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Original contribution

Dysregulation of the mammalian target of rapamycin pathway in chromophobe renal cell carcinomas☆

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Summary Targeted therapy in advanced clear cell renal cell carcinomas (RCC) is now an established modality. The latter is in stark contrast to non–clear cell subtypes. We explored the translational support for the use of antagonists of the mammalian target of rapamycin (mTOR) and the vascular endothelial growth factor pathways in chromophobe RCC. The immunoexpression of PTEN, phos-AKT, phosphorylated S6 (phos-S6), 4EBP1, p27, c-MYC, and HIF-1α was evaluated in 33 patients with chromophobe RCC who were treated by partial/radical nephrectomy without adjuvant therapy. PTEN was lower in tumor than in normal kidney ($P < .001$), and loss of PTEN expression was found in 67% of the tumors. In tumor tissues, phos-S6 and 4EBP1 were higher than in normal kidney ($P \leq .005$). Conversely, scores of p27 were lower in tumor than in normal kidney ($P < .001$). Finally, scores of phos-AKT, c-MYC, and HIF-1α were not significantly different in tumor and in normal kidney. Overall mortality and cancer-specific mortality were 9% and 0%, respectively. Multifocal tumors had higher levels of PTEN, phos-AKT, and HIF-1α ($P \leq .01$). None of the clinicopathologic variables (age, ethnicity, gender, pT stage, tumor size, multifocality, and positive surgical margins) was associated with outcome. Similarly, none of the tested biomarkers predicted overall mortality, either in unadjusted or adjusted Cox regression models. In summary, our study provides new evidence of dysregulation of the mTOR pathway in chromophobe RCC. Immunohistochemistry for mTOR pathway and hypoxia-induced pathway members lacked prognostic significance in our cohort.

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1. Introduction

Dysregulation of the mammalian target of rapamycin (mTOR) and hypoxia-induced pathways has been consistently identified in clear cell renal cell carcinomas (RCC) [1,2]. Currently, inhibitors of the mTOR and vascular endothelial growth factor (VEGF) pathways are being used in patients with advanced clear cell RCC, either as first-line options or in refractory disease [3,4]. However, clinical experience with targeted agents is limited in non–clear cell RCC [3,5,6]. In addition, previous studies have analyzed the expression status and prognostic significance of members of the mTOR and hypoxia-induced pathways in RCC [2,7-9], but none has focused exclusively on chromophobe RCC. Moreover, chromophobe RCC, which accounts for approximately 5% of all RCC, has no established therapy for the less frequently aggressive examples of this type [10]. Thus, studies providing preclinical support for the use of targeted therapies in chromophobe RCC are of value.

In this study we have evaluated the immunohistochemical expression of upstream (PTEN and phosphorylated-AKT) and downstream (phosphorylated-S6 and 4EBP1) effectors of the mTOR pathway in chromophobe RCC, as well as mTOR-related proteins p27 and c-MYC. Considering the interplay between the mTOR and the hypoxia-induced pathways [11], we have also evaluated HIF-1α, a key member of the latter. First, we compared the expression of these proteins between normal kidney and tumor tissue. Second, we analyzed the associations between immunohistochemical expression and pathologic features of the primary tumor. Third, we evaluated the prognostic impact that these biomarkers may have on the outcome of patients with chromophobe RCC.

2. Material and methods

The present study includes tissue samples from 33 consecutive patients with chromophobe RCC treated at the Johns Hopkins Medical Institutions (Baltimore, MD) between January 2004 and December 2006. All patients were treated by partial/radical nephrectomy without adjuvant therapy. After approval by the institutional review board, a retrospective study was performed with outcome assessment based on chart review of clinical and pathological data. Histologic slides were retrieved and reviewed by two urologic pathologists (R.A. and G.J.N.) for confirmation of the original diagnosis and pathologic staging, in compliance with the American Joint Committee on Cancer 2009 classification [12]. Using a previously described procedure, 1 tissue microarray (TMA) was built [13]. Four cores of tumor tissue and 4 cores of paired normal kidney tissue were spotted from each specimen. Patients were followed-up from the date of surgery until the endpoint was reached or the study ended (range, 28-86 months; mean, 55.5 months; median, 55 months). Patients who did not present the event of interest at the end of the study were considered as censored. For outcome analysis, endpoints included cancer-related death and all-cause death.

