



# Activation of mTOR in renal cell carcinoma is due to increased phosphorylation rather than protein overexpression

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**Abstract.** The altered expression and activation of the mammalian target of rapamycin (mTOR) promotes the invasiveness and metastatic potential in a variety of malignancies. The aim of the present pilot study was to determine mTOR expression in clear cell renal cell carcinoma (RCC) and to evaluate mTOR activation and phosphorylation at Ser2448. Tissue microarray immunohistochemistry and Western blot analysis of tumor and benign tissue from 10 patients subjected to tumor nephrectomy were investigated. Staining of mTOR and phosphorylated-mTOR (p-mTOR) was documented and determined as percentage of the maximum. Western blots were evaluated densitometrically. Ratios of tumor versus benign tissue were calculated and compared by the Wilcoxon/Kruskal-Wallis test. Immunohistochemical expressions of mTOR and p-mTOR were 49 and 40% in benign renal parenchyma, whereas it was 20 and 42% in tumor tissue. Ratios of tumor versus benign tissue revealed a reduction to 0.44 for mTOR and corresponding elevation to 1.29 for p-mTOR ( $p < 0.05$ ). The rate of p-mTOR to mTOR was 1.19 in benign, whereas it was 5.30 in tumor tissue. Western blot densitometry detected lower expressions of mTOR in tumor compared to benign tissues. Ratio of p-mTOR to mTOR were significantly different in benign versus tumor tissue (0.86 vs. 1.37;  $p < 0.04$ ). The observation that RCC specimens exhibit higher levels of p-mTOR in RCC compared to benign renal parenchyma indicate the role of mTOR phosphorylation in RCC tumor development and progression. This study found a concomitant reduction of the RCC mTOR protein expression, which suggests that elevated levels of activated p-mTOR result predominantly from an increased mTOR phosphorylation

rather than from protein overexpression. These pilot study results may contribute to the clarification of mTOR-pathway regulation processes in RCC on the way to the protein profiling-predicted targeted therapy.

## Introduction

Renal cell carcinoma (RCC) is the most frequent kidney tumor lesion with ~30,000 new diagnosed patients within the European Union and over 38,000 new cases in the USA. Approximately 15,000 (European Union) and 12,000 (USA) disease related deaths are reported annually (1). After showing their therapeutic effectiveness in first- and second-line regimens new antiangiogenic drugs have been approved in Europe and in the USA for the treatment of metastatic RCC (mRCC). At present the main targets are multiple tyrosine kinase inhibitors and specific inhibitors of mammalian target of rapamycin (mTOR) (2,3). Among the mTOR inhibitors temsirolimus showed anti-tumor activity in a phase III study compared to interferon- $\alpha$  (IFN)- $\alpha$  (3,4). Everolimus proved beneficial in a phase III study in patients with metastatic RCC, who had progressed on vascular endothelial growth factor-targeted therapy (5) and deforolimus was tested in a phase I study in patients with advanced malignancies with a similar toxicity and pharmacokinetic profile (6).

The mTOR pathway has a central role in the regulation of cell growth, protein degradation and angiogenesis. Its regulation is frequently altered in tumors. The mTOR protein consists of two molecular complexes called mTORC1 and mTORC2. The subunit mTORC1 additionally contains the rapamycin-sensitive RAPTOR (regulatory associated protein of mTOR) protein. The mTORC1 complex is activated by AKT which promotes mTORC1 by phosphorylation of the tuberous sclerosis complex (TSC1/2) and activation of the RHEB (Ras homolog enriched in brain) protein. The downstream targets of mTORC1 are the p70-S6 Kinase 1 (S6K1) and the translation initiation factor 4E binding protein (4E-BP1), which is activated by mTOR and then dissociates from the eukaryotic translation factor (eIF-4E) (7). The activated form of S6K1 stimulates protein synthesis of elongation and initiation factors and ribosomal proteins required for the start of the S phase of the cell cycle (8). The phosphorylation and activation of mTOR at Ser2448 is blocked by mTOR inhibitors depending on the AKT activation status (9). This phosphorylated subunit of mTORC1 is

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commonly described as a biomarker of mTOR activation (9,10). The second complex called mTORC2 contains instead of RAPTOR protein the rapamycin-insensitive companion of mTOR (RICTOR) protein and interacts with AKT/PKB by phosphorylation with AKT/PKB. However, in RCC the mTOR pathway and the activation of mTOR are not completely defined.

