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Mutations in TSC1, TSC2, and MTOR Are Associated with Response to Rapalogs in Patients with Metastatic Renal Cell Carcinoma

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Abstract

Purpose: We examined the hypothesis that mutations in mTOR pathway genes are associated with response to rapalogs in metastatic renal cell carcinoma (mRCC).

Experimental Design: We studied a cohort of mRCC patients who were treated with mTOR inhibitors with distinct clinical outcomes. Tumor DNA from 79 subjects was successfully analyzed for mutations using targeted next-generation sequencing of 560 cancer genes. Responders were defined as those with partial response (PR) by RECIST v1.0 or stable disease with any tumor shrinkage for 6 months or longer. Nonresponders were defined as those with disease progression during the first 3 months of therapy. Fisher exact test assessed the association between mutation status in mTOR pathway genes and treatment response.

Results: Mutations in *MTOR, TSC1*, or *TSC2* were more common in responders, 12 (28%) of 43, than nonresponders, 4 (11%) of 36 ($P = 0.06$). Mutations in *TSC1* or *TSC2* alone were also more common in responders, 9 (21%), than nonresponders, 2(6%), ($P = 0.05$). Furthermore, 5 (42%) of 12 subjects with PR had mutations in *MTOR, TSC1*, or *TSC2* compared with 4 (11%) of 36 nonresponders ($P = 0.03$). Eight additional non-mTOR pathway genes were found to be mutated in at least 4 of 79 tumors (5%); none were associated positively with response.

Conclusions: In this cohort of mRCC patients, mutations in *MTOR, TSC1*, or *TSC2* were more common in patients who experienced clinical benefit from rapalogs than in those who progressed. However, a substantial fraction of responders (24 of 43, 56%) had no mTOR pathway mutation identified. *Clin Cancer Res; 22(10); 2445–52. ©2016 AACR.*

See related commentary by Voss and Hsieh, p. 2320

Introduction

The PI3K–mTOR signaling pathway is one of the two main growth factor stimulated signaling cascades that regulate cell growth in many cell types. mTORC1 and mTORC2 are distinct complexes that each contain the serine/threonine kinase mTOR (1). The kinase activity of mTORC1 is regulated by both growth factor signaling and nutrient availability through distinct Ras family GTPases (2–5). Growth factor regulation of mTORC1 occurs largely through regulation of the GTPase–activating protein (GAP) activity of the TSC1/TSC2 protein complex for the ras family member RHEB (4, 6). Both PI3K and MAPK signaling cascades lead to phosphorylation of TSC2, reducing its GAP activity (7, 8). Nutrient sensing occurs through the Rag GTPases and is regulated by a protein complex termed GATOR1 (GAP activity towards Rags) that functions as a GAP for the Rag proteins (9). Both RHEB and Rag proteins regulate activation of mTORC1, which consists of mTOR, raptor, and mLST8 (1). Activated

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Translational Relevance

mTOR inhibitors, everolimus and temsirolimus, are approved in metastatic renal cell carcinoma (mRCC), but only a small subset of patients derives clinical benefit. Recent data have suggested that mutations in mTOR pathway genes might be associated with response to rapalogs in several malignancies, including RCC. We evaluated a large international cohort of mRCC patients with available pretreatment specimens who were treated with mTOR inhibitors and had distinct clinical outcomes. We found that mutations in *MTOR*, *TSC1*, or *TSC2* were more common in patients who experienced a response from rapalogs than in those with rapid progression. This association was even stronger in the subset of patients with partial response. Identification of biomarkers of response to mTOR inhibitors may lead to improved patient selection.

mTORC1 phosphorylates multiple downstream proteins, leading to complex metabolic and anabolic effects including synthesis of nucleotides, lipids, amino acids, biogenesis of ribosomes, and cap-dependent translation of cellular mRNAs (10, 11). The regulation of cap-dependent translation by mTORC1 is mediated by the mTOR-dependent phosphorylation (and inhibition) of 4E-BP1/2, an inhibitory eIF-4E binding protein (12).

mTORC1 is activated in cancer cells through multiple mechanisms, including growth factor and receptor tyrosine kinase signaling events. Genetic events that activate mTORC1 include activating mutations in *PIK3CA*, the gene encoding the catalytic subunit of PI3K; inactivating mutation or deletion of *PTEN*; activating mutation or amplification of one of the three AKT isoforms, *AKT1*, *AKT2*, *AKT3*; and inactivating mutation or deletion of either *TSC1* or *TSC2* (13). In addition, mutation or loss of *DEPDC5*, *NPRL2*, *or NPRL3*, which encode protein components of the GATOR1 complex, also lead to high-level activation of mTORC1 (9).

