Potential Histologic and Molecular Predictors of Response to Temsirolimus in Patients with Advanced Renal Cell Carcinoma

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Abstract

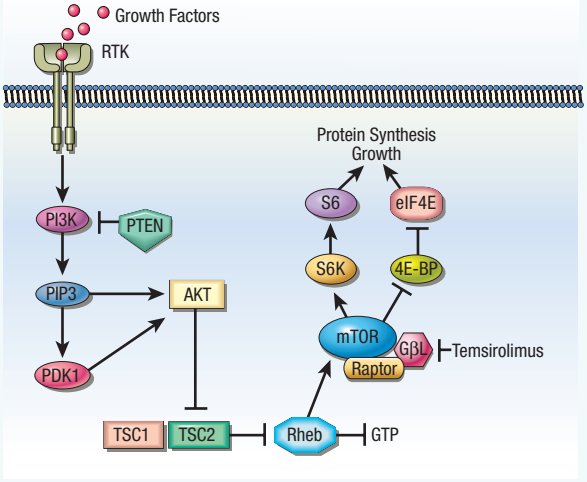
Purpose: Similar to other molecularly targeted agents, temsirolimus, an inhibitor of mammalian target of rapamycin, has shown promising activity in advanced renal cell carcinoma. However, only a subset of patients appears to derive significant tumor responses. In an effort to identify potential predictors of response to temsirolimus, tumor samples from a subset of patients within a randomized phase II trial of temsirolimus in advanced renal cell carcinoma were studied. **Patients and Methods:** Paraffin-embedded tissue sections from patients who had received temsirolimus were immunostained with antibodies to carbonic anhydrase IX, phospho-S6, phospho-Akt (pAkt), and phosphotase and tensin homologue. Expression levels were correlated with objective response (partial response [PR], minor response [MR]) and clinical benefit (PR, MR, SD ≥ 4 cycles) to temsirolimus. In addition, von Hippel–Lindau (*VHL*) mutational analysis was performed and correlated with response. **Results:** Tissue specimens were obtained from 20 patients who were evaluable for both tumor response and staining for phospho-S6 and carbonic anhydrase IX. In addition, 19 specimens were evaluable for pAkt, and 18 for phosphotase and tensin homologue. *VHL* mutational analysis was performed on 16 samples. Five patients achieved an objective response (1 PR/4 MRs) to temsirolimus. There was a positive association of phospho-S6 expression ($P = .02$) and a trend toward positive expression of pAkt ($P = .07$) with response to temsirolimus. No patient without high expression of either phospho-S6 or pAkt experienced an objective tumor response. There was no correlation of carbonic anhydrase IX and phosphotase and tensin homologue expression or VHL status with response to temsirolimus. **Conclusion:** These results suggest that phospho-S6 and pAkt expression are promising predictive biomarkers for response to temsirolimus that are worthy of further exploration for use in patient selection models for mammalian target of rapamycin inhibitors.

Introduction

Therapies for metastatic renal cell carcinoma have traditionally been limited. Recently, several novel agents targeting specific molecular aberrations have demonstrated promising efficacy in renal cell carcinoma. Whereas a large proportion of patients appear to derive some degree of clinical benefit from these targeted therapies, responses have rarely been complete or maintained off of therapy. Therefore, identification of baseline characteristics that predict for significant clinical responses to specific targeted therapies is a high priority.

One promising targeted agent is temsirolimus (CCI-779), an ester of the immunosuppressant rapamycin and an inhibitor mammalian target of