In this study, the mechanism of mTOR phosphorylation was exemplarily investigated in RCC by Western blot (WB) analysis and tissue microarray (TMA) immunohistochemistry (IHC).

## Materials and methods

**Patients.** Tissue from ten randomly selected patients (mean age:  $66.3 \pm 2.6$  years), who underwent nephrectomy for RCC at the University of Tuebingen, Germany between 2003 and 2004 was analyzed. Tissue was formalin-fixed, paraffin-embedded and in parallel fresh frozen samples from pathologically representative tumor regions and corresponding benign renal tissues were obtained. All tumors were classified as clear cell carcinomas according to the WHO classification (11). The study cohort consisted of 6 men and 4 women. Tumor staging was performed according to the 2002 UICC TNM classification system and was stage pT1 in 8 patients and pT3 in 2 patients (12). Nuclear grading according to the Fuhrman classification was G1 in one patient and G2 in the others (13). Median tumor size was 5.75 cm. Patients were node negative and had no evidence of metastatic disease. WB and IHC analysis of all the patients were performed simultaneously.

**Tissue microarrays.** Tissue specimens were formalin-fixed, dehydrated and paraffin embedded. 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 (14,15).

**Immunohistochemistry.** Staining of mTOR and p-mTOR (Ser2448) was performed according to the following protocol. Sections (5  $\mu$ m) 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. RCC specimens with strong expression levels confirmed by Western blot analysis were used as positive controls. For negative controls the primary antibody was replaced by non-immune serum. TMA slides were evaluated in a blinded manner by two investigators.

Staining was classified according to a semi-quantitative IHC reference scale: the relative amount (0-100%) of tumor cells stained together with the staining intensity (0-3+) resulted

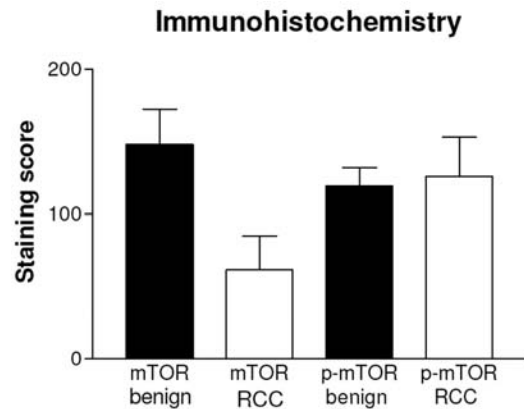


Figure 1. Staining scores of the immunohistochemical expression of mTOR and p-mTOR in benign and tumor tissues.

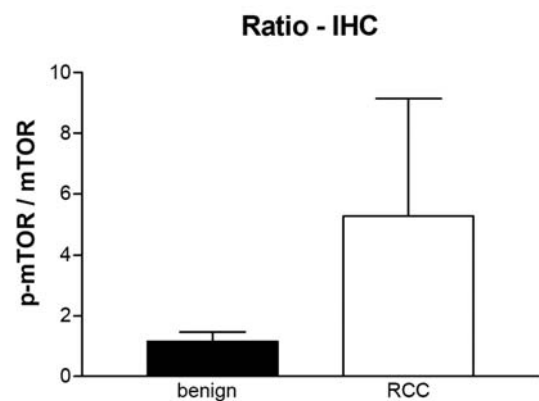


Figure 2. Ratio of the phosphorylated form of mTOR to mTOR in benign and tumor tissues.

in a score from 0-300 as previously described (16,17). Results were determined as percentage of the maximum value.

**Preparation of whole cell lysates.** Frozen samples (300-500 mg) were cut into small pieces and rubbed through cell strainer (40- $\mu$ m mesh) to obtain single cell suspensions. Cells were collected in ice-cold PBS and pelleted by centrifugation. Lysates were prepared by ultrasound sonifier ELB buffer (50 mM Tris, 250 mM NaCl, 0.1% NP-40, 1 mM EDTA, 20% glycerol) containing 5 mM DTT and protease inhibitor cocktail (Complete Mini, Roche, Germany). Cell debris was eliminated by centrifugation (10 min, 14,000 rpm, 4°C). Total protein concentration was determined by BioRad protein assay (BioRad Laboratories, Richmond, CA).

