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ORIGINAL ARTICLE – CANCER RESEARCH

mTOR and mTOR phosphorylation status in primary and metastatic renal cell carcinoma tissue: differential expression and clinical relevance

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Abstract

Purpose Impaired regulation of the Akt/mammalian target of rapamycin (mTOR) pathway has been implicated in mechanisms related to neoplastic transformation in renal cell cancer (RCC) through enhancement of cell proliferation and survival and mTOR activation has been reported to occur due to phosphorylation of mTOR. To further determine the relevance of mTOR expression and activation and to analyze their putative role as a biomarker for systemic treatment in metastatic RCC, we investigated the expression of mTOR and phospho(p)-mTOR in primary RCC and metastases and correlated levels with pathological variables and clinical outcome.

Methods Tissue microarrays (TMA) from paraffin-embedded tissue from 342 patients with primary clear cell renal cell carcinoma and 90 patients undergoing surgical resection for metastases were immunohistochemically stained for mTOR and p-mTOR and expression was quantified with immunoreactivity scores. Clinical patient characteristics and follow-up were recorded. Comparative evaluation of protein expression levels and association of expression with clinical variables and survival was performed.

Results mTOR staining revealed differential expression in benign, primary and RCC metastasis (average staining score: 1.64, 0.78, and 1.44, respectively). Average staining of p-mTOR was 0.99 in benign kidney tissue, 0.73 in primary RCC and 1.14 in RCC metastasis tissue. Elevated mTOR expression in primary RCC tissue was associated with the presence of tumor necrosis, while a high level of p-mTOR was significantly correlated with advanced T-stage, high Fuhrman grade, the presence of tumor necrosis and sarcomatoid features. An elevated ratio of p-mTOR/mTOR was significantly correlated with advanced stage and sarcomatoid histology. mTOR expression was not predictive of overall survival (OS), while high p-mTOR levels were associated with impaired OS ($p = 0.0046$) and cancer-specific survival ($p = 0.0067$). In univariate analysis, advanced stage (HR 3.78), high Fuhrman grade (HR 4.0), the presence of tumor necrosis (HR 1.99), and sarcomatoid features (HR 5.12) were significant predictors of OS. Moreover, elevated levels of p-mTOR (HR 1.67) and an elevated ratio of p-mTOR/mTOR ratio (HR 1.73) were significantly predictive of OS. In the multivariate regression model only the presence of locally advanced tumors (HR 2.44) was of independent prognostic value for OS, while there was a trend for impaired OS for patients with a high p-mTOR (HR 1.27, $p = 0.21$).

Conclusions Phosphorylated mTOR is differentially expressed in localized RCC and metastasis. Elevated phosphorylation of mTOR is associated with aggressive pathologic features and unfavorable outcome. Whether these findings portend to relevance for mTOR inhibition treatment for metastatic RCC should be objective of further investigations.

Keywords mTOR · Metastasis · Everolimus · Phosphyorylation · Kidney · Clear cell

Introduction

Renal cell carcinoma (RCC) represents the most frequent renal malignancy, accounting for 90% of newly diagnosed renal cancers (Ljungberg et al. 2011).

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Impaired regulation of the Akt/mammalian target of rapamycin (mTOR) pathway has been implicated in mechanisms related to neoplastic transformation through enhancement of cell proliferation and survival (Lin et al. 2006). Supported by well-conducted Phase III clinical trials within the past decade, pharmacological inhibition of mTOR has become standard of care for patients with metastatic RCC (Grgic et al. 2011; Hudes et al. 2007).

Functionally, rapamycin inhibits mTOR by binding to FK506-binding Protein 12 (FKBP-12) (Choi et al. 1996) in the cytoplasm conjoining to an inhibitory complex which can bind to the FKB12-rapamycin-binding- (FRB-) domain at the C-terminal part of mTORC1-protein (Choi et al. 1996). This ultimately blocks an association of mTOR with raptor (Oshiro et al. 2004).

Regular activation of mTOR-complex-1 (mTORC1) is largely attributed to phosphorylation of Ser^{2448} (Copp et al. 2009), which leads to concomitant activation of mTOR downstream eukaryotic initiation factor binding protein-1 (4E)-binding protein (BP) and p70S6K (Haghighat et al. 1995; Hara et al. 1997), who ultimately initiate further processing of Cyclin D1 (Rosenwald et al. 1995), Vascular endothelial growth factor (VEGF), Fibroblast Growth Factor (FGF), and Hypoxia inducible factor alpha (HIF-1α) (Hudson et al. 2002).

Several retrospective studies revealed significant associations between expression levels or phosphorylation status of mTOR and its downstream targets with clinical and pathological factors in RCC and also RCC prognosis (Hager et al. 2012, 2011; Lin et al. 2006; Pantuck et al. 2007).