Figure 1 The Mammalian Target of Rapamycin Signaling Pathway



Abbreviations: 4E-BP = the eukaryotic initiation factor 4E binding protein; GβL = G protein β protein subunit-like; GTP = guanosine-5′-triphosphate; PDK1 = pyruvate dehydrogenase kinase, isoenzyme 1; PI3K = phosphoinositide kinase-3; Rheb = Ras homologue enriched in brain; RTK = receptor tyrosine kinase; TSC = tuberous sclerosis complex 2

rapamycin (mTOR) kinase activity. Temsirolimus demonstrated antitumor activity and encouraging progression-free and overall survival (OS) in a phase II study randomizing patients with advanced renal cell carcinoma to receive 3 different doses.[1] Overall, 111 patients were enrolled and 110 patients went on to receive therapy: 36 patients at 25 mg/m^2, 38 patients at 75 mg/m^2, and 37 patients at 250 mg/m^2. The vast majority of patients (91%) had received ≥ 1 previous therapy and 51% had ≥ 2 previous therapies. Although the objective response rate was only 7%, 26% of patients experienced minor responses (MRs) and another 17% of patients had stable disease (SD) lasting ≥ 6 months. The median time to tumor progression and median survival were 5.8 months and 15 months, respectively. Interestingly, patients in intermediate- and poor-prognostic populations, as determined by Memorial Sloan-Kettering Cancer Center (MSKCC) criteria, had a 1.6- to 1.7-fold longer median survival than the historic control of patients treated with first-line interferon (IFN)–α.[1,2] As neither toxicity nor efficacy was significantly affected by temsirolimus dose-level, a dose of 25 mg intravenous (I.V.) weekly was selected for further study as a single agent.

Based on these results, a randomized 3-arm phase III trial comparing temsirolimus versus IFN-α alone versus the combination of temsirolimus was performed and preliminary analysis was recently reported.[3] Overall, 626 previously untreated patients with metastatic renal cell carcinoma and poor risk features (≥ 3 of 6 risk factors; 5 MSKCC risk factors and > 1 metastatic site) were enrolled and randomized in a 1:1:1 fashion to receive either IFN-α up to 18 MU subcutaneously 3 times per week, temsirolimus 25 mg I.V. weekly, or the combination of temsirolimus 15 mg I.V. weekly and IFN-α 6 MU subcutaneously 3 times per week. The OS of patients treated with temsirolimus alone was statistically longer than those treated with IFN-α alone (10.9 months vs. 7.3 months; hazard ratio, 0.73; P = .0069). There was no statistical difference between patients treated with IFN-α alone and the combination of IFN-α and temsirolimus. Temsirolimus is the first molecularly targeted agent to demonstrate a statistically significant survival benefit in first-line therapy of patients with metastatic renal cell carcinoma. With the use of temsirolimus in renal cell carcinoma therapy expected to expand, particularly in patients with high-risk features, development of patient selection strategies for therapy with mTOR inhibitors is of great interest.

Potential predictive biomarkers for response to temsirolimus include expression of signaling substrates upstream and downstream from mTOR. The inhibition of downstream effectors of mTOR, specifically the 40S ribosomal protein p70S6 kinase and eukaryotic translation initiation factor 4E binding protein-1, causes G1 phase cell cycle arrest.[4] As shown in Figure 1, the mTOR pathway is downstream from the PI3-kinase and Akt pathway. PI3-kinase is most frequently activated by growth factors binding to receptor tyrosine kinases, resulting in generation of phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 binds directly to the pleckstrin homology domain of Akt and mediates its localization to the cell membrane where it is then activated by phosphorylation, in part by phosphoinositide dependent kinase–1. PIP3 levels, and therefore activity of the PI3-kinase/Akt pathway, are regulated by the phosphotase and tensin homologue (*PTEN*) tumor suppressor gene. Although *PTEN* mutations are believed to be rare in renal cell carcinoma, *PTEN* gene expression has been shown to be downmodulated in ≥ 20%-30% of renal cell carcinoma, presumably by epigenetic silencing.[5,6] Decreased *PTEN* expression has also recently been shown to be an independent negative prognostic factor for disease-specific survival in metastatic renal cell carcinoma.[7] In vitro studies have demonstrated that cells deficient in PTEN are particularly sensitive to the cytostatic effects of temsirolimus.[8] Akt activates mTOR through phosphorylation of tuberous sclerosis complex–2, resulting in the inhibition of its GTPase-activating protein activity toward the small GTPase Ras homolog enriched in brain. Relief of this GTPase-activating protein activity enhances the GTP loading of Ras homolog enriched in brain, resulting in enhanced activation of mTOR. Once activated, mTOR acts through its downstream effectors to regulate protein synthesis and cell cycle progression. More specifically, activation of mTOR also increases hypoxia inducible factor (HIF)–1α expression, both at the levels of messenger RNA translation and protein stabilization.[9,10] Inhibition of mTOR in cell lines possessing biallelic deletion of tuberous sclerosis complex–2 gene (resulting in mTOR activation) leads to normalization of HIF levels and partial downregulation of vascular endothelial growth factor (VEGF).[11] Therefore, the activity of temsirolimus in sporadic clear-cell renal cell carcinoma, the majority of which possess biallelic alterations in the von Hippel–Lindau (*VHL*) gene resulting in accumulation of HIF-1α and HIF-2α, might be mediated at least in part by its activity against HIF. It was recently shown in a mouse renal cell carcinoma xenograft model that loss of *VHL* predicted for sensitivity to the effects of temsirolimus likely through the inhibition of mTOR-dependent translation of HIF-1α and HIF-2α.[12]