2.1. Immunohistochemistry

Immunohistochemistry was performed for the following proteins: PTEN, phosphorylated AKT (phos-AKT), phos-S6, 4EBP1, c-MYC, p27, and HIF-1α. Immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections using the PowerVision Poly-HRP IHC Detection Systems (Leica Microsystems, Bannockburn, IL). Sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval with a buffer solution using a steamer. Sections were then incubated with the appropriate primary antibody. Following the application of an anti-Rabbit or antimouse poly-HRP secondary (except for c-MYC, for which the Dako Catalyzed Signal Amplification System Kit was used), the slides were developed using 3-3′-diaminobenzidine chromogen and counterstained with hematoxylin. Proper cell lines were used as external controls, and internal controls were checked for negative and positive immunohistochemical expression. For HIF-1α, the protocol described by Tickoo et al was used [14]. Table 1 lists information regarding antibodies and vendors.

2.2. Scoring system

Immunohistochemistry staining was evaluated by 2 urologic pathologists (A.C. and G.J.N.) using a previously

Table 1 Summary of antibodies used for immunohistochemical analysis

	Vendor	Clone	Pre-treatment	Dilution
PTEN	Cell Signaling	D4.3	EDTA, 45 min	1:100
phos-AKT [a]	Cell Signaling	736E11	EDTA, 45 min	1:50
phos-S6 [b]	Cell Signaling	Polyclonal	EDTA, 45 min	1:200
4EBP-1	ProSci	Polyclonal	Citrate, 25 min	1:250
c-MYC	Epitomics	Y69	EDTA, 45 min	1:300
p27	Transduction Lab	57	Citrate, 25 min	1:4000
HIF-1α	Novus Biologicals	NB100-123	Heat (oven) at 62°C, 60 min	1:1600

[a] Phosphorylation site at Ser473.
[b] Phosphorylation site at Ser235/236.

validated methodology [2]. Both tumor cells and normal epithelial cells from renal tubules were evaluated for pattern of staining (nuclear versus cytoplasmic), extent (percentage of positive cells) and intensity (0 to 3+). Nuclear staining was considered positive for phos-AKT, c-MYC, p27, and HIF-1α. Cytoplasmic stain was considered positive for PTEN, phos-S6, and 4EBP1. An H-score was calculated for each TMA spot, as the sum of the products of the intensity (0 for negative, 1 for weakly positive, 2 for moderately positive, and 3 for strongly positive) by the extent of immunoexpression (0%-100%). The overall score used for subsequent statistical analysis was the pooled mean from the 4 spots of normal kidney and of tumor tissue.

2.3. Statistical analysis

Scores of paired normal kidney and tumor were compared using the Wilcoxon matched-pairs signed-ranks test. Differences in scores of tumor tissue were stratified by clinicopathologic variables and compared using the Spearman rank correlation coefficient (ρ), the Mann-Whitney U test, the Kruskal-Wallis test, or the Fisher exact test depending on the data type. Unadjusted and adjusted Cox models were built to evaluate the hazard ratio of clinicopathologic variables and biomarker expression as prognosticators of outcome. A 2-tailed $P < .05$ was required for statistical significance. Data were analyzed using Stata/SE 12 (StataCorp Inc, College Station, TX).

3. Results

Clinicopathologic features of all patients are shown in Table 2. In chromophobe RCC, overall mortality and cancer-specific mortality were 9% and 0%, respectively.

3.1. Biomarker expression in chromophobe RCC and paired normal kidney

Figs. 1 and 2 show the expression patterns of PTEN, phos-AKT, phos-S6, 4EBP1, c-MYC, p27, and HIF-1α in normal kidney and chromophobe RCC. Overall, striking differences were noted in the scores of most biomarkers between normal kidney and tumor tissue (Table 3). Briefly, PTEN was lower in tumor tissue than in normal kidney, and loss of PTEN expression (H-score <10) was found in 22 (67%) patients. Levels of phos-S6 and 4EBP1 were significantly higher in tumor tissues than in normal kidneys. Conversely, p27 levels were significantly lower in tissue tumors than in normal kidneys. Levels of phos-AKT were slightly higher in normal kidneys than in tumor tissues, with the opposite trend for c-MYC; nevertheless, differences in absolute levels were insignificant for both aforementioned markers. Regarding HIF-1α levels, differences between normal kidneys and tumor tissues were not significant.