**Western blotting.** Protein of each sample (150  $\mu$ g) was loaded for SDS-PAGE on 6/12% gels. Proteins were electroblotted onto nitrocellulose membranes Optitran BA-S 83 (Schleicher & Schüll, Germany). Membranes were blocked with blocking buffer containing 5% non-fat dry milk or 1% BSA in TBS, containing 0.1% Tween-20. Membranes were incubated with the described primary antibodies of mTOR and p-mTOR and diluted (1:1000) in blocking buffer at 4°C overnight. Horseradish peroxidase conjugated secondary antibodies were diluted 1:2000 to 1:4000 and ELC (Amersham Biosciences)

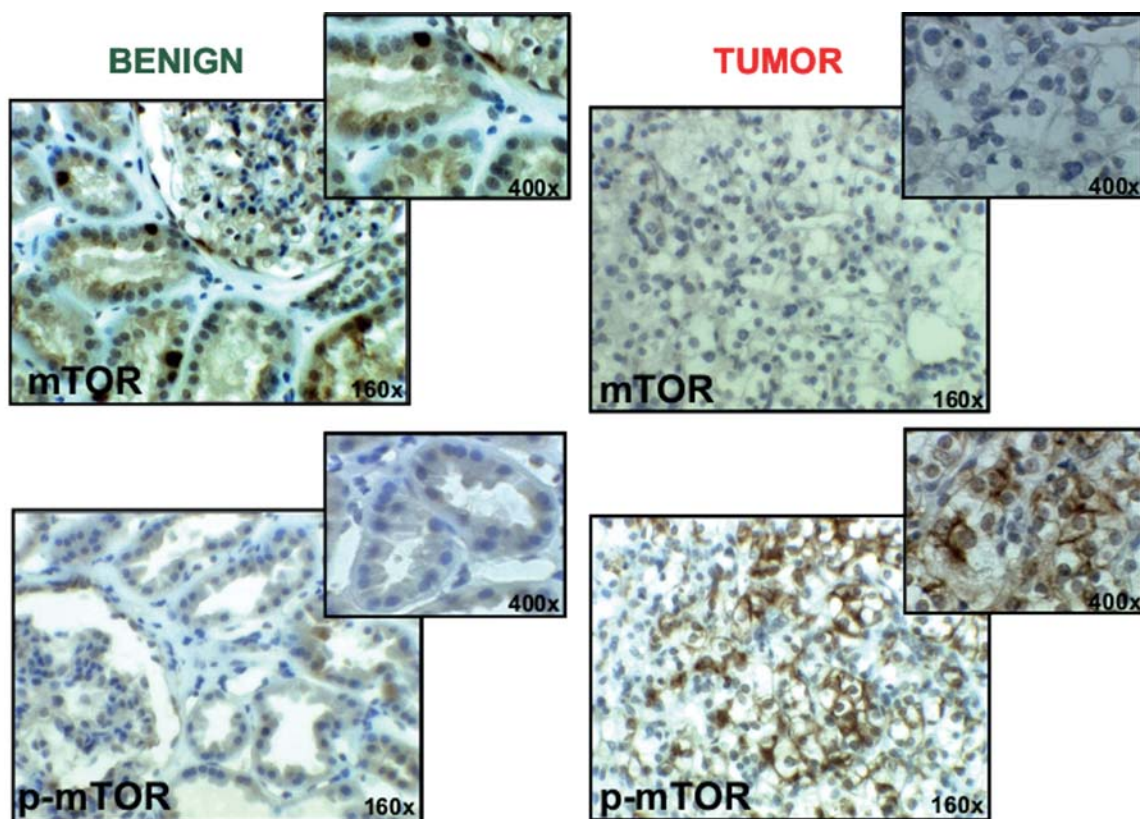


Figure 3. Representative figures of the immunohistochemical staining of mTOR and p-mTOR in benign renal parenchyma and renal cell carcinoma tissues. Staining showed a predominant cytoplasmic localization of mTOR and p-mTOR and was stronger in benign tissue for mTOR and in tumor tissue for p-mTOR.