In an earlier publication, we demonstrated that in clear cell renal cell carcinoma (ccRCC) mTOR activity is linked to the activation via phosphorylation of the mTOR protein rather than to protein overexpression (Kruck et al. 2010). However, these preliminary results and the evidence from other studies investigating correlation between mTOR activation and RCC prognosis are largely based on the analysis of primary RCC specimen of patients with localized or metastatic RCC. To draw more in depth conclusions on putative biomarkers for treatment response to, e.g. mTOR targeted therapy, it is of high importance to analyze both the primary tumor and metastatic tissue of RCC, since genetic heterogeneity of metastases may reflect clonal populations within the primary carcinoma (Turajlic and Swanton 2016).

We hypothesized that the expression and the ratios of mTOR and phosphorylated (p)-mTOR positively correlate between primary ccRCC and metastatic tissue of ccRCC, but with a potentially stronger expression of p-mTOR in metastatic ccRCC tissue.

Patients and methods

Tissue microarrays (TMA) from formalin-fixed, paraffin embedded tissue from 342 patients undergoing surgical treatment for primary renal cell carcinoma (between 1993 and 2010) and 90 patients undergoing surgical resection for metastases (between 2004 and 2013) treated at the Department of Urology, University of Tuebingen, Germany were created. After histological evaluation of hematoxylin and eosin-stained slides, the TMA slides were constructed with a tissue arrayer (Beecher Instruments, Silver Springs, MD) as described previously elsewhere (Kononen et al. 1998).

For primary tumors, representative tumor regions and corresponding benign renal tissues were obtained. For RCC metastasis cores from different tumor-bearing blocks were analyzed when available, to provide information on intratumoral heterogeneity of expression patterns. All tumors were classified as clear cell carcinomas according to the WHO classification. Tumor staging was performed according to the 2002 UICC TNM classification (LH and Wittekind 2002). Clinical patient characteristics and follow-up were recorded.

For the IHC-staining of mTOR and p-mTOR (Ser2448), sections (5 µm) were transferred to slides (Superfrost-Plus, Langenbrinck, Teningen) and deparaffinized. Slides were incubated with rabbit polyclonal antibodies against mTOR (Cell Signaling Technology, Beverly, MA, dilution 1:50) and p-mTOR (Ser2448, Cell Signaling, dilution 1:50). After 12 h of incubation sections were incubated with a secondary biotinylated anti-mouse IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, USA) and for p-mTOR with an IHC-specific monoclonal rabbit antibody (Cell Signaling Technology) for 60 min. The DAB system (Vector) was used for visualization and slides were counterstained with hematoxylin. Human placenta served as negative control.

Staining was classified by two independent reviewers (D.S. and S.R.) according to a semi-quantitative IHC reference scale, the relative amount (0.0–1.0) of tumor cells stained together with the staining intensity (0–3 +) resulted in a score from 0 to 3 as previously described. Staining patterns for mTOR and p-mTOR are illustrated in Figs. 1 and 2.

Statistical analysis was performed using MedCalc. (Version 12.5, Ostend, Belgium). Expression levels of mTOR, p-mTOR and the ratio of p-mTOR/mTOR were evaluated as continuous variables and additionally as categorized variables in relation to the median expression (> median/< median) value.

For the comparison of expression patterns in normal tissue and RCC, Mann–Whitney *U* test (when 2 independent