Rapamycin and related drugs collectively called rapalogs bind to FKBP12 to inhibit mTORC1 kinase activity (14–16). In a randomized phase 3 trial of 626 previously untreated patients with metastatic RCC and poor risk features who were randomized to either temsirolimus, a prodrug ester of rapamycin, IFNα, or both, single-agent temsirolimus-treated patients showed significantly longer overall survival (10.9 vs. 7.3 vs. 8.4 months; $P = 0.0069$; ref. 17). Similarly, everolimus (RAD001), another derivative of rapamycin, showed significant improvement in progression-free survival (PFS) in comparison with placebo (PFS 4.9 vs. 1.9 months, $P < 0.0001$) in a large randomized phase 3 trial in metastatic RCC patients who had failed treatment with VEGF-targeted tyrosine kinase inhibitor (18, 19). On the basis of these findings, inhibitors of mTORC1 are a standard therapy in RCC (20).

Unfortunately, clinical experience has shown that only a subset of RCC patients derives substantial benefit from mTORC1 inhibitor treatment. Complete responses to these drugs are extremely rare. Among those deriving significant benefit from rapalogs are those showing partial response (PR), and those that have extended disease control, even if not meeting the criteria for a PR.

Understanding the molecular basis of response to targeted therapies has gained high prominence recently as a method to both understand response and categorize patients for their likelihood of response. Mutations in *TSC1/TSC2/MTOR* have been shown to be associated with response to rapalog treatment in several cancer types, including a small set ($n = 5$) of patients with RCC (21–27).

Here we assess the hypothesis that mutations in selected mTOR pathway genes can predict response to rapalog therapy by performing molecular genetic analysis on a cohort of 79 RCC patients who were roughly evenly divided between those who demonstrated benefit from rapalog therapy versus those who had progression within 3 months of initiation of rapalog therapy.

Materials and Methods

Patients

We identified 97 mRCC patients treated with rapalogs with available pretreatment tumor tissues and distinct clinical outcomes. Eighteen patients were excluded because of an insufficient amount of DNA or assay failure. Seventy-nine mRCC patients with successful assay results were included in this study. These included 28 patients treated on the trial of temsirolimus versus IFNα versus both drugs (17) as well as 51 samples from patients treated with mTOR inhibitors between October 2007 and June 2013 at both U.S. and non-U.S. institutions. Patients were selected to include subjects that had either responded or rapidly progressed on rapalog therapy. For this study, we defined response as either PR (by RECIST v1.0), or stable disease (SD) with any tumor shrinkage (no growth) for at least 6 months. Nonresponders were patients showing progressive disease (PD) within the first 3 months of therapy (usually at first restaging), without marked toxicity leading to treatment discontinuation. All patients were treated with standard dosage of rapalogs: temsirolimus ($n = 41$ at 25 mg i.v. weekly) or everolimus ($n = 38$ at 10 mg orally daily).

Clinicopathologic data was obtained either from Pfizer through a data transfer agreement, or collected retrospectively from the institutions at which treatment was given, and included treatment received and best response to rapalog. Uniform data collection templates were used for all subjects. Institutional Review Board approval was obtained locally before tissue acquisition, processing, and provision of clinical information.

Tissue collection, DNA extraction, and next-generation sequencing

Formalin-fixed paraffin-embedded (FFPE) tissue sections and/or blocks were assessed for availability of material for sequencing. All material processing and sequencing were done without the knowledge of patients' treatment assignments or outcomes. Hematoxylin and eosin stained slides were reviewed by an expert genitourinary pathologist (S. Signoretti) and tumor areas containing at least 50% of tumor cells were selected for DNA extraction.