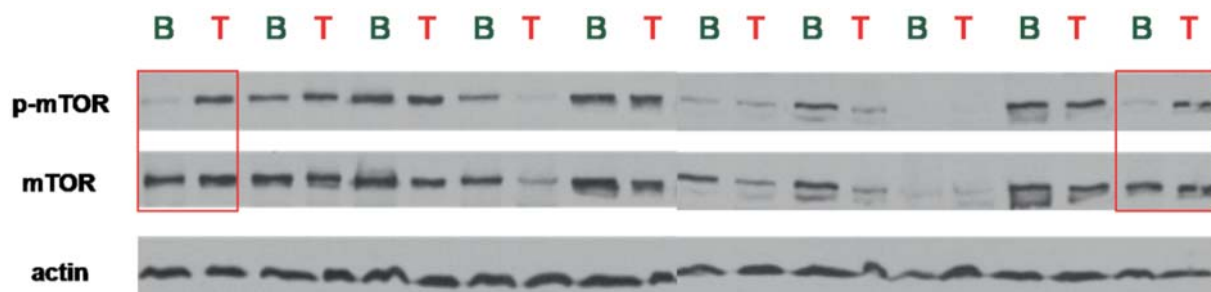


Figure 4. Western blot expressions of mTOR and p-mTOR in benign renal parenchyma and renal cell carcinoma tissue samples (B, benign renal parenchyma; T, tumor tissue).

was used for immunodetection. Blots were evaluated densitometrically using Scion program.

**Calculations.** Protein expressions of mTOR and p-mTOR as demonstrated as staining scores or densitometric values were detected in tumor and corresponding benign tissue and compared using the Wilcoxon/Kruskal-Wallis tests with the software JMP 5.1 (SAS, Cary, USA). The significant difference was set at  $p < 0.05$ .

## Results

**Immunohistochemistry.** Immunohistochemistry showed a predominant cytoplasmic localization of both mTOR and p-mTOR protein while nuclear staining was weak.

Expression of mTOR was stronger in benign kidney tissue than in RCC tissue [ $148 \pm 24$  (49%) vs.  $61 \pm 24$  (20%)] while p-mTOR expression was less in benign tissue compared to clear cell carcinoma tissue [ $119 \pm 13$  (40%) vs.  $126 \pm 27$  (42%)] (Fig. 1). Comparing the ratios between RCC and benign tissue, the mTOR level was decreased (0.44,  $p < 0.05$ ), while the ratio of p-mTOR was increased (1.29,  $p < 0.05$ ). Thereby the rate of p-mTOR to mTOR was higher in RCC (5.30) than in benign (1.16) tissue (Fig. 2). Representative results of the immunohistochemical staining are shown in Fig. 3.

**Western blot analysis.** Western blot analysis detected lower expression of mTOR in tumor tissue compared to benign (Fig. 4). Densitometric data showed a considerably higher



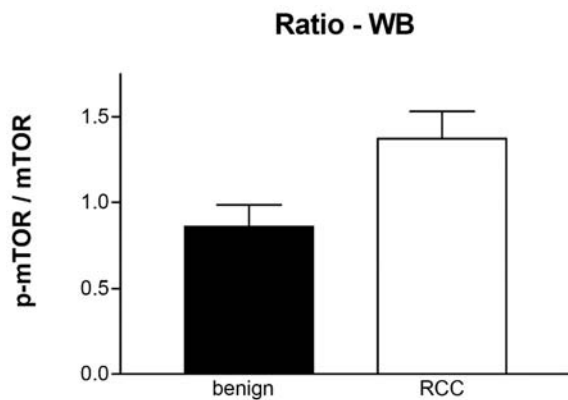


Figure 5. Ratios of p-mTOR to mTOR in benign and tumor tissue as determined by Western blot densitometry analysis.

ratio of p-mTOR to mTOR in tumor tissues (1.36) in comparison to benign renal parenchyma (0.86,  $p < 0.04$ ; Fig. 5).