Table 2 Demographic and clinicopathological features of 33 patients with chromophobe RCC treated by nephrectomy

	No. of cases
Age at nephrectomy, y	
Mean (SD)	59.8 (12.5)
Median (IQR)	61 (18)
Range	34, 80
Ethnicity (%)	
Caucasian	27 (82)
African-American	4 (12)
Other	2 (6)
Gender (%)	
Male	18 (55)
Female	15 (45)
Pathologic stage T (%) [a]	
T1a	15 (48)
T1b	4 (13)
T2a	7 (23)
T2b	0 (0)
T3a	5 (16)
Tumor size, cm	
Mean (SD)	4.7 (3.2)
Median (IQR)	4 (4.85)
Range	1, 16
Multifocality (%) [b]	3 (9)
Positive surgical margins (%) [c]	1 (3)

Abbreviation: IQR, interquartile range.

[a] In 2 cases, staging was not possible due to fragmentation of the specimen.

[b] In 1 case determination of multifocality was not possible.

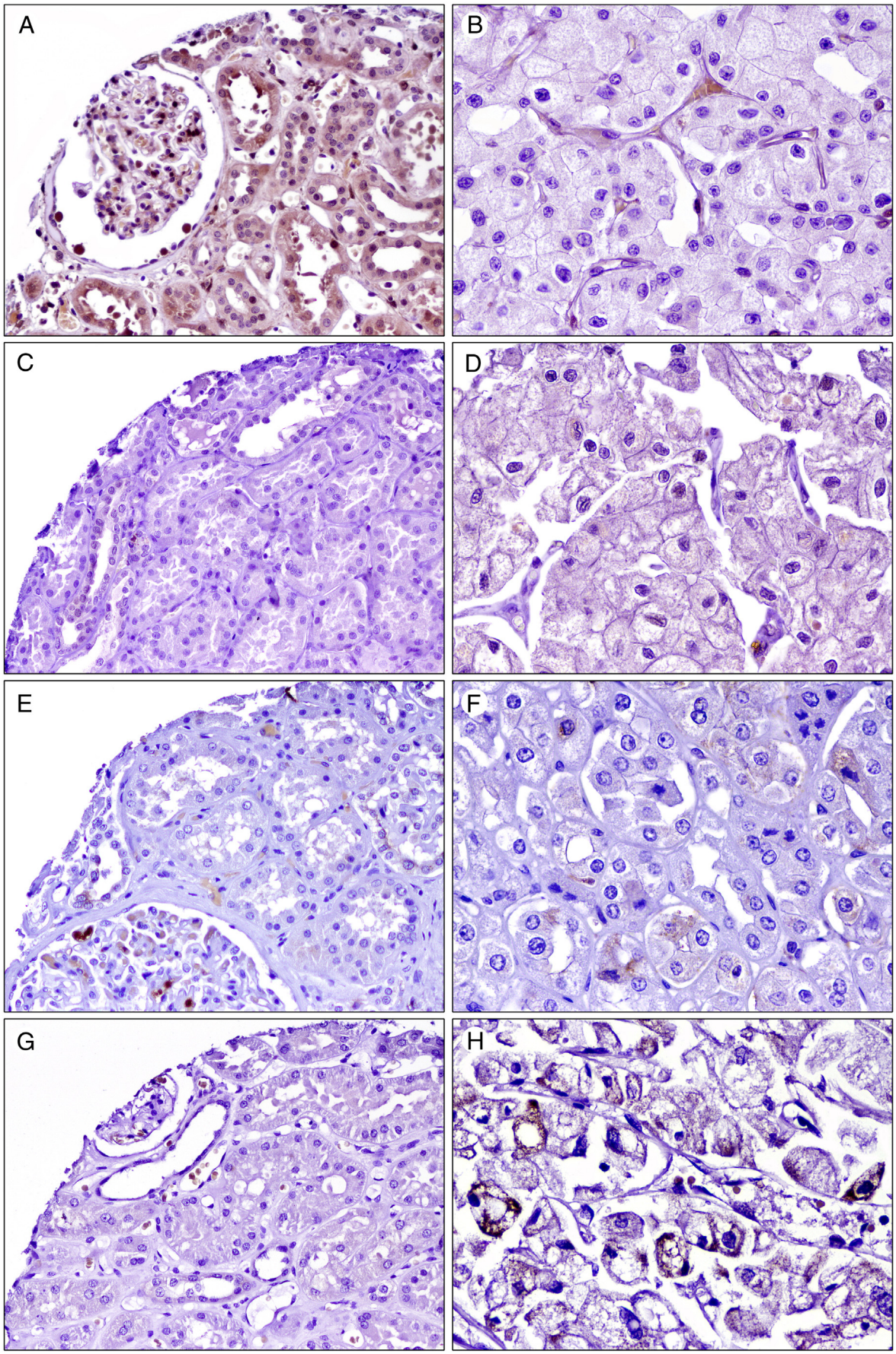
[c] In 3 cases determination of the status of the surgical margins was not possible.