In addition to molecular biomarkers related to targeted path-

ways, other tumor markers might be worthy of exploration as predictive biomarkers. We have recently shown in retrospective analysis that certain histologic features along with carbonic anhydrase IX expression in renal cell carcinoma tumor specimens can predict for response to high-dose interleukin (IL)–2.[13,14] Carbonic anhydrase IX might be of particular interest in this case as its expression is under the control of HIF-1α, a putative target of temsirolimus. In an effort to explore the predictive value of the pretreatment expression of signaling substrates of targeted pathways in addition to pathologic markers previously explored in other contexts, we evaluated renal cancer specimens collected from patients treated on the randomized phase II trial of temsirolimus in patients with advanced renal cell carcinoma and correlated with response and other clinical endpoints.

Patients and Methods

Patients

Tissue blocks were collected from 20 patients who had participated in the randomized phase II temsirolimus at the Beth Israel Deaconess Medical Center and for whom both tissue and clinical data were available. All patients had previously provided informed consent to participate in this clinical trial. Approval for the current investigation linking clinical data with pathologic investigations was obtained from the institutional review board of the Dana-Farber Cancer Institute/Harvard Cancer Center. Responses in the randomized phase II trial were classified according to World Health Organization criteria. In addition, patients were classified as having experienced clinical benefit if they experienced a best response of MR, partial response (PR), or SD for ≥ 4 cycles of therapy. Patients with SD < 4 cycles were not classified as having experienced clinical benefit.

Immunohistochemistry

Specimens were stained using the following antibodies: carbonic anhydrase IX (MN-75)[15] phospho-Akt (Ser473), phospho-S6 ribosomal protein (Ser235), and PTEN. Slides were prepared from paraffin-embedded specimens, 5 μm thick, and stained with hematoxylin and eosin. Sections were dewaxed, soaked in alcohol, and after microwave treatment in antigen unmasking solution for 10 minutes, incubated in 3% hydrogen peroxide for 15 minutes to inactivate endogenous peroxidase. Sections were incubated with the appropriate antibody and detection was performed using DAKO EnVision+™ System horseradish peroxidase detection kit. Semiquantitative assessment of antibody staining was done by a single pathologist blinded to the clinicopathologic variables. As described by Bui et al, specimens in which > 85% of tumor cells stained for carbonic anhydrase IX were labeled as high carbonic anhydrase IX expressors, whereas those in which ≤ 85% of cells expressed carbonic anhydrase IX were labeled as low carbonic anhydrase IX–expressing tumors.[16] Expression of phospho-S6 was scored based on a composite of staining intensity (graded 0 = 1+, 1 = 2+, 2 = 2-3+, and 3 = 3+) and percentage of tumor cells staining positive (1 = 1%-29%, 2 = 30%-69%, 3 = 70%-100%). Patients were then classified as low (product of intensity and staining percentage score = 0-1), intermediate (2-3), and high (4-9) expressors of phospho-S6. Phospho-Akt (pAkt) expression was similarly scored based on a composite of intensity (graded 0 = 1+, 1 = 1-2+, and 2 = 2+ or 2-3+) and percentage of tumor cells positive (1 = 1%-29%, 2 = 30%-69%, 3 = 70%-100%). Patients were then classified as low (product of intensity and staining percentage score = 0), intermediate (1-2), and high (3-6) expressors of pAkt. *PTEN* expression was scored based on a product of intensity (graded 2 = 2+, 2.5 = 2-3+, and 3 = 3+) and percentage of tumor cells staining positive (1 = 1%-25%, 2 = 26%-50%, 3 = 51%-75%, 4 = 76%-100%). *PTEN* expression was then classified as either low (product = 2-6) or high (7.5-12).

