targeted proteomics helped to guide therapy in addition to elucidating the mechanisms of resistance, and response.

Case report

A 58-year-old Caucasian man presented to our institution after undergoing unsuccessful treatment for recurrent refractory metastatic ChRCC elsewhere. Two years prior, he was diagnosed with a pT3aN0M0 ChRCC after nephrectomy for a renal mass found after presentation with hematuria. He did well subsequently with no evidence of disease for 21 months, after which new metastases to lung, liver, and anterior abdominal wall were noted.

At this point the patient presented to our institution. Genomic profiling to identify hotspot mutations in 46 genes was performed on the tumor sample from the nephrectomy 2 years prior. It failed to show any mutations in *KIT*, *TP53*, or *PTEN* genes, which have been reported in sporadic, and inherited forms of ChRCC.^{2,14,15} He underwent resection of the abdominal wall implants and partial lobectomy of the right lobe of liver. Seven months later he was noted to have retroperitoneal, and more liver lesions, and was started on pazopanib. Sixteen months later a drop down metastasis was noted in his pelvis and gemcitabine was added for four cycles. Tumor response was determined using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 by positron emission tomography/computed tomography (PET/CT) scans or CT scans obtained about every six to eight weeks. Subsequently he developed progressive disease, so was then started on carboplatin AUC 5, paclitaxel 175 mg/m², and bevacizumab. Three months later, he was noted to have lung, liver, and pelvic metastases.

At this juncture he was enrolled in a trial of vandetanib in combination with everolimus, NCT01582191 (Phase I). Vandetanib 100 mg daily, and everolimus 2.5 mg daily were given

orally in a 28 day cycle. Unfortunately his tumor progressed at the first restaging on mTOR based therapy.

We performed morphoproteomic analysis¹⁶ using immunohistochemical probes to determine the level of expression of different proteins, their state of activation, and subcellular compartmentalization, in the cells from the tumor, and the tumor microenvironment, thereby identifying the cell signaling pathways functional in his tumor and hence, resistance and response mechanisms. Methodology and immunohistochemical probes used have been published previously.¹⁶⁻¹⁹ The results are shown in table 1, figure 1 and figure 2.

Given his major burden of disease was in the liver, he was enrolled in a trial of hepatic arterial infusion (HAI) of oxaliplatin, oral capecitabine, with systemic bevacizumab, NCT01213238 (Phase I). Oxaliplatin 140 mg/m² was given by HAI on day 1 of a 21 day cycle, capecitabine had a starting dose of 500 mg/m² by mouth twice daily, on days 1 - 14 of a 21 day cycle, and bevacizumab 10 mg/kg was given intravenously on day 1 of a 21 day cycle. He tolerated therapy reasonably well and showed prolonged response for nine cycles with this regimen (figure 3 showing pre- and post-therapy staging scans), after which he developed progressive disease and was taken off the protocol.

Discussion

Given the rarity of this sub-group of tumor, and relative under-representation in clinical trials, there is a lack of consensus on the best regimen for treating metastatic ChRCC. This case illustrated how morphoproteomic analysis can help elucidate the response mechanisms and guide therapy in such difficult cases when there are no actionable aberrations in a clinical next generation sequencing panel.

The findings in this case are in line with our clinical experience with RCC, and reflect preclinical data suggesting how complex pathways drive resistance, and response in these tumors. From unpublished experience (personal communication Dr. Robert E. Brown) with metastatic RCC and resistance signatures to rapalogs, it has been found that rapalogs inhibit mTORC1, and upregulate mTORC2 pathway components (e.g., p-Akt [Ser473]) in the context of IGF signaling²⁰ along with constitutive activation of the ERK, and STAT3 prosurvival/antiapoptotic pathways²¹. The tumor in our patient showed these adaptive mechanisms of mTORC2 pathway activation against a background of a constitutively activated IGF signaling, ERK, and STAT3 pathways, thereby explaining the resistance to everolimus.

Oxaliplatin works via p53 through a complex molecular mechanism. In colorectal cancer (CRC) cell lines with wild type-p53, oxaliplatin has been shown to increase the levels of p53, and promote apoptosis through multiple mechanisms including upregulation of the pro-apoptotic Bax gene²². Oxaliplatin also likely worked through the inhibition of HIF expression, and p-Akt, which has been demonstrated in hepatocellular cancer cell lines²³. Functional p53 has also been shown to mediate sensitivity to 5-fluorouracil, the active metabolite of capecitabine²⁴. In esophageal cancer and CRC cell lines, oxaliplatin²⁵, and

capecitabine²⁶ respectively have been shown to inhibit the phosphorylation of STAT3, thereby interfering with the prosurvival/antiapoptotic STAT3 pathway which was upregulated in this tumor. Presence of wild type-p53, and activation of STAT3 pathway could partially explain the response to oxaliplatin and capecitabine combination, whereas presence of hypoxia response markers and p-Akt expression further supported the use oxaliplatin.