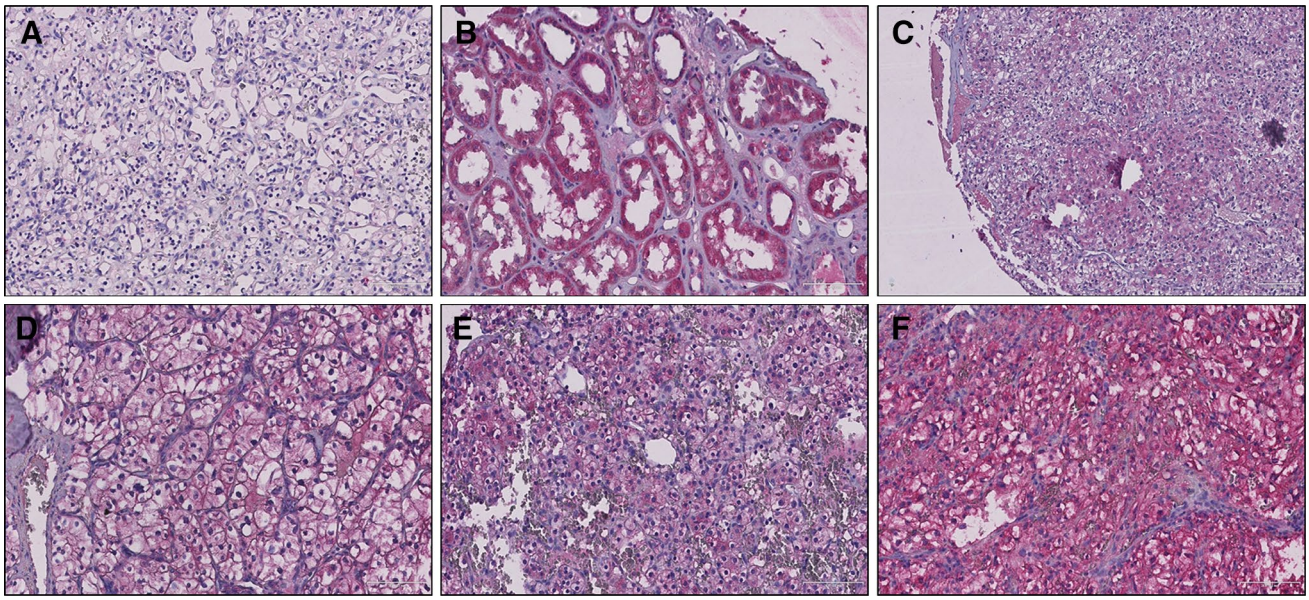

Fig. 1 Characteristic staining patterns for mTOR **a** RCC: mTOR negative; **b** Benign tissue: intense staining proximal tubuli; **c** RCC: perivascular intensified mTOR expression; **d** RCC: mTOR staining intensity 1; **e** RCC: mTOR staining intensity 2; **f** RCC: mTOR staining intensity 3

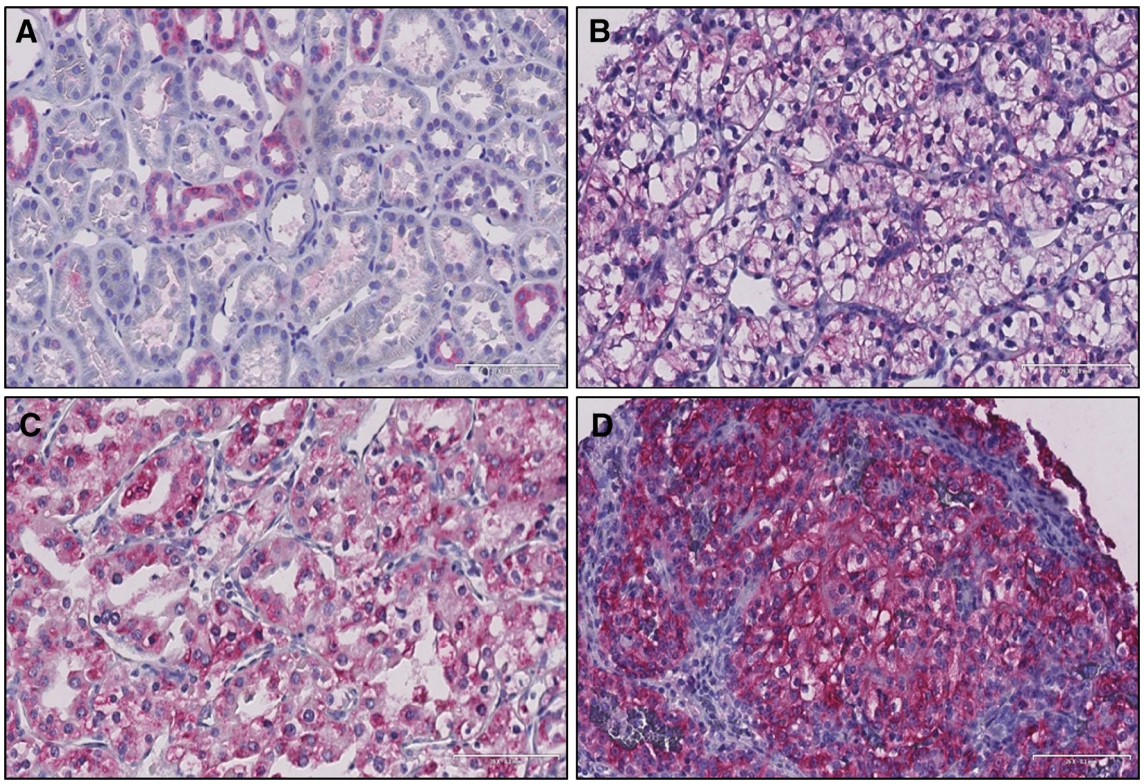

Fig. 2 Characteristic staining patterns for p-mTOR. **a** Benign tissue, **b** RCC: p-mTOR staining intensity 1; **c** RCC: p-mTOR staining intensity 2; **d** RCC: p-mTOR staining intensity 3

groups were compared) or the Kruskal–Wallis test (when more than 2 independent groups were compared) was applied. Univariate analyses were performed using Chi-square test and univariate Cox regression analyses. Kaplan–Meier analyses were performed to evaluate overall survival (OS) and cancer-specific survival (CSS), differences between subgroups were evaluated using Log-rank test. Multivariate testing was performed using Cox regression analyses. Statistical significance was regarded as $p < 0.05$.

Results

Median age at diagnosis of primary RCC was 64.17 years. Patient characteristics for primary RCC are shown in Table 1. In patients with metachronous diagnosis of RCC metastasis, median time from diagnosis of primary RCC to mRCC was 36.35 months. Median age at RCC metastasectomy was 66.49 months. The clinical characteristics of metastases are summarized in Table 2. Follow up information for OS was available for 337/342 (98.59%) primary RCC patients. A total of 136 (40.40%) of patients deceased during follow-up. Median OS for the primary RCC collective was 181.12 months (101.31–237.18). Information for CSS was available for 335/342 (97.95%) patients and 73 (21.79%) patients died in association with RCC.