Von Hippel–Lindau Sequencing

VHL sequencing was performed in collaboration with the DNA Sequencing Laboratory of the Harvard Partners Genome Center, which is part of the Harvard Partners Center for Genetics and Genomics. DNA was extracted from paraffin-embedded tissue sections using the QIAamp® DNA Micro Kit. In order to enrich for tumor cells, tissue sections were stained with hematoxylin and eosin and microdissected under the microscope using a 27 G needle syringe. DNA samples from both frozen and paraffin-embedded tumor samples with known mutational status were utilized as controls. DNA was amplified using a polymerase chain reaction–based whole genome amplification protocol. Amplification of exons 1-3 (and flanking regions) of the *VHL* gene was subsequently performed using sets of primers designed with an automated primer selection program. Bidirectional resequencing of the amplicons was then performed. The amplicon sequences were reviewed and variants identified for each individual. Each variant was to be characterized with respect to location, sequence environment (4 bases either side), type of variation, and if it is in coding sequence, the codon number and amino acid change. Because of the recent demonstration that artificial sequence alterations are frequently detected with the use of formalin-fixed tissues, PCR products from 4 independent amplifications were sequenced for each case.[17,18]

Statistical Analysis

Comparisons of degree of clinical benefit according to categorized antibody staining levels used Jonckheere-Terpstra tests for doubly-ordered RxC tables to test an ordered alternative hypothesis that degree of staining levels was associated with degree of clinical outcome versus the null hypothesis that clinical benefit was the same at all staining levels. Kaplan-Meier curves of OS were plotted for time to progression and OS from time of initiation of temsirolimus. The analysis used SAS version 9. Two-sided *P* values were reported throughout.

Results

Demographics

Demographics and response characteristics of the 20 patients included in this analysis are described in Table 1. Seventeen patients (85%) were men, 15 patients (75%) and 1 patient (5%) were classified as having intermediate and poor prognoses, respectively, based on the MSKCC clinical criteria.[2] In the overall randomized phase II trial, there was 1 complete response,

Table 1 Patient Demographics (N = 20)

Characteristic	N (%)
Male Sex	17 (85)
Previous Nephrectomy	18 (90)
Response	
PR	1 (5)
MR	4 (20)
SD ≥ 4 cycles	5 (25)
SD	3 (15)
PD	7 (35)
Survival	
Alive	1 (5)
Dead	19 (95)
MSKCC Risk Group	
Favorable	4 (20)
Intermediate	15 (75)
Poor	1 (5)
Median Time to Progression, Months (Range)	3.6 (3.6-35.7)
Median OS, Months (Range)	12.2 (2.6-43.8)

Table 2 Pathologic Evaluation (N = 20)

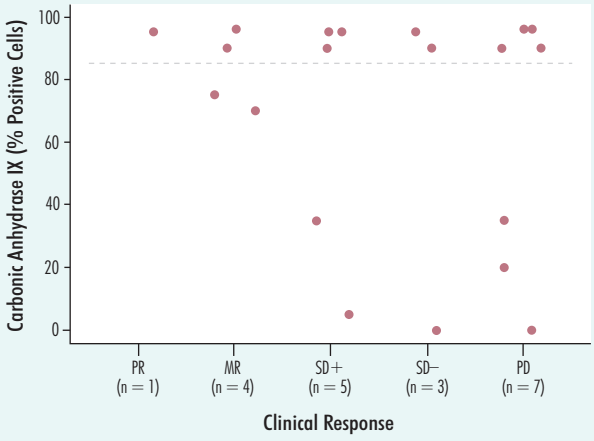
Characteristic	N (%)
Specimen	
Primary	12 (60)
Metastatic	8 (40)
Clear-Cell Features	17 (85)
Alveolar Features	15 (75)
Granular Features	16 (80)
Pseudopapillary Features	5 (25)