The expression of VEGF-A, and activation of MAPK/ERK pathway in this tumor supported the addition of bevacizumab to oxaliplatin and capecitabine for this patient. We had previously demonstrated how regimens similar to this one, containing capecitabine, and platinum agents exhibit synergy with VEGF/R inhibition across a wide range of cancers²⁷. Apart from tumor neovascularization, evidence from several preclinical studies point towards a more multifaceted role of VEGF/R towards promotion of neoplasia. In different cancer cell lines VEGF has been shown to provide antiapoptotic signals, promote epithelial-mesenchymal transition, and metastasis through direct and indirect mechanisms²⁸⁻³⁰. Bevacizumab binds to VEGF, thereby preventing it from binding to, and activating its cognate receptors. VEGF stimulation has also been shown to promote ERK phosphorylation, and cell proliferation, while bevacizumab removed this effect by blockade of ERK1/2 activation^{31,32} thus likely disrupting the activated MAPK/ERK pathway in this tumor. It has also been hypothesized that bevacizumab-induced “normalization” of vascular architecture produces a transient, and long term hemodynamic phenomenon which improves chemotherapy delivery to tumor tissue²⁷. These mechanisms help to explain how bevacizumab contributed towards the response, and has consistently shown benefit in clinical studies.

There were some limitations with our approach. This NGS platform did not test for FLCN, or TERT mutations which have also been reported in ChRCC.² However, we do not have targeted therapies for these aberrations. In addition the proteomic panel was a targeted proteomic panel with IHC based approach and the level of protein expression may change with therapy. We did not have proteomics data on the initial tumor sample, and the subsequent tumor sample which progressed during the second phase I trial. Those information might have helped us to study the evolution, and better characterize this tumor.

Conclusion

In conclusion, this case illustrates how semi-quantitative assessment of cell signaling pathways using morphoproteomics and immunohistochemistry can help elucidate response and resistance mechanisms when the clinical next generation sequencing panel are negative. In addition they have the potential to guide therapy for rare or resistant cancers without established treatments.

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Clinical Practice Points

- Metastatic chromophobe renal cell carcinoma (ChRCC) is often resistant to systemic therapy, has poor survival, and very limited data on therapeutic options.
- We report a patient with recurrent metastatic ChRCC who was refractory to mTOR inhibitor therapy but showed a prolonged response to a combination of cytotoxic chemotherapy with oxaliplatin, oral capecitabine, and intravenous bevacizumab.
- We used targeted exome next generation sequencing and morphoproteomics to help elucidate the mechanisms of resistance and response, in order to guide therapy.
- We found predominance of the mTORC2 pathway against a background of constitutively activated IGF signaling, MAPK/ERK, and STAT3 pathways, which explained the resistance to mTOR inhibitor.
- The presence of wild type p53, HIF expression, p-Akt, activated STAT3 pathways in this tumor supported the use of oxaliplatin and capecitabine. While VEGF-A expression, and activation of ERK pathway guided the choice for bevacizumab.
- This case illustrates how proteomics based profiling can help elucidating response and resistance mechanisms for rare cancers in the absence of actionable genetic aberrations.
- These response signatures can help guide therapy in similar cases of refractory ChRCCs.

Figure 1. Analysis of chromophobe renal cell cancer

Immunohistochemical staining showed **a.** expression of p-IGF-1 receptor phosphorylated on tyrosine 1165/1166 at up to 2+ (on a scale of 0 to 3+) in the cytoplasmic compartment and up to 1+ on the plasmalemmal aspect, **b.** absence of expression (0 signal intensity) of p-c-Met (Tyr1234/1235) in the tumor cells, **c.** expression with nuclear translocation of p-ERK 1/2 (Thr 202/Tyr204) in nearly one-third of the tumor cells, **d.** Up to 3+ nuclear expression of p-mTOR (Ser 2448), **e.** expression of p-Akt (Ser 473) up to 1+ in the plasmalemmal and nuclear compartments, **f.** Overnight negative control, **g.** Absence of mutant p53 expression in viable tumor, and **h.** expression of mutant p53 in perinecrotic tumor cells.