The examination of the staining patterns revealed a different expression of mTOR in benign tissue, primary RCC and RCC metastasis tissue with an average staining score of 1.64 in benign, 0.78 in primary RCC and 1.44 in RCC metastasis. Staining was significantly lower in primary RCC tissue compared to benign kidney tissue. Interestingly, mTOR expression was significantly elevated in RCC metastasis tissue as compared to primary RCC tissue. Phosphorylation of the mTOR protein was detectable in benign kidney tissue,

Table 1 Characteristics of patients/primary tumors ($n = 342$)

	Levels/summary statistics	No.	%
Sex	Male	231	67.5%
	Female	111	32.5%
Age (years) at diagnosis of primary RCC	Median (range)	64.17 (17.12–90.32)	
T	1a	126	36.8%
	1b	77	22.5%
	2a	5	1.5%
	2b	1	0.3%
	3a	108	31.6%
	3b	22	6.4%
	3c	3	0.9%
	4	0	
N	0	322	94.2%
	1	19	5.6%
	2	1	0.3%
M	0	288	84.2%
	1	53	15.5%
	X	1	0.3%
R	0	319	93.3%
	1	22	6.4%
	NA	1	0.3%
Sarcomatoid features	No	319	93.3%
	Yes	23	6.7%
Tumor necrosis	No	219	64.0%
	Yes	123	36.0%
Primary tumor size (cm)	Median (range)	4.8 (0.3–18)	
Follow-up time (months) from date of diagnosis of primary ccRCC	Median (95%CI)	63.02 (55.27–72.84)	
Cancer-specific death	No	262	78.2%
	Yes	73	21.8%
Overall mortality	No	201	59.6%
	Yes	136	40.4%

Table 2 Characteristics of metastasis/local recurrence specimens

	Levels/summary statistics	*n* =	%
Total number of metastases		150	
Total number of patients with mRCC		90	
Number of mRCC patients with clinical data		84	93.3%
Metastasis	Synchronous	25	29.8%
	Metachronous	59	70.2%
Age (years) at metastasis resection	Median (range)	66.49 (30.81–80.77)	
Time (months) from diagnosis of primary RCC to metastasis resection	Median (95%CI)	36.35 (25.01–59.94)	
Metastatis site	Adrenal gland	11	7.3%
	Bone	22	14.7%
	Bowel	4	2.7%
	Corpus uteri	1	0.7%
	Diaphragm	1	0.7%
	Larynx	2	1.3%
	Liver	7	4.7%
	Local recurrence	7	4.7%
	Lung	44	29.3%
	Lymph node	19	12.7%
	Muscle	1	0.7%
	NA	3	2.0%
	Pancreas	3	2.0%
	Peritoneum	2	1.3%
	Skin	2	1.3%
	Soft tissue	17	11.3%
	Spleen	1	0.7%
	Sympathetic trunk	1	0.7%
	Testicle	1	0.7%
	Thyroid	1	0.7%

primary RCC and RCC metastasis tissue. Average staining of p-mTOR was 0.99 in benign kidney tissue, 0.73 in primary RCC and 1.14 in RCC metastasis tissue. Significant differences of expression were observed between primary RCC and benign tissue with a lower rate of phosphorylation in primary RCC. If RCC metastasis tissue was compared to primary RCC, mTOR phosphorylation was significantly higher in RCC metastasis. Expression values for mTOR and p-mTOR in primary RCC, benign and metastasis tissue are illustrated in Table 3 and Fig. 3. No significant differences were observed between primary and secondary metastatic RCC. Figure 4 illustrates expression differences between pulmonary and non-pulmonary metastasis.

Association of expression levels of mTOR and p-mTOR with pathological findings in RCC is shown in Table 4. Analysis of mTOR overexpression (> median) in primary RCC tissue demonstrated an association with the presence of tumor necrosis, while other clinicopathologic factors such as pathological stage, Fuhrman grade, tumor diameter and the presence of sarcomatoid features were not correlated with mTOR expression. In contrast, the presence of high phosphorylation of mTOR (> median) was significantly correlated with a higher T-stage, high Fuhrman grade, presence of tumor necrosis, and sarcomatoid features. With regard to the ratio of phosphorylated mTOR and non-phosphorylated mTOR (p-mTOR/mTOR) there was a significant correlation of a higher ratio with locally advanced tumors (T-stage), and the presence of sarcomatoid features.

Figure 5 illustrates Kaplan–Meier analyses in dependence of high (> median) or low (< median) mTOR and p-mTOR expression scores. Patient OS and CSS were not different in patients with a high or low expression of mTOR. In contrast, patients with high p-mTOR expression showed significantly impaired OS ($p = 0.0046$) and CSS ($p = 0.0067$) as compared to patients with low p-mTOR level.