7 PRs, and 29 MRs according to World Health Organization response criteria for an overall response rate of 33%.[1] Objective responses occurred in 25% of the patients in this analysis including 1 PR and 4 MRs. There were also 5 patients who experienced clinical benefit in the form of tumor regression or SD which was maintained over ≥ 4 cycles of therapy (2 separate disease evaluations by computed tomography scan). At the time of this analysis, 1 of 20 patients remains alive with an overall median survival of 12.2 months after initiation of temsirolimus.

Pathology Specimen Characteristics

The characteristics of the pathology specimens examined are summarized in Table 2. Twelve specimens (60%) were obtained from the renal primary tumor with the remainder coming from metastatic lesions. No specimens taken from both the primary and metastatic lesions of the same patient were available. Eighteen specimens were assessed as clear-cell histology with 15, 16, and 5 of these being classified as having alveolar, granular, or papillary features, respectively. Thirteen specimens

Figure 2 Carbonic Anhydrase IX Expression and Clinical Response to Temsirolimus



(65%) and 7 specimens (35%) were classified as intermediate and poor prognosis based on the previously reported pathologic model developed as a predictor of response to IL-2–based immunotherapy.[13] Three (2 MRs, 1 PR) of 13 patients (23%) with intermediate pathologic risk and 1 MR of 7 patients (14%) with poor pathologic risk experienced objective responses. Six of 13 patients (46%) with intermediate pathologic risk and 4 of 7 patients (57%) with poor pathologic risk experienced clinical benefit from temsirolimus.

Association of Carbonic Anhydrase IX Expression with Response to Temsirolimus

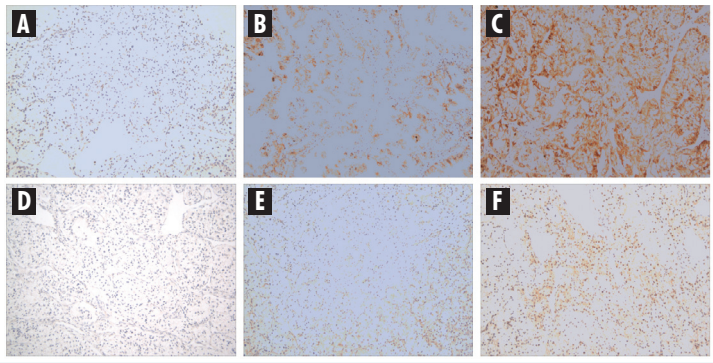
Carbonic anhydrase IX staining was performed on all 20 tumor samples. Figure 2 depicts the relationship between carbonic anhydrase IX expression and response to temsirolimus. Using the previously described definition of high carbonic anhydrase IX expression as > 85% of tumor cells positive, there was no correlation between carbonic anhydrase IX expression and objective response or clinical benefit to temsirolimus (P = .92). Two of 8 patients (25%) with low carbonic anhydrase IX and 3 of 12 patients (25%) with high carbonic anhydrase IX were responders. Four of 8 patients (50%) with low carbonic anhydrase IX and 6 of 12 patients (50%) with high carbonic anhydrase IX experienced clinical benefit. Patients with very low expression of carbonic anhydrase IX (< 50%) did not appear to respond to temsirolimus. The median OS in the high carbonic anhydrase IX–expressing group was 20.3 versus 10 months in the low expressors.