3.2. Clinicopathologic features and biomarker expression

Table 4 summarizes the association between clinicopathologic features and immunohistochemical expression of mTOR and hypoxia-induced pathway members. Levels of PTEN, phos-AKT, and HIF-1α were higher in multifocal tumors. HIF-1α levels were also higher in African-American patients. Additionally, phos-S6 levels were higher in larger tumors: phos-S6 positive tumors showed a mean size of 5.8 cm compared to phos-S6 negative tumors with a mean size of 1.8 cm. Finally, c-MYC levels were slightly higher in pT1 tumors compared to higher pT stages. No other significant association was observed between clinicopathologic features and biomarker expression.

3.3. Predictors of outcome in chromophobe RCC

Since none of the patients died of cancer, outcome analyses were restricted to all-cause death as the endpoint. None of the clinicopathologic features was associated with overall mortality (Table 5). Hazard ratios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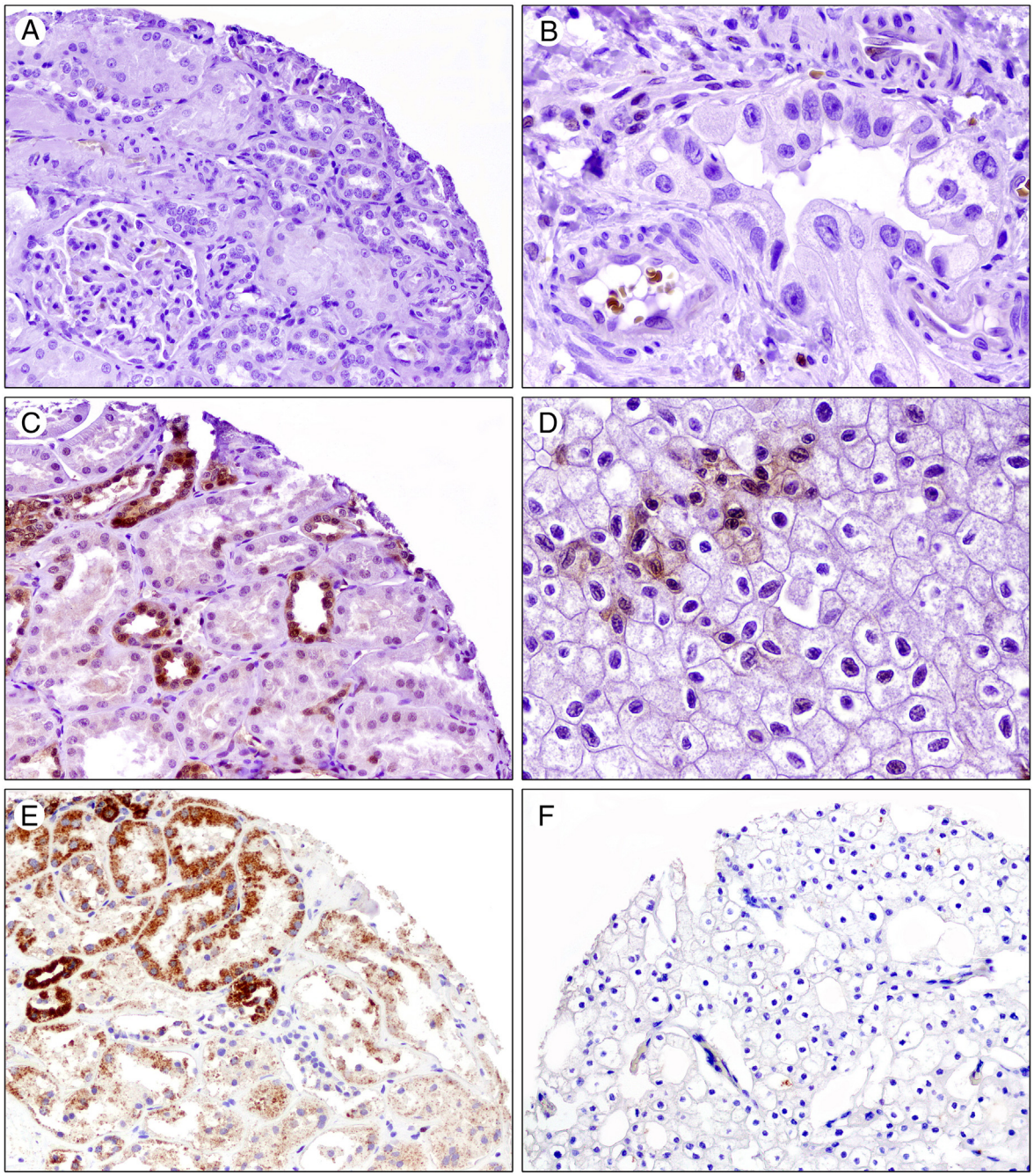