In univariate analysis higher T-stage (HR 3.78), higher Fuhrman grade (HR 4.0), the presence of tumor necrosis (HR 1.99), and sarcomatoid features (HR 5.12) were significant predictors of OS. High p-mTOR (HR 1.67) and an elevated p-mTOR/mTOR ratio (HR 1.73) were also significantly predictive of impaired OS, while high mTOR expression had no significant predictive value. In the multivariate

Table 3 Comparison of expression levels of mTOR and p-mTOR

		(1) Benign tissue $n=237$	(2) Primary RCC $n=301$	(3) RCC metastasis $n=158$
mTOR	Min IRS	0	0	0.15
	Max IRS	3	3	3
	Avg IRS	1.6422	0.7847	1.4424
	95% CI for avg	1.5470–1.7375	0.7079–0.8616	1.3253–1.5595
	Median IRS	2	0.6154	1.4
	95% CI for median	1.6667–2.0000	0.5548–0.6713	1.2000–1.5000
	Different from factor nr ($p<0.005$*)	(2)	(1) (3)	(2)
p-mTOR	Min IRS	0	0	0
	Max IRS	3	3	2.8500
	Avg IRS	0.9898	0.7328	1.1378
	95%CI for avg	0.9133–1.0663	0.6537–0.8119	1.0194–1.2562
	Median IRS	0.8536	0.5	1.0500
	95% CI for median	0.7518–0.9066	0.4221–0.5870	0.8000–1.2000
	Different from factor nr ($p<0.005$*)	(2)	(1) (3)	(2)

avg average, *CI* confidence interval, *IRS* immunoreactivity score

*From Kruskal–Wallis analysis

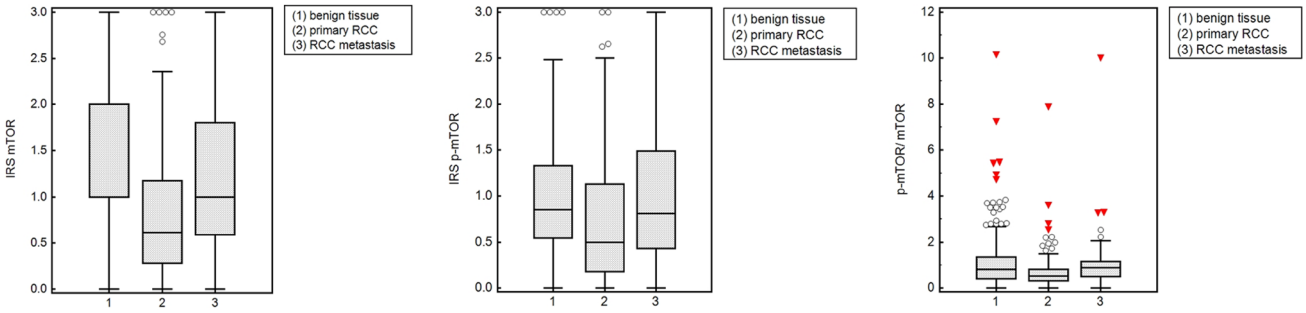

Fig. 3 Comparison of immunoreactivity scores (IRS) and p-mTOR/mTOR ratios in benign tissue, primary RCC, and RCC metastasis

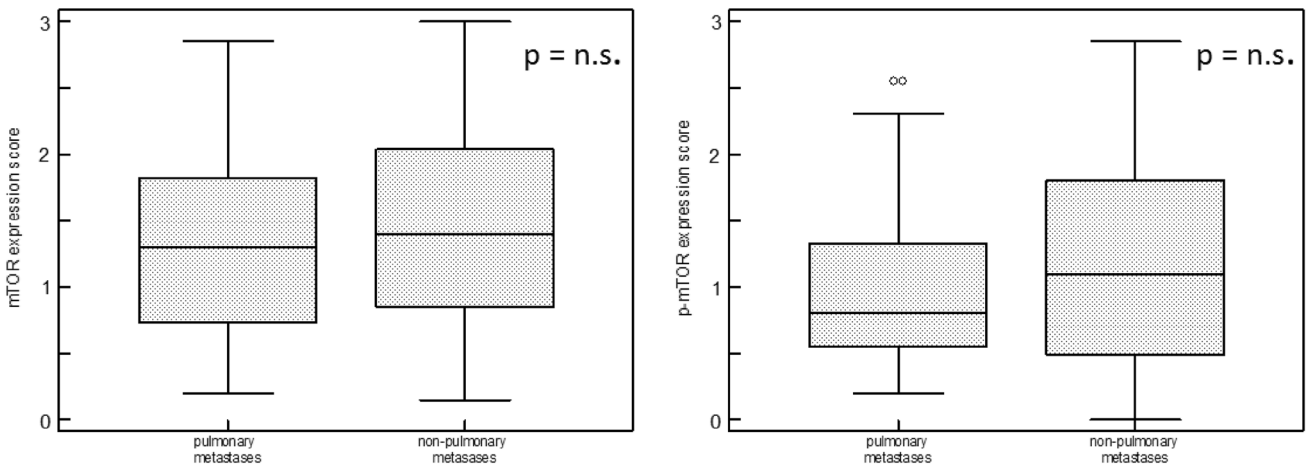

Fig. 4 Comparison of expression scores of mTOR and p-mTOR in dependence of metastasis localization