Targeted sequencing

For each tumor specimen, DNA was extracted from the selected tumor areas using the QIAamp DNA FFPE Tissue Kit (Qiagen). DNA was then subjected to targeted exon capture and sequencing using the Oncopanel_v3 cancer gene panel at the Center for Cancer Genome Discovery (CCGD) at the Dana-Farber Cancer Institute (DFCI; Boston, MA). OncoPanel_v3 consists of the coding exons of 560 genes of known or potential importance in cancer. Genes in the mTOR and related signaling pathways that are included in this capture set are: *PIK3C2B*, *PIK3CA*, *PIK3CG*,

PIK3R1, *PTEN*, *TSC1*, *TSC2*, *MTOR*, *RHEB*, *RPTOR*, *NPRL2*, *NPRL3*, *NF1*, *NF2*, *FLCN*, *RICTOR*, *DEPDC5*, and *STK11*. All genes commonly mutated in clear cell RCC are also included in this panel: *VHL*, *PBRM1*, *SETD2*, *KDM5C*, *BAP1*, *TP53*, *ATM*, and *ARID1A* (28).

Sequencing libraries were prepared, as previously described, starting from 200 ng of genomic DNA with inclusion of a unique bar-code for each sample to enable pooling (29). Libraries were quantified using qPCR (Kapa Biosystems, Inc.), pooled in equimass amounts to 500 ng total, and captured using the Oncopanel_v3 baitset using the Agilent SureSelect hybrid capture kit. The captured libraries were again quantified using qPCR, and sequenced on a Hiseq 2500 sequencer (Illumina Inc.) in 2 × 100 nucleotide (nt) paired end read mode. Primary sequence data were deconvoluted using index sequences to individual sample files and converted to FASTQ format using Picard tools. Reads were aligned to the human genome using bwa-0.5.8c (Burrows–Wheeler Alignment; ref. 30) and filtered to eliminate reads of low quality and duplicates. The data were then analyzed for sequence variants using tools from the Genome Analysis Toolkit (GATK; ref. 31), including IndelGenotyperV2 and UnifiedGenotyper, to identify indels and single-nucleotide variants. A second approach was used in parallel to analyze the sequence data, with capture of read calls at all positions using SAMtools Pileup (32) followed by custom processing in Python and Matlab to determine base call frequency at each position in each read orientation for *AKT1*, *AKT2*, *AKT3*, *MTOR*, *PIK3CA*, *PTEN*, *RHEB*, *TSC1*, and *TSC2*. These data were then filtered to eliminate variant calls observed in only a single read orientation, or seen in multiple samples to exclude artifacts derived from the sequencing process. All variants observed at a frequency of $\geq$ 2% were directly reviewed using the Integrative Genomics Viewer (33) to identify bona fide variant calls and exclude sequencing or alignment artifacts.

Samples were excluded if the mean depth of read coverage for the 560 genes was < 36× or if there was a high duplicate read rate. The mean, median, and range of mean depth of read coverage for the 79 remaining samples were 129, 125, and 36–414, respectively. The mean, median, and range of percentage of target bases with read depth > 30× was 95%, 98%, and 60%–99%, respectively.

Variants observed at any frequency in the 1000 Genomes variant server (34), the NHLBI Exome Variant Server (35), or the ExAC Exome Aggregation Consortium browser (36) were not considered further, as they were likely germline, nonfunctional variants. SNVs and indels that were identified as novel and/or of possible significance in *TSC1*, *TSC2*, *MTOR*, or *PTEN* were confirmed by Sanger bidirectional sequencing when seen observed at $\geq$5%–10% allele ratio; and amplicon next-generation sequencing (NGS) for those observed at < 5%–10% allele ratio. Amplicon NGS was performed by NGS analysis of individual amplicons, leading to the generation of 10,000 to 1,000,000 sequencing reads. These read files as well as the primary original read files were interrogated using Unix grep with a 20 nt sequence matching the wild-type allele and the mutant allele, to determine the precise frequency of mutant and wild-type reads. Missense and potential splice site variants in *TSC1/TSC2* were compared with findings in the LOVD Tuberous Sclerosis mutation database to help assess pathogenicity. Missense variants were also assessed using PolyPhen2 (37) and SIFT (38). Missense variants in *MTOR* were assessed by comparison with the set of missense variants reported to cause mTORC1 activation (39). Thus, only variants in *TSC1/TSC2* that were thought to be inactivating, and those in *MTOR* that were thought to be activating were included in the subsequent analyses. For all other genes studied, variants were included in this analysis if they were not identified in any of the SNP variant servers listed above, and appeared to be inactivating based upon PolyPhen2 and SIFT analyses.