Fig. 2 Immunohistochemical expression of mTOR-related and hypoxia-induced pathways members in normal kidney (left column; original magnification ×200) and chromophobe RCC (right column; original magnification ×400). A and B, Low to null nuclear c-MYC expression. C and D, Nuclear p27 expression. E and F, Low to null nuclear HIF-1α expression.

for overall mortality were not significantly different when considering clinicopathologic features and biomarker levels either in unadjusted or adjusted Cox models (Table 6).

4. Discussion

In this study, we analyzed the immunohistochemical expression of several members of the mTOR and hypoxia-

Fig. 1 Immunohistochemical expression of mTOR pathway members in normal kidney (left column; original magnification ×200) and chromophobe RCC (right column, original magnification ×400). A, Cytoplasmic PTEN expression in normal cells of the renal tubules. B, Loss of PTEN expression in tumor cells. C and D, Nuclear phos-AKT expression. E and F, Cytoplasmic phos-S6 expression. G and H Cytoplasmic 4EBP1 expression.

Table 3 Comparison of mean (SD) levels of biomarker expression in chromophobe RCC and paired normal kidney

	Chromophobe RCC	Normal kidney	*P* [a]
PTEN	13.2 (28.6)	199.6 (28.1)	<.001
phos-AKT	5.1 (5.8)	7 (4.5)	.04
phos-S6	4.2 (13.4)	0 (0)	.005
4EBP1	67.4 (79.3)	0 (0)	<.001
c-MYC	0.1 (0.2)	0.2 (0.7)	.08
p27	11.7 (17.3)	59.4 (21.5)	<.001
HIF-1α	0.9 (5.2)	0 (0)	.32

[a] Using the Wilcoxon matched-pairs test.

induced pathways in chromophobe RCC. To the best of our knowledge, this is the first series to evaluate the prognostic utility of members of the mTOR and hypoxia-induced pathways focusing exclusively on chromophobe RCC. PTEN scores were lower in tumor tissues than in normal kidney, and loss of immunohistochemical expression of PTEN was observed in two-thirds of the patients. We have recently demonstrated that loss of PTEN immunohistochemical expression in prostate cancer is associated in most cases with *PTEN* genomic loss [15]. Whether similar genetic alterations are responsible for observed PTEN loss of expression in chromophobe RCC remains to be determined. In addition, downstream effectors phos-S6 and 4EBP1 were higher in tumor tissues than in normal kidney. In addition, p27 scores were higher in normal kidney than in tumor tissues, while scores of phos-AKT, c-MYC, and HIF-1α were not significantly different in absolute values. Our results suggest that dysregulation of the mTOR pathway might be involved in the oncogenesis of chromophobe RCC. Our finding of higher PTEN, phos-AKT, and HIF-1α levels in multifocal tumors and higher phos-S6 levels in larger tumors merits further investigation in larger cohorts. Except for the aforementioned associations, immunohistochemical expression of these mTOR and hypoxia-induced members failed to be associated with clinicopathologic surrogates of more aggressive disease such as higher stage disease. Not surprisingly, adverse outcome was a rare event in our cohort; the low overall mortality and null cancer-related death could limit the validity of our results. The low mortality rate we observed in our study is consistent with data from the Surveillance Epidemiology and End Result program [10].