Table 4 Association of expression levels of mTOR and p-mTOR to pathological findings in RCC

	mTOR > median n/y (189 vs 150)	p-mTOR > median n/y (163 vs 149)	p-mTOR/mTOR > median n/y (74 vs 167)
Clinical stage > T2	$p = 0.9169$	$p = \mathbf{0.0001}$	$p = \mathbf{0.0010}$
Fuhrman-Grade > G2	$p = 0.7752$	$p = \mathbf{0.0030}$	$p = 0.0597$
N+	$p = 0.2187$	$p = 0.1581$	$p = 0.4061$
M+	$p = 0.5506$	$p = 0.2341$	$p = 0.3565$
Tumor necrosis	$p = \mathbf{0.0166}$	$p = \mathbf{0.0065}$	$p = 0.1469$
Sarcomatoid features	$p = 0.5837$	$p = \mathbf{0.0080}$	$p = \mathbf{0.0425}$
Diameter > median	$p = 0.3758$	$p = 0.2570$	$p = 0.1937$

regression model only the presence of high T-stage (HR 2.44) was of independent prognostic value for OS (Table 5a, b).

Discussion

Phosphorylation status of mTORC1 at mTOR Ser2448 represents an established marker for the activation of mTOR and PI3K-signaling in RCC (Altomare et al. 2004; Chiang and Abraham 2005; Choe et al. 2003). Earlier studies revealed that both mTOR and hypoxia-induced pathways are activated in primary and metastatic ccRCC (Schultz et al. 2011). Since clonal heterogeneity challenges the concept of targeted monotherapy, identification of potential biomarkers for targeted agents is difficult. However, recent genomic studies on RCC primary tumors and metastases show that clonal convergence can occur within the mTOR pathway which might have implications for biomarker development or individual treatment selection (Voss et al. 2014).

In a pilot trial, Kruck et al. investigated protein expression patterns of mTOR und p-mTOR in ten patients with primary ccRCC by immunohistochemistry and Western-blot analysis in comparison with corresponding benign tissue. Here, mTOR und p-mTOR were mainly localized in the cytoplasm. In analogy to findings from the present investigation, a significantly decreased mTOR expression and overexpression of p-mTOR was noted when benign and RCC tissues were compared ($p < 0.05$). In consequence, the increased activity of mTOR in primary RCC tissue was described as related to phosphorylation rather than to an overexpression of mTOR in primary RCC (Kruck et al. 2010). This constellation of different mTOR expression could also be observed in metastasic tissue in the present investigation. A further analysis of mTOR protein expression and its activation by phosphorylation in three different tissue compartments of benign kidney tissue, primary RCC, and a large cohort of RCC metastasis tissue (with $n = 90$ samples) was undertaken. Noteworthy, the expression of mTOR and p-mTOR was significantly higher in RCC metastasis as compared to primary RCC.

This fact may be of importance for the systemic treatment of metastatic RCC patients with mTOR inhibitors as it underlines that the target molecule of everolimus and temsirolimus is present in its native (mTOR) and activated (p-mTOR) status in the metastasis of RCC, especially in a significantly higher amount compared to primary RCC tissue.

Moreover, with regard to the ratio of p-mTOR to mTOR (p-mTOR/mTOR) there was a significant correlation of a higher ratio with locally advanced tumors (T-stage) and the presence of sarcomatoid features, which reflects a pathologic parameter of tumor aggressiveness.

Despite the biological function of mTOR and p-mTOR, a predictive value of phosphorylated mTOR protein for OS and CSS could be determined as both, OS and CSS was impaired in patients with a higher p-mTOR protein in univariate analysis. However, p-mTOR could not be confirmed as an independent predictor of OS in a multivariate model.

Earlier studies found that not all RCC tumor types are equally amenable to mTOR targeted treatment, while a majority of patients was found to have at least one component of the mTOR pathway impacted. The mTOR pathway was shown to be more significantly altered in ccRCC, high-grade tumors, and tumors with poor prognostic features, indicating a putiative selection parameter for mTOR inhibition. The authors further concluded that patients with nonclear-cell tumors and highly activated pathway may also be candidates for targeted therapy (Pantuck et al. 2007).

Other authors performed analysis of mTOR and its related proteins, like 4E-binding protein, p70S6K, p-TSC2 with regard to their predictive capacity in localized and metastatic RCC. In one study, tumor size, HIF-1α, and p-S6 expression were found to be independent predictors of both CSS and tumor progression in primary ccRCC (Schultz et al. 2011). Hager et al. evaluated p-AKT, p-mTOR, and PTEN expression in a tissue microarray of primary RCC and corresponding metastases, and normal renal parenchyma. Metastases in most subcellular compartments showed