Assessment of copy number variation from exome sequencing data

Copy number (CN) variants were identified using RobustCNV, a tool developed by the CCGD at DFCI (Paul Van Hummelen; personal communication). RobustCNV includes a normalization step in which systematic bias in mapping depth is reduced or removed using a two-step process. First, robust regression is used to fit tumor mapping depth values against mapping depths from a panel of normals (PON) sampled with the same capture bait set. Observed values are normalized against predicted values calculated from the fitted model and expressed as log2 ratios. In the second step, remaining GC bias is removed using a loess fit (40). Normalized coverage data is then segmented using circular binary segmentation (41) with the DNAcopy Bioconductor package and default settings. Finally, segments are assigned gain, loss, or normal-copy calls using a sample-specific cutoff calculated by multiplying the postnormalized median of within-segment standard deviations by a tuning factor (set to 0.5). Because this cutoff is likely to lead to high level of false positives in samples with noisy CNV data, we removed 12 samples with large variance (median + 1 standard deviation = 0.37) to increase the reliability of the CNV calls within the sample cohort. In this study, a set of 18 copy-normal samples was used in the PON. Samples were verified as being copy-normal through a leave-one-out strategy where each sample was analyzed against the other putative normal samples using the normalization approach outlined above. The results of this analysis were then manually reviewed and samples were filtered to include only the final set of 18 copy-normal samples in the PON. CN variants identified in *PTEN*, *TSC1*, *TSC2*, *DEPDC5*, *NPRL2*, *STK11*, and *NPRL3* were then validated by visual inspection of the data using the Spotfire software platform (http://spotfire.tibco.com/).

Statistical analysis

To investigate the association of selected mTOR pathway gene mutations with response to rapalog therapy, all patients with successful sequencing were included. Four distinct mutation categories were defined on the basis of the mutational status of the prespecified individual genes (mutated/wild-type) as (i) any mutation in *MTOR*, *TSC1*, *TSC2*; (ii) any mutation in *MTOR*, *TSC1*, *TSC2*, *PTEN*; (iii) any mutation in *TSC1*, *TSC2*; and (iv) any mutation in *MTOR*, *TSC1*, *TSC2*, *PTEN*, *PIK3CA*. The primary endpoint was response status (responders vs. nonresponders) to rapalog therapy. A secondary analysis explored the association between mutation and response in the subset of patients who had either PR or PD only.

Patient characteristics were descriptively summarized by number and proportion (categorical data) or by mean, standard deviation, and range (continuous data) according to response status. Comparisons between mutational status according to response to mTOR inhibitors were evaluated using a one-sided Fisher exact test for each mutation category with hypothesis that a mutation category was positively associated with treatment

Table 1. Patient characteristics

Characteristics	Response status: Nonresponder ($n = 36$) n (%)	Response status: Responder ($n = 43$) n (%)	Total ($n = 79$)
Gender			
Female	11 (31)	11 (26)	22 (28)
Male	25 (69)	32 (74)	57 (72)
Age at diagnosis of metastatic disease (years)			
Mean (Standard Deviation)	56.6 (10.0)	58.0 (10.0)	57.4 (10.0)
[Min, Max]	[35, 78]	[42, 79]	[35, 79]
Histology			
Clear cell RCC	31 (86)	38 (88)	69 (87)
Nonclear cell RCC	4 (11)	5 (12)	9 (11)
Unclassified RCC	1 (3)	0 (0)	1 (1)
Prognostic risk score (MSKCC)			
Good	4 (11)	8 (19)	12 (15)
Intermediate	13 (36)	25 (58)	38 (48)
Poor	19 (53)	10 (23)	29 (37)
Treatment			
Everolimus	14 (39)	24 (56)	38 (48.1)
Temsirolimus	22 (61)	19 (44)	41 (51.8)
Best response status			
PD	36 (100)	0 (0)	36 (46)
PR	0 (0)	12 (28)	12 (15)
SD	0 (0)	31 (72)	31 (39)

response. Similarly exploratory analysis assessed non-mTOR gene mutations in *ARID1A*, *ATM*, *BAP1*, *KDM5C*, *PBRM1*, *SEDT3*, *TP53*, and *VHL* with treatment response. An exploratory analysis was also performed to assess the association of CN alterations with response.