Table 4 Clinicopathologic features and immunohistochemical expression of biomarkers in chromophobe RCC [a]

	PTEN	phos-AKT	phos-S6	4EBP1	c-MYC	p27	HIF-1α
Age [b]	.82	.60	.72	.62	.13	.86	.61
Ethnicity [c]	.52	.99	.33	.20	.89	.16	.03
Gender [d]	.71	.90	.24	.76	.36	.18	.36
pT Stage [c]	.76	.43	.24	.24	.08	.45	.33
Tumor size [b]	.44	.30	.06	.34	.49	.15	.18
Multifocality [d]	.04	.01	.66	.47	.75	.36	.002
Surgical margins [d]	.23	.64	.59	.69	.85	.91	.85

[a] Values provided correspond to *P* values.
[b] Using the Spearman's rank correlation coefficient (ρ).
[c] Using the Kruskal-Wallis test.
[d] Using the Mann-Whitney *U* test.

Table 5 Clinicopathologic features and overall mortality in 33 patients with chromophobe RCC

	Overall mortality		*P*
	Yes	No	
Age at nephrectomy, years			
Mean (SD)	64.7 (14.1)	59.3 (12.5)	.47
Ethnicity (%)			.46
Caucasian	2 (7)	25 (93)	
African-American	1 (25)	3 (75)	
Other	0 (0)	2 (100)	
Gender (%)			.58
Male	1 (6)	17 (94)	
Female	2 (13)	13 (87)	
Pathologic stage T (%) [a]			.60
T1a	3 (20)	12 (80)	
T1b	0 (0)	4 (100)	
T2a	0 (0)	7 (100)	
T2b	0 (0)	0 (0)	
T3a	0 (0)	5 (100)	
T3b	0 (0)	0 (0)	
Tumor size, cm			.12
Mean (SD)	2.4 (1.1)	4.9 (3.3)	
Multifocality (%)	3/29 (10)	0/3 (0)	.99
Positive surgical margins (%)	3/29 (10)	0/1 (0)	.99

[a] In 2 cases staging was not possible due to fragmentation of the specimen.

Our results are comparable to what we have previously found in clear cell and papillary RCC using the same immunohistochemical approach and scoring system [2,16]. Patterns of immunohistochemical expression were similar among clear cell, papillary, and chromophobe RCC in normal kidneys and tumor tissues. PTEN was lower and phos-S6 and 4EBP1 were higher, indicating an activation of the mTOR pathway. The similarities suggest that current therapies for clear cell and papillary RCC involving mTOR inhibitors might also be beneficial for patients with chromophobe RCC. Nonetheless, experience with mTOR inhibitors in non–clear cell RCC is scant, mostly because patients with non–clear cell subtypes are excluded from clinical trials evaluating these drugs [5]. Recently, a small number of case reports have described the results of administering mTOR inhibitors to patients with advanced chromophobe RCC [10,17-19]. In these 4 cases, mTOR inhibitors (temsirolimus in 3 cases, everolimus in 1 case) were used as the 2nd or 3rd line of therapy in patients with recurrence post-nephrectomy. Patients were alive with stable disease 3 to 5 years after the development of metastases. Our

Table 6 Cox models for clinicopathologic features and immunohistochemical expression of biomarkers as prognosticators of overall mortality in chromophobe RCC [a]

	HR	*P*
Unadjusted Cox models [b]		
Age, years	1.04 (0.94, 1.15)	.48
Ethnicity [c]	1.04 (0.94, 1.15)	.48
Gender	0.43 (0.04, 4.71)	.49
Tumor size, cm	0.56 (0.24, 1.36)	.20
PTEN	1.01 (0.99, 1.04)	.30
phos-AKT	0.93 (0.72, 1.19)	.56
phos-S6	1.02 (0.97, 1.07)	.41
phos-4EBP1	1.01 (0.99, 1.02)	.11
p27	1.02 (0.97, 1.07)	.46
Adjusted Cox models [d]		
PTEN	1.04 (0.95, 1.15)	.40
phos-AKT	0.91 (0.69, 1.21)	.52
4EBP1	1.02 (0.99, 1.05)	.18