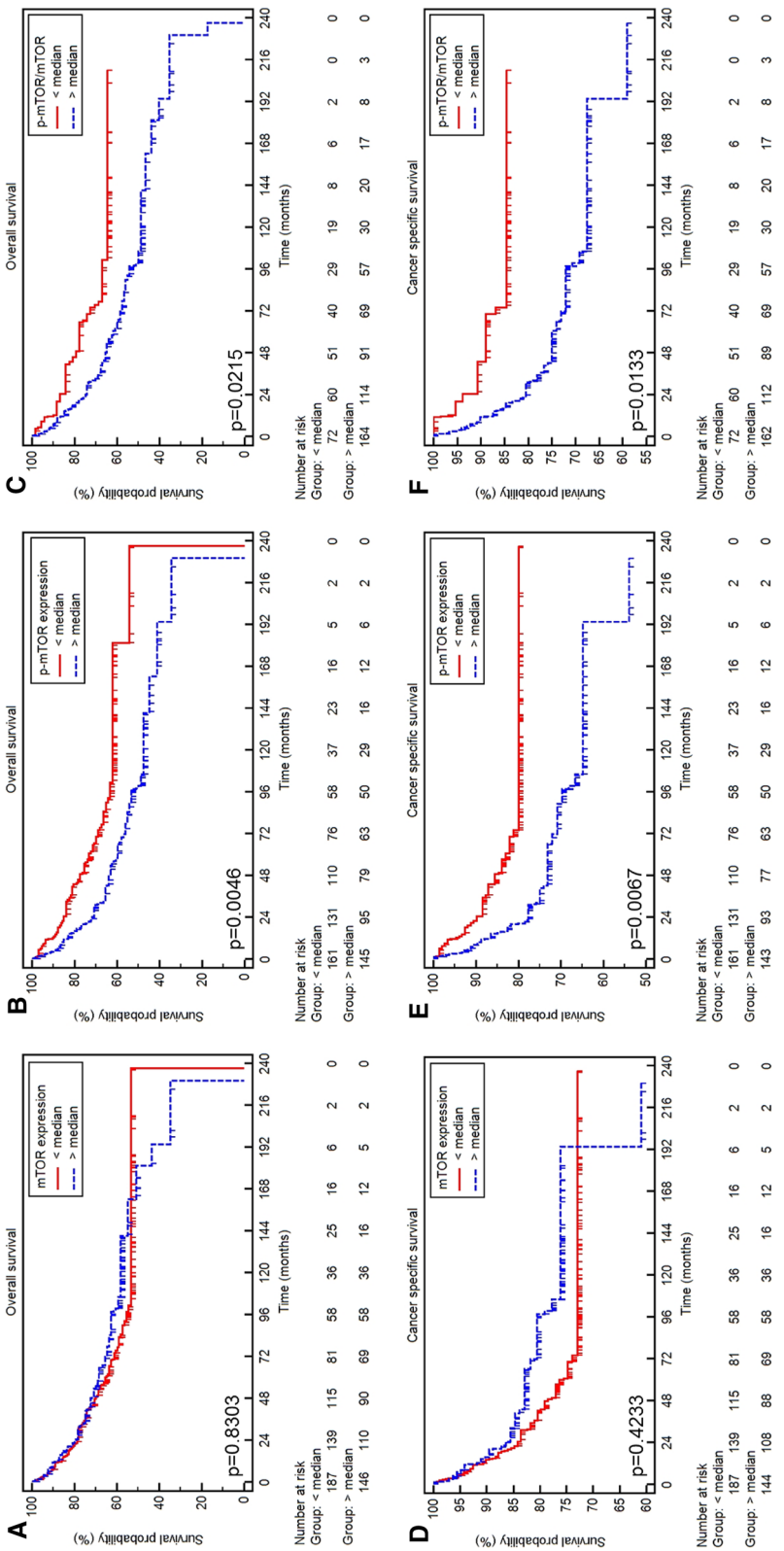

Fig. 5 Kaplan–Meier survival analyses for overall survival (**a–c**) and cancer-specific survival (**d–f**); *p* from log-rank test

Table 5 Cox regression analysis for overall (a) and cancer-specific survival (b)

Covariate	Univariate			Multivariate		
	p	Exp(b)	95% CI of Exp(b)	*p*	Exp(b)	95% CI of Exp(b)
(a) Overall survival						
Clinical stage > T2	< **0.0001**	3.7841	2.6644–5.3744	**0.0002**	2.4402	1.5295–3.8932
Fuhrman-Grade > G2	< **0.0001**	4.0261	2.7604–5.8721	0.1072	1.5797	0.9083–2.7472
Tumor necrosis	**0.0001**	1.9877	1.4180–2.7864	0.1415	1.3216	0.9131–1.9130
Sarcomatoid features	< **0.0001**	5.1171	3.1536–8.3032	0.0983	1.7169	0.9075–3.2483
Diameter > median	< **0.0001**	2.0952	1.4811–2.9638	0.4504	1.1748	0.7748–1.7811
mTOR > median	0.8304	0.9632	0.6847–1.3549			
p-mTOR > median	**0.005**	1.6673	1.1685–2.3792	0.2078	1.2689	0.8776–1.8347
p-mTOR/mTOR > median	**0.0232**	1.7341	1.0806–2.7828	0.2241	1.3597	0.8306–2.2257
(B) Cancer-specific survival						
Clinical stage > T2	< **0.0001**	10.6951	5.8556–19.5343	< **0.0001**	4.6684	2.2488–9.6915
Fuhrman-Grade > G2	< **0.0001**	8.1183	5.0889–12.9512	0.0546	1.8881	0.9908–3.5980
Tumor necrosis	< **0.0001**	3.3515	2.0944–5.3633	**0.0175**	1.8454	1.1159–3.0520
Sarcomatoid features	< **0.0001**	8.6108	5.0018–14.8238	0.0888	1.833	0.9154–3.6705
Diameter > median	< **0.0001**	4.443	2.5545–7.7275	0.1579	1.5778	0.8405–2.9618
mTOR > median	0.4241	0.8235	0.5128–1.3226			
p-mTOR > median	**0.0077**	1.9364	1.1936–3.1415	0.4359	1.2222	0.7396–2.0196
p-mTOR/mTOR > median	**0.0165**	2.4102	1.1784–4.9294	0.2227	1.6116	0.7513–3.4572