All statistical analyses were performed using SAS v.9.2 (SAS Institute Inc.) and R 3·2·1 (The R Foundation for Statistical Computing).

Results

Patient characteristics

Patient characteristics are summarized in Table 1 (Supplementary Table S1 provides complete details). Forty-three (54%) of 79 patients were responders. No complete responses were seen in this cohort. Thirty-eight (48%) were treated with everolimus and 41 (52%) received temsirolimus. Histologic subtypes included clear cell RCC ($n = 69$, 87%) and nonclear cell RCC ($n = 10$, 13%). Forty-nine of 79 (62%) had been previously treated, prior to rapalog therapy, and 44 of those 49 (89%) received VEGF-targeted therapy (Table 2). None had prior rapalog therapy.

Mutation findings

NGS on FFPE DNA was used to analyze 560 genes for mutations (see Materials and Methods for details), including 18 genes involved in the PI3K–mTOR signaling pathway, and 8 genes not in that pathway that are commonly mutated in RCC. A summary of mutation findings for all 79 samples is presented in Fig. 1 (see also Supplementary Table S2 for mutations in all genes).

Table 2. Previous treatment experiences

Prior treatment	Nonresponders $n = 21$ n (%)	Responders $n = 28$ n (%)	Total ($N = 49$) n (%)
VEGF-TT	19 (90)	25 (89)	44 (89)
Sunitinib	14 (66)	20 (71)	34 (69)
Sorafenib	5 (23)	4 (14)	9 (18)

NOTE: 30 of the 79 patients studied here received a rapalog as their initial treatment, and are not included in this table.

We first assessed our primary hypothesis that inactivating mutations in *TSC1* or *TSC2* and activating mutations in *MTOR* would be associated with response. Mutations in those three genes were found in 12 (28%) of 43 responders and in 4 (11%) of 36 nonresponders ($P = 0.06$; Table 3). *TSC1* and *TSC2* mutations considered alone were also shown to be associated with response (21% for responders vs. 6% from nonresponders; $P = 0.05$; Table 3). When including all activating mutations in *MTOR* or *PIK3CA*, and all inactivating mutations in *TSC1* or *TSC2*, or *PTEN*, there was a significant association between response and mutation status; 19 (44%) responders had mutations in at least one of these 5 genes, whereas 8 (22%) nonresponders had such mutations (one sided $P = 0.03$, OR, 2.73).

When we restricted the analysis to the subset of patients with PR or PD status only, patients with mutations in any of *TSC1*, *TSC2*, or *MTOR* were more common in the PR group (5/12 PR vs. 4/36 PD, $P = 0.03$; Table 4).

As an exploratory analysis, we assessed the association between response and mutation status in all genes that were found to be commonly mutated in this set of samples [minimum mutation frequency of 4 of 79 patients (5%)]: *VHL*, *PBRM1*, *SETD2*, *KDM5C*, *BAP1*, *TP53*, *ATM*, and *ARID1A* (Supplementary Table S3). Out of 8 genes tested for association with response, only *ATM* showed a possible association with response. *ATM* mutations were observed in 5 of the nonresponder patients and none of the responders, suggesting a negative association with response ($P = 0.02$). Further investigation or independent analyses are needed to confirm this potential association.

CN variation and association with response

Genomic loss events leading to CN variation are common in RCC. Therefore, we also examined the frequency and association between CN loss and responses to rapalogs in this population. CN status for 7 genes (*PTEN*, *TSC1*, *TSC2*, *DEPDC5*, *NPRL2*, *STK11*, *NPRL3*) that are negative regulators of mTOR signaling was