Figure 2. Analysis of tumor after patient failed vandetanib and everolimus combination
a. The mitotic index is 6 mitotic figures per 10 high power fields. Immunohistochemical staining showed **b.** the antiapoptotic protein, Bcl-2 was expressed at up to 3+ in the cytoplasm of the tumor cells, **c.** nuclear immunopositivity for p-STAT3 (Tyr705) up to 3+. Analysis for angiogenic pathway proteins showed **d.** mild expression (up to 1+) of hypoxia-inducible factor (HIF)-1 alpha, confined to the cytoplasm, **e.** moderate expression of HIF-2 alpha (up to 2+) in the cytoplasmic compartment, and **f.** moderate expression (up to 2+) of vascular endothelial growth factor (VEGF)-A isoform on the plasmalemmal aspect. **g.** CD44 was variably expressed from 0 to 3+ on the plasmalemmal aspect of the tumor cells. There was greater plasmalemmal expression in the viable tumor cells adjacent to the necrotic tumor cells consistent with the influence of the hypoxic microenvironment. **h.** Glioma-

associated oncogene protein (Gli) 2 showed variable expression in the tumoral nuclei (up to 3+), once again most pronounced in the viable tumor cells adjacent to the necrotic region.

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Figure 3.
CT scan of the abdomen showing response to hepatic arterial infusion therapy in the liver metastases. **a.** pre-therapy scans showing large liver metastases. **b.** post- therapy scans showing reduced tumor bulk

Table 1

Results of morphoproteomic analysis

Molecular pathway	Patient's tumor	Microanatomical region	Implication/Inference	Figure
Protein analyte	Score ^a	Compartment ^b		
Upstream signal transducers:				
p-IGF-1R (Tyr 1165/1166)	±2+/0-1+	C/P	The insulin-like growth factor (IGF) pathway was constitutively activated as shown by moderate expression of p-IGF-1 receptor (R) phosphorylated on tyrosine 1165/1166 in the cytoplasmic compartment and weak expression on the plasmalemmal aspect.	1a
p-c-Met (Tyr 1234/1235)	0		The c-Met pathway was not activated, as evidenced by the absence of expression (0 signal intensity) of p-c-Met (Tyr1234/1235) in his tumor cells.	1b
Downstream effectors and pathways of convergence:				
p-ERK 1/2 (Thr202/Tyr204)	0-3+/0-1+	N/C-P	The MAPK/ERK pathway was constitutively activated by virtue of the expression with nuclear translocation of p-ERK 1/2 (Thr 202/Tyr204). The mTOR pathway was also constitutively activated, with strong nuclear expression of p-mTOR (Ser 2448). This coupled with the concomitant weak expression of p-Akt (Ser 473) in the plasmalemmal and nuclear compartments indicated a component of mTORC2 pathway activation and signaling ³³⁻³⁵ . This was consistent with downstream signaling through the IGF pathway ²⁰ in this tumor.	1c
p-mTOR (Ser 2448)	0-3+/0-±	P/N		1d
p-Akt (Ser 473)	0-1+/0-1+	P/N		1e
Nuclear cell cycle-related analytes ^a				
p53	<5%	N	The tumor showed predominantly wild type p53 expression in the majority of the tumor. A minor component of the tumor cell nuclei (<5%) in the perinecrotic zone showed mutant p53 expression likely secondary to oxidative stress.	1g, 1f
Mitotic index ^c	6	N		2a
Antiapoptotic /Tumorigenic /Angiogenic/Chemo-resistance Factors:				
Bcl-2	±3+	C	There was strong expression of antiapoptotic protein, Bcl-2 in the cytoplasm of the tumor cells. The STAT3 pathway was constitutively with up to 3+ nuclear immunopositivity for p-STAT3 (Tyr705). Angiogenic pathway protein analysis showed weak expression of hypoxia-inducible factor (HIF)-1 alpha confined to the cytoplasm, moderate expression of both HIF-2 alpha in the cytoplasmic compartment and vascular endothelial growth factor (VEGF)-A on the plasmalemmal aspect, consistent with the role of the former in maintaining the VEGF levels in renal cell carcinoma cells ^{36,37} . There was variable expression of Sirt1.	2b
p-STAT3 (Tyr 705)	0-3+	N		2c
Sirt1	0-3+	N		
HIF-1 alpha	±1+	C		2d
HIF-2 alpha	0-2+	C		2e
VEGF-A	0-2+	P		2f
Stemness Markers /Sonic Hedgehog-TGF-beta {Smad3} EMT Pathway				
CD44	0-3+	P	Minor component greater adjacent to tumor necrosis	2g
Gli2	0-3+/±	N/C	Nuclear positivity minor component and greatest adjacent to tumoral necrosis	2h

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Chromogenic signals were evaluated by bright-field microscopy and semi-quantified with regard to percentage of cells stained (0–100%) and the staining intensity (0: non-staining, 1+: weak staining, 2+: moderate staining, and 3+: strong staining).

Compartmentalization of proteins have been denoted by C = cytoplasm, N = nuclear, P = plasmalemma

Mitotic index calculated by counting the number of mitotic figures per 10 high power fields