Exp(b) hazard ratio, *CI* confidence interval

comparable and stronger expression for p-AKT, p-mTOR, and PTEN than primary RCC and normal tissue, which was even more pronounced in patients with high-risk Memorial Sloan-Kettering Cancer Center (MSKCC) score. Along with the findings from the present investigation, this is interesting with regard to sensitivity to mTOR inhibitor therapy in metastasized RCCs with alterations in the PI3K/AKT pathway (Hager et al. 2011). The same group evaluated p-S6 expression as a putative surrogate of responsiveness mTOR inhibiton treatment in an analogous experimental scenario. While p-S6 overexpression was more frequently found in metastases than in primary RCC, interestingly, overexpression of p-S6 was detected in about one-third of cases in metastases without concomitant overexpression in their primary tumors. Patients with p-S6 overexpression in metastatic primary RCC specimen but also in metastases showed a tendency to shorter OS (Hager et al. 2012). Darwish et al. evaluated diverging immunohistochemical staining of multiple parameters of the mTOR signaling cascade (p-S6, p-mTOR, mTOR, p-AKT), hypoxia inducible factor-1alpha, Raptor, phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K), and phosphorylated 4E-binding protein-1 (p-4EBP1) of 419 primary RCC and found a higher cumulative number of altered biomarkers to be significantly associated with more aggressive pathologic features and inferior outcome (Darwish et al. 2013). Nishikawa et al. identified p-4EBP1 ($p < 0.001$, HR 4.08), C-reactive-protein ($p = 0.010$, HR 3.64) and pathological stage ($p = 0.035$, HR 2.64) as independent predictors of recurrence free survival in non-metastatic RCC after surgery (Nishikawa et al. 2014b). Additionally, in mRCC, the relevance of 4E-BP1 was confirmed by an analysis of RCC specimen of patients undergoing first- or second line treatment with mTOR-inhibitors by the same group. Here, 4E-BP1 expression was predictive of response to mTOR-inhibition treatment and 4E-BP1 and bone metastasis appeared to be independently associated with PFS on multivariate analysis (Nishikawa et al. 2014a).

In a small cohort of 18 mRCC patients before everolimus treatment, Li et al. observed that patients with positive expression of p-mTOR showed a better clinical benefit rate (71.4% versus 0%, $p = 0.023$) and PFS time (11.3 vs 3.7 months, $p = 0.001$) than patients with negative expression. However, no association of expression levels of p-4EBP1 and p-AKT were seen with regard to efficacy of everolimus treatment (Li et al. 2014). Since these observations are only preliminary and mTOR inhibition treatment is currently not considered a first-line therapy option in mRCC, further evaluation appears valuable to overcome present limitations in putative biomarker driven treatment selection for individualized systemic mTOR-inhibition. However, while distinct expression patterns for primary RCC and resected RCC metastases can be concluded from the present analysis, no distinct comparison of matched primary RCC and metastases was obtainable during the present study, which represents a major limitation. However, it must be remarked that for the evaluation of benign renal tissue corresponding

tissue from tumor-free paraffin blocks from RCC patients was analyzed. The expression patterns in benign renal tissue from healthy patients might, therefore, differ from the patterns observed in the present study. Finally, the single-center design and the therefore reduced sample size must also be taken into consideration.

Conclusions

The present study confirmed that activated (phosphorylated) mTOR is present to a greater extent in more aggressive tumors and also in RCC metastases. Both, the predictive relevance and association to adverse clinical factors are attributed to high p-mTOR status rather than mTOR overexpression. These findings may be of relevance with regard to systemic treatment with drugs targeting mTOR.

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Author contributions Protocol development: SK, SR, JB. Data collection and experiments: SR, DS, JH, VS. Data analysis and interpretation: SR, DS, JB, VS. Study supervision: AS, JB. Manuscript writing/editing: SR, JB, SK, AS.

Compliance with ethical standards

Conflict of interest JB: consultancies, honoraria or study participation from Bayer, BMS, Eisai, Eusa, Immatics, Ipsen, Novartis, Pfizer and Roche. AS: consultancies, honoraria or study participation from Bayer, BMS, Eisai, Immatics, Ipsen, Novartis, Pfizer and Roche.

Ethical standards The study was approved by the institutional review board and conducted in accordance with the Helsinki and START protocol. We take responsibility to the integrity of the data and accuracy of the reported study. All authors have made a substantial contribution to the information or material submitted for publication and approved the final version.

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