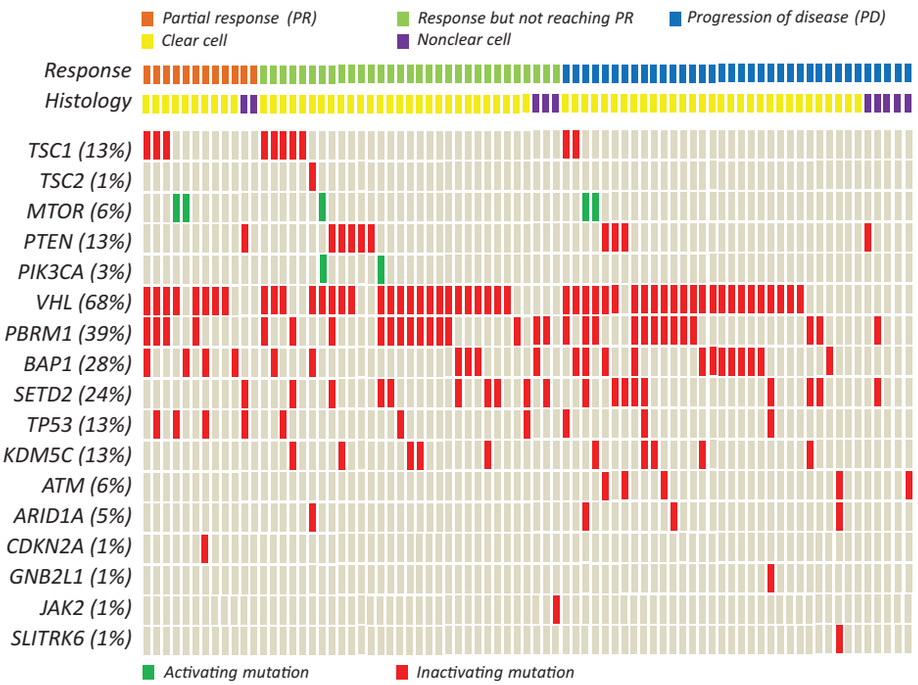

Figure 1.
Comutation plot of subjects and mutations. A plot of all subjects (left to right) with their respective responses, histology, and mutations is shown.

determined and association with response was assessed (Supplementary Tables S1 and S4). No indication of association between CN loss and response to rapalogs for any of these genes was observed ($P > 0.30$ for all), with the exception of *DEPDC5* for which single CN loss was associated with lack of response to rapalogs ($P = 0.02$).

Detailed scrutiny of mutation findings in responder patients

We examined the degree of response measured using RECIST criteria and the duration of response for these patients, as well the precise nature of the mutation present in *TSC1/TSC2/MTOR* in responders versus nonresponders to assess whether there was any association between a particular mutation and degree of response to rapalogs (measured as percent reduction in tumor size, or the duration of response). Inactivating mutations in *TSC1/TSC2* of all kinds (missense, nonsense, deletions, splice site) were observed in responding patients, at allele frequencies as low as 7% (Supplementary Table S2). Two patients with *TSC1* mutations who were progressors both had inactivating (out-of-frame) deletion mutations at relatively high allele frequency (Supplementary Table S2). Two of the 5 *MTOR* mutations have been shown directly to be activating with respect to mTOR kinase activity (39), and were both seen in responders. The other 3 *MTOR* mutations were in mutation hotspot regions where nearby mutations have been shown to cause activation of mTOR, but were not directly studied (39); 1 was seen in a responder and 2 were in progressors. Furthermore, we found no association between mutation in any of these three genes and either the degree or duration of response.

Discussion

Tumor genetic analyses to identify mutations correlating with response to kinase inhibitor therapy has a rich history, including the most important discovery in therapy for lung adenocarcinoma in the past 30 years, the discovery of activating kinase mutations in *EGFR* and their correlation with response to the EGFR kinase inhibitors erlotinib and gefitinib (42). However, despite the success of several targeted therapies for mRCC, no genetic or other biomarkers have been identified and validated to predict

Table 3. Association of mTOR pathway mutation status and response

Mutation category	Mutations	Nonresponders ($n = 36$) n (%)	Responders ($n = 43$) n (%)	Fisher exact P[a]
MTOR, TSC1, TSC2	No (ref)	32 (89)	31 (72)	0.06
	Yes	4 (11)	12 (28)	
MTOR, TSC1, TSC2, PTEN	No (ref)	28 (78)	25 (58)	0.05
	Yes	8 (22)	18 (42)	
TSC1, TSC2	No (ref)	34 (94)	34 (79)	0.05
	Yes	2 (6)	9 (21)	
TSC1, TSC2, MTOR, PTEN, PIK3CA	No (ref)	28 (78)	24 (56)	0.03
	Yes	8 (22)	19 (44)	

[a]A one-sided Fisher exact test was used to assess the association between mutation and response status, with the hypothesis that a mutation was positively associated with being a responder.