[a] Values provided correspond to hazard ratios (HR) with 95% confidence intervals in parenthesis.
[b] Variables were entered separately. Pathologic stage, multifocality, surgical margins, c-MYC, and HIF-1α were excluded due to extremely low HR values.
[c] Comparing African-American versus white and others.
[d] Adjusted for age, ethnicity, gender, and tumor size. HR for phos-S6, p27, c-MYC, and HIF-1α were excluded due to extremely low values.

results lend support to the rationale of these studies, by demonstrating immunohistochemical evidence of dysregulation of the mTOR pathway in chromophobe RCC.

Contrary to what we have found in clear cell and papillary RCC [2,16] HIF-1α levels were not significantly increased in chromophobe RCC compared to normal kidney. In advanced chromophobe RCC, antagonists of the VEGF pathway have been studied more extensively than mTOR inhibitors, although the results have been rather inconclusive [10]. In a 2008 study published by Choueiri et al 3 of 12 patients with metastatic chromophobe RCC achieved partial responses with sorafenib or sunitinib [20]. Later, in 2010, Stadler et al reported a large series of patients with advanced RCC treated with sorafenib that included 20 patients with chromophobe RCC [21]. In the Stadler et al series, 1 patient had a partial response, 17 had stable disease for at least 8 weeks, and 2 had progressive disease. These studies indicate that antagonists of the VEGF pathway have some activity in chromophobe RCC. Nevertheless, patients with non–clear cell RCC have fewer responses to antagonists of the VEGF pathway compared to clear cell RCC [6,22]. Interestingly, the 4 previously mentioned patients, who were treated with mTOR inhibitors, all received sunitinib or sorafenib as the first or second line of treatment without an objective response [10,17-19]. Our results of lack of HIF-1α overexpression in chromophobe RCC support the low clinical responses that antagonists of the VEGF pathway have in this scenario.

The prognostic value of immunohistochemistry for members of the mTOR and hypoxia-induced pathways to predict outcome of patients with RCC has been previously evaluated [7-9]. In an earlier study, we focused on the analysis of 176 patients with clear cell RCC [2]. We found that phos-S6, 4EBP1, p27, and HIF-1α were significant predictors of disease-specific survival and tumor progression in univariate analysis, with phos-S6 and HIF-1α retaining statistical significance in multivariate analysis. Later, we evaluated 54 patients with papillary RCC using the same methodological approach, but none of the analyzed biomarkers were useful to predict overall mortality, cancer-specific mortality, or tumor progression [16]. Evaluation of a larger multi-institutional cohort of chromophobe RCC in which the less frequent poor outcome events can be adequately represented will be required to fully address the prognostic role, if any, of the immunohistochemical evaluation of members of the mTOR and hypoxia-induced pathways in non–clear cell RCC.

The use of TMAs instead of whole tissue sections for evaluation of immunohistochemical expression must be acknowledged as a limitation for the current study. Regarding this issue, although it would be preferable to use whole sections in determining the pattern of expression and prognostic usefulness of a biomarker, TMAs provide a convenient way to evaluate a considerable number of cases under the same conditions and using the same protocol for immunohistochemistry. This diminishes the impact that external conditions might have in the development of the stain and facilitates the interpretation of the immunohistochemical expression. Given the staining heterogeneity of some biomarkers, the use of TMA instead of whole sections could have some impact on the detected expression levels. However, several studies have supported the high throughput value of the TMA usage and the adequate representation of the overall expression levels using multiple TMA spots [23].

In summary, we have analyzed the expression status and prognostic significance of members of the mTOR and hypoxia-induced pathways in 33 patients with chromophobe RCC. We found lower PTEN expression and higher phos-S6 and 4EBP1 expression in tumor tissues compared to normal kidneys. Our study provides evidence of dysregulation of the mTOR pathway in chromophobe RCC, with no significant activation of the hypoxia-induced pathway.

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