Association of Phospho-S6 Expression with Response to Temsirolimus

Twenty tumor specimens were stained for phospho-S6 expression and graded as described. Representative stains are shown in Figure 3A-3C. There was a positive association between higher phospho-S6 expression and clinical response to temsirolimus as defined as PR, MR, or SD over ≥ 4 cycles of therapy (P = .02; Table 3). There were no objective responses in the patients with low phospho-S6 expression and all 4 patients had progressive

disease. The objective response rates were 20% (1 of 5 patients) and 36% (4 of 11 patients) in the intermediate and high phospho-S6 expression groups, respectively. Three of 5 patients (60%) with intermediate expression of phospho-S6 and 7 of 11 patients (64%) with high expression of phospho-S6 experienced clinical benefit from temsirolimus. Median OS was 17.3 versus 9.1 months among patients with high S6 expression versus those with intermediate or low S6 expression (P = .02). There was no significant difference noted in expression of phospho-S6 in the metastatic versus primary tumor samples. Two of 7 patients with high phospho-S6 expression in metastatic lesions and 2 of 6 patients with high S6 expression in nephrectomy samples experienced objective tumor responses.

Figure 3 Representative Stains of Renal Cell Carcinoma Tumor Specimens



(**A**) Low phospho-S6; (**B**) intermediate phospho-S6; (**C**) high phospho-S6; (**D**) low phospho-Akt; (**E**) intermediate phospho-Akt; (**F**) high phospho-Akt.

Association of Phospho-Akt Expression with Response to Temsirolimus

Nineteen tumor samples were stained for pAkt expression and graded as described. Representative stains are shown in Figure 3D-3F. One tumor specimen from a patient who had experienced a clinical response (MR) was insufficient for further staining. There was a trend toward a positive association between higher pAkt expression and response to temsirolimus (P = .07; Table 4). No patients with low pAkt expression experienced an objective response. Two of 10 patients (20%) with intermediate pAkt expression and 2 of 4 patients (50%) with high pAkt expression experienced objective responses. Five of 10 patients (50%) with intermediate pAkt expression and 3 of 4 patients with high pAkt expression experienced clinical benefit from temsirolimus. Three of 4 patients with high pAkt expression also had high expression of phospho-S6. However, only 3 of 11 patients with high phospho-S6 had high expression of pAkt. As with phospho-S6 expression, no significant difference was noted between pAkt staining in metastatic versus primary tumor samples.

Association of PTEN Expression with Response to Temsirolimus

Eighteen tumor samples were stained for *PTEN* expression. The majority of patients (12 of 18 patients) demonstrated high and presumably intact *PTEN* expression. Two of 6 patients (33%) with low *PTEN* expression and 2 of 12 patients (17%) with high *PTEN* expression experienced objective responses to temsirolimus. Two of 6 patients (33%) with low *PTEN* expression and 7 of 12 patients (58%) with high *PTEN* expression experienced clinical benefit (P = .55). There was no clear association between low *PTEN* expression with high expression of either pAkt or phospho-S6.

Association of von Hippel–Lindau Mutational Status and Response to Temsirolimus

VHL mutational analysis was able to be performed on 16 tumor samples. Table 5 displays the results of the analysis and the relationship between *VHL* mutational status and clinical benefit. Five of 16 tumor samples analyzed had reproducible *VHL* mutations and 5 did not show any sequence variation. In the remaining 6 tumor samples, we detected multiple nonreproducible sequence alterations that were interpreted as artifactual. One of 5 patients (20%) with a *VHL* mutation and 3 of 11 patients (27%) without a *VHL* mutation had an objective response. Three of 5 patients with a detectable mutation in *VHL* experienced clinical benefit

Table 3 Expression of Phospho-S6 and Response to Temsirolimus

Expression	PR	MR	SD+	SD–	PD
Low (n = 4)	0	0	0	0	4
Intermediate (n = 5)	0	1	2	1	1
High (n = 6)	1	3	3	2	2

Abbreviations: SD+ = SD ≥ 4 cycles, SD– = SD < 4 cycles

Table 4 Expression of Phospho-Akt and Response to Temsirolimus

Expression	PR	MR	SD+	SD–	PD
Low (n = 5)	0	0	1	1	3
Intermediate (n = 10)	0	2	3	2	3
High (n = 4)	1	1	1	0	1