Table 4. Subgroup analysis ($N = 48$): association of mTOR pathway mutation status and response in best response groups (PR vs. PD)

		Best response group		
		PD ($n = 36$)	PR ($n = 12$)	
Mutation category	**Mutations**	***n* (%)**	***n* (%)**	**Fisher exact *P*[a]**
MTOR, TSC1, TSC2	No (ref)	32 (89)	7 (58)	0.03
	Yes	4 (11)	5 (42)	
MTOR, TSC1, TSC2, PTEN	No (ref)	28 (78)	6 (50)	0.07
	Yes	8 (22)	6 (50)	
TSC1, TSC2	No (ref)	34 (94)	9 (75)	0.09
	Yes	2 (6)	3 (25)	
TSC1, TSC2, MTOR, PTEN, PIK3CA	No (ref)	28 (78)	6 (50)	0.07
	Yes	8 (22)	6 (50)	

[a]A one-sided Fisher exact test was used to assess the association between mutation and response status, with the hypothesis that a mutation was positively associated with being a responder.

response to these agents, including VEGFR inhibitors and rapalogs.

mTORC1 and mTORC2 are kinase complexes containing mTOR which are located deep within signaling pathway cascades, downstream of both PI3K and MAPK signaling pathways. The relative location of mTORC1 and mTORC2 within these signaling pathways, and their involvement in a variety of feedback or counter-regulatory effects, might suggest that their oncogenic effects are not as potent in comparison with a receptor tyrosine kinase such as EGFR. Nonetheless in mouse models, loss of TSC1/TSC2, proximate upstream regulators of mTORC1 acting through the RHEB GTPase, contributes to the development of a variety of cancers, including lung, kidney, prostate, and mesothelioma (43, 44). Furthermore, *TSC1* and *TSC2* are the causative genes of tuberous sclerosis complex, a tumor suppressor gene syndrome in which a variety of progressive tumors that require therapeutic intervention are often identified.

In addition, through the NCI The Cancer Genome Atlas (TCGA) program, inactivating mutations in *TSC1/TSC2* have been identified at low frequency in a wide variety of the common cancers, highest in bladder cancer (45). In addition, activating mutations in *MTOR* have also been identified in many different cancer types at low frequency, and at somewhat higher levels in RCC and endometrial carcinoma (cBio, http://www.cbioportal.org/; ref. 46).

Furthermore, several case reports or small series have reported on the association between *TSC1/TSC2/MTOR* mutations and response to rapalog therapy in cancer. The first association of this kind was the response to rapalog therapy seen in PEComa, a rare sarcoma subtype in which *TSC2* mutations are common (25, 26). Subsequently, a good response to everolimus (PFS > 13 months) was reported in an RCC patient with an inactivating *TSC1* splice mutation (27). Later, an exceptional response to everolimus was reported in a single patient enrolled in a bladder cancer trial (subject remains in CR on everolimus > 4 years after initiation of therapy; ref. 21). However, other patients on that trial with *TSC1* mutations showed little or no evidence of response (21). More recently, exceptional responses to rapalog therapy have been seen in patients with *MTOR* and *TSC2* mutations, in bladder and anaplastic thyroid cancer, respectively (23, 24).

TSC1 and *MTOR* mutations have also been reported recently in a series of 5 patients with RCC and exceptional responses to mTOR inhibitors (22). Of 5 patients studied, 2 had inactivating mutations in *TSC1*, 1 had an activating mutation in *MTOR*, and in 2 no mutations in the mTOR pathway were identified. Hence, this study already suggested that in RCC, good responses to rapalog therapy might occur without an identifiable underlying genetic event.

Here we explored the hypothesis that mutations in *MTOR*, *TSC1*, or *TSC2* are associated with response to rapalog therapy. Despite collection of a substantial cohort, our findings were of marginal statistical significance. Mutations in these genes were more common in RCC patients who responded to rapalog therapy (12/43) than nonresponders (4/36; OR, 3.05; $P = 0.06$); and 5 of 12 subjects with PR had mutations in *MTOR*, *TSC1*, or *TSC2* versus 4 of 36 primary refractory patients (OR, 5.28; $P = 0.03$). Of note, in some of the responders, *TSC1/TSC2* mutations were detected at low allele frequencies (as low as 7%), suggesting that they represent subclonal driver events that are both biologically and clinically relevant. Careful scrutiny of the extent and duration of response failed to suggest a correlation between truly exceptional responses and *TSC1/TSC2/MTOR* mutations. Considering the larger set of genes whose mutation might be associated with rapalog response, we found that mutations in *TSC1/TSC2/MTOR/PIK3CA/PTEN* were associated with response (OR, 2.73; $P = 0.03$). However, even considering this larger set of genes, it is notable that 24 of 43 (56%) rapalog responders had no molecular finding to explain their response. We speculate that the good responses seen without *TSC1/TSC2/MTOR* mutation may be due to nongenetic mechanisms of mTOR dependence in these cases, including upstream signaling events and epigenetic effects (13). Further investigation is required. Mutations in *ATM* and heterozygous deletions of *DEPDC5* were negatively associated with response in exploratory analyses, and also require further analysis.

Even though we evaluated a large number of RCC subjects treated with mTOR inhibitors, our study has limitations. First, this is a relative select cohort of patients where we focused on the "extremes" of clinical outcomes. Second, intratumor heterogeneity is well known in RCC, and may have compromised our ability to detect important mutations in the samples available to us (22, 47). However, studies of heterogeneity have reported that different cancer specimens derived from a single patient often have convergent mutations that result in activation of the same gene/pathway in different tumor samples (22, 47). In addition, our analysis of a single cancer specimen reflects clinical practice in that multiple biopsies from different sites are rarely available, and decisions on choice of therapy generally need to be made based on analysis of a single sample. Third, although we filtered our mutation findings to identify those we thought activating or inactivating in terms of their effects, some of the assessments were based on computational analyses, and we did not have direct evidence of functional effect in several cases (note that these

functional effect assessments were made blinded to clinical outcome). Finally, mutations may have been missed because of the limited depth of read coverage and/or relatively low percent tumor content in some cases.

Mutations in several genes were identified at rates somewhat different from what was reported from the TCGA analysis of RCC (28). The most striking of these differences was for TSC1 for which we identified 10 mutations (13%), whereas fewer than 1% were identified in the TCGA analysis. This may reflect many differences in the two populations, including presence of metastatic disease and selection of rapalog responders in our cohort.

In conclusion, mutations in *MTOR*, *TSC1*, or *TSC2* were more common in RCC patients who experienced clinical benefit from everolimus or temsirolimus than in those with rapid progression in this large cohort of mRCC patients. However, the majority of mRCC patients with response to rapalogs had no mutation identified in these three genes or in *PIK3CA* or *PTEN*, and hence remain unexplained at the molecular level. These findings suggest that a personalized medicine approach may have value in RCC when an appropriate (inactivating or activating) mutation is identified in 1 of these 5 genes, and make selection of an mTOR inhibitor for treatment a higher clinical priority than in patients without such mutations. However, lack of a mutation in these 5 genes does not exclude the possibility of a good, durable response to mTOR inhibitor therapy in mRCC.

Disclosure of Potential Conflicts of Interest

T.K. Choueiri is a consultant/advisory board member for Novartis and Pfizer. L. Albiges is a consultant/advisory board member for Bayer, Bristol-Meyers Squibb, Novartis, Pfizer, and Sanofi. N. Agarwal is a consultant/advisory board member for Argos, Exelixis, and Pfizer. M.D. Michaelson is a consultant/advisory board member for Exelixis, Novartis, and Pfizer. C. Porta reports receiving speakers bureau honoraria from Bayer-Schering, Bristol-Meyers Squibb, Novartis, and Pfizer and is a consultant/advisory board member for Astellas, Bayer-Schering, Bristol-Meyers Squibb, Exelixis, Novartis, Pfizer, Pierre Fabre, and Roche-Genentech. S.K. Pal and D.Y.C. Heng are consultant/advisory board members for Novartis and Pfizer. D.F. McDermott is a consultant/advisory board member for Bristol-Meyers Squibb, Exelixis, Genentech, Merck, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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