Abbreviations: SD+ = SD ≥ 4 cycles, SD– = SD < 4 cycles

Table 5 Von Hippel–Lindau Mutational Analysis and Response to Temsirolimus

Analysis	N	PR	MR	SD+	SD–	PD
VHL Mutation	5	1	0	2	0	2
VHL Wild-type	11	0	3	3	2	3

Abbreviations: SD+ = SD ≥ 4 cycles, SD– = SD < 4 cycles

Table 6 Composite Expression of Phospho-S6 and Phospho-S6 and Response to Temsirolimus

Expression	Low Phospho-S6 Expression	Intermediate Phospho-S6 Expression	High Phospho-S6 Expression
Low pAkt	0 of 3	0 of 1	0 of 1
Intermediate pAkt	0 of 1	0 of 3	2 of 6
High pAkt	0	1 of 1	1 of 3

Expressed as number of objective responses (MR/PR)/total number with specific composite expression.

from temsirolimus therapy compared with 6 of 11 patients without a documented *VHL* mutation. All 5 patients with a *VHL* mutation and 5 of 11 patients without a *VHL* mutation had high carbonic anhydrase IX expression.

Discussion

Temsirolimus has resulted in tumor regressions and delays in median time to progression in patients with advanced renal cell carcinoma as well as improved OS in patients with poor prognostic features. However, similar to other targeted agents in renal cell carcinoma therapy, many patients experienced clinical benefit but only a small group exhibited tumor response. In order to direct appropriate therapies to specific patients, robust predictive models must be developed to identify those patients who are likely to experience significant tumor responses with particular therapies. Because of the small overall sample size and smaller number of objective tumor responses, this exploratory analysis seeks to identify potential predictive biomarkers worthy of further investigation. Therefore, the predictive factors identified will hopefully help direct future retrospective analyses as well as clinical trial design with temsirolimus or other mTOR inhibitors.

As previous analyses have shown that certain histologic features and carbonic anhydrase IX expression might predict for response to IL-2 therapy,[13,14] these pathologic markers were chosen for the initial analysis. Because functional loss of *VHL* is believed to be a central feature of clear-cell renal cell carcinoma, this initial analysis also included examination of *VHL* mutational status. The data reported herein did not find a significant correlation of IL-2 pathologic predictive risk group, high carbonic anhydrase IX expression, or *VHL* mutational status (in contrast to animal models) with response to temsirolimus. However, there does appear to be a trend that patients with very low expression of carbonic anhydrase IX (< 50%) did not respond to temsirolimus. Because of the regulation of carbonic anhydrase IX by HIF, this trend might be consistent with the concept that the activity of temsirolimus is at least partially mediated through inhibition of HIF. Whereas the small sample size makes any definitive conclusions impossible, the lack of a strong correlation also suggests a partially HIF-independent pathway of response to temsirolimus. The observed lack of correlation between *VHL* mutational status and response to temsirolimus is also consistent with data from the phase III placebo-controlled trial of sorafenib (a multitargeted kinase inhibitor with activity against VEGF receptor 2 and platelet-derived growth factor receptor) in advanced renal cell carcinoma. It was recently reported that there was no significant correlation with *VHL* status and response to sorafenib.[19] This could suggest that *VHL* mutational status might not be an accurate predictor of HIF activity. Under hypoxic conditions, VHL function would be expected to have less relevance and HIF translation and stability could instead be dependent upon the mTOR-p70RSK-S6 pathway. Whereas functional loss of *VHL* with upregulation of HIF likely remains a critical element in renal tumor formation, the current data does not support *VHL* mutational status and expression of the HIF-regulated protein carbonic anhydrase IX as predictive biomarkers for mTOR inhibitors.

In a recent phase II trial of temsirolimus in glioblastoma multiforme, expression of phospho-S6 kinase, but not phospho-Akt or *PTEN*, in pretreatment tissue specimens was found to correlate with likelihood of radiographic response.[20] In contrast, the data reported herein demonstrate a similar correlation between phospho-S6 expression but also trend toward correlation between pAkt expression and response to temsirolimus. High phospho-S6 expression was also associated with a greater median survival relative to patients with lower phospho-S6 expression, suggesting that phospho-S6 could have a role as a prognostic factor as well as a predictive marker. Table 6 displays the composite expression of phospho-S6 and pAkt versus objective response in patients in whose specimens both stains were performed. These data suggest that patients without high expression of either phospho-S6 of pAkt might be unlikely to experience an objective response to temsirolimus compared with other patients (0 of 8 vs. 4 of 11 responses). Also taking into account the correlations of phospho-S6 and pAkt expression with clinical benefit, these findings suggest a potential selection model for patients who should or should not be treated with temsirolimus. Because of the small sample size and intrinsic subjectivity of grading intensity of immunohistochemical staining, these correlations must be strengthened in a larger analysis. Nevertheless, both pAkt and phospho-S6 show promise as predictive biomarkers and should be considered for future studies.

As tumor response in the phase II study was largely measured in metastatic disease, it would be expected that expression of these biomarkers in metastatic lesions might be more predictive of response. In this small study sample, we did not observe a difference in the predictive utility of phospho-S6 and pAkt expression in tissue from metastatic lesions versus the primary tumor. A larger study might be required to detect this difference. However, as biopsy samples for metastatic lesions in renal cell carcinoma are often difficult to obtain, the primary tumor removed during nephrectomy might be the most practical and often the only available source of tissue for analysis.

Interestingly, there were more patients expressing high levels of phospho-S6 (55%) than those expressing high levels of phospho-Akt (21%). It is possible that the phosphorylation site on Akt is simply less stable than that of the S6 ribosomal protein and, therefore, less reliable on paraffin-embedded sections. However, in vitro studies have demonstrated that S6K phosphorylation does not always correlate with Akt activity in mammalian cells, suggesting that this difference in expression frequency might be real.[21] This difference might be because of

the fact that other pathways, particularly the mitogen-activated protein kinase (MAPK) pathway through the p90 ribosomal S6 kinase, can phosphorylate the S6 ribosomal protein. However, it is also possible that there are additional mechanisms of either mTOR or S6 kinase activation independent of Akt and MAPK. Larger sample sizes will be needed to explore the correlation of phospho-S6 with other tumor markers, including those regulated by HIF and the MAPK pathway.

Similar to the previous study of temsirolimus in glioblastoma multiforme, there was no clear correlation between *PTEN* and clinical response to temsirolimus. Although the overall frequency of decreased *PTEN* expression (33%) observed in this study was similar to that reported in the literature, no clear trends emerged with respect to response to temsirolimus.

Conclusion

In the past few years, several novel therapies have shown promising efficacy in the therapy of metastatic renal cell carcinoma, with sorafenib and sunitinib recently joining IL-2 as Food and Drug Administration–approved therapies for this group of patients. However, as only subsets of patients experience significant tumor responses to each therapy, efforts in determining which patients should get which therapies will be critical in the future. Despite the small sample size, this analysis shows that phospho-S6 and phospho-Akt expression hold promise as predictive biomarkers for response to inhibitors of mTOR and are worthy of further exploration in larger analyses in which measurement of these substrates can be refined. If these larger analyses are, in turn, promising, these predictive biomarkers should be validated prospectively in a clinical trial. This analysis also demonstrates that surrogates for activation of pathways targeted by a particular therapy could be promising predictive biomarkers in patients with renal cell carcinoma. Similar studies could be performed with other therapies, particularly those targeting VEGF and VEGF receptor, in the hope of developing a more general selection model to help choose initial therapies and sequence of treatment. Finally, this analysis calls into question the fidelity in vivo of some of the major pathways felt to play a role in renal cell carcinoma in vitro, including discrepancies between pAkt expression and *PTEN* loss with phospho-S6 expression. Whether these observations result from technical issues with immunohistochemistry from paraffin-embedded tissue samples or suggest the existence of alternative pathways for mTOR activation requires further investigation.

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