## Discussion

Currently, the anti-tumor mechanisms of targeting angiogenesis are the main focus in the treatment of mRCC. The upstream and downstream targets of VEGFR, or parallel targets with VEGFR led to the development of new multi-kinase inhibitors (18). These different growth receptors (e.g. VEGFR, PDGFR- $\beta$ ), which effect the signaling pathway of AKT/mTOR, are a major signaling axis in kidney cancer. The specific inhibition of mTOR has anti-angiogenic effects in addition to and in combination with the inhibition of numerous pathways, effective on cellular metabolism, growth and progression. In the clinic the mTOR inhibitor temsirolimus proved beneficial and significantly enhanced survival of mRCC patients (4). It is the recommended first line treatment for patients with advanced mRCC and a poor prognosis according to the Motzer score (1). Another mTOR inhibitor everolimus (RAD001) proved beneficial in a clinical phase II and III study and is regarded as a second line option after failure of tyrosine kinase treatment (2,5). Furthermore, numerous studies provided significant insights into the mTOR pathway and underlined the relevance of mTOR as potential target of anti-cancer therapy (7).

The biological basis of mTOR activation starts with the activation by growth factor receptors and the PI3-kinase-AKT/PKB pathway. The two molecular subunits of mTOR called mTORC1 and mTORC2 are activated by AKT through inhibiting tuberous sclerosis complex TSC1/2 (7). The disruption of the TSC complex leads to mTOR activation by Rheb (19). The downstream targets S6K1 and 4E-BP1 are stimulated through phosphorylation of mTORC1 at Ser2448. Ser2448 is known to be blocked by sirolimus depending on the AKT activation status (9). The dissociation of 4E-BP1 from eIF-4E leads to cap-dependent messenger (mRNA) translation of c-Myc, cyclin D1, ornithine decarboxylase and hypoxia-inducible factor (HIF). The HIF overexpression upregulates a number of growth factors including the key regulators of angiogenesis, the vascular-endothelial growth factor (VEGF), PDGF and transforming growth factor (TGF). Normally HIF

is degraded by the von-Hippel-Lindau (VHL) gene, but the mutation of the VHL gene in clear cell RCC leads to HIF- $\alpha$  overexpression with the result of increased levels of VEGF, PDGF and TGF. Therefore, at least one important part of the synthesis of HIF is regulated by mTOR (7).

The goal of this study was to evaluate the status of mTOR expression and phosphorylation in renal cell carcinoma and corresponding non-tumorous kidney tissue. We hypothesized that the activation of the mTOR signaling pathway in clear cell RCC is caused by hyperphosphorylation of expressed mTOR rather than through an increased protein expression. For a better comparability of immunohistochemical results between the samples, a tissue-microarray analysis was performed. Decreased mTOR levels with corresponding increased p-mTOR expression were found in RCC tissue compared to benign kidney tissue. This resulted in significantly higher ratios for p-mTOR compared to mTOR in tumor tissue and was confirmed by immunohistochemistry and Western blot analysis.

In RCC the altered regulation of the upstream proteins of the mTOR pathway (phospho-AKT, PTEN, p27, and pS6) in advanced RCC were independent prognostic factors of disease-specific survival (20). The benefit of mTOR targeted therapy might be the greatest in patients with a highly activated mTOR pathway and mTOR protein. In both benign and tumor tissue, mTOR and p-mTOR were expressed. The altered ratio of p-mTOR to mTOR in RCC indicated the higher activation level of mTOR in tumor tissue. This was caused by hyperphosphorylation and not by protein overexpression. This higher ratio of p-mTOR to mTOR in RCC tissue points out that the phosphorylation of mTOR in RCC seems to be one important step in the activation and progression of RCC. The simple analysis of the protein expression status of mTOR and p-mTOR might not be sufficient enough to correlate with the disease progression. Thereby, regulation of phosphorylation of the mTOR protein at Ser(2448) seems to be a point of convergence for the regulatory effects of upstream signals in the AKT/mTOR axis with the activation of downstream events. Therefore, the mTOR to p-mTOR ratio might serve as a sensitive surrogate parameter to predict survival or response to mTOR targeted therapy. The simple change of expression status of mTOR or p-mTOR may not lead to the progression of RCC. Rather, the higher activation status of p-mTOR compared to mTOR seems to be important.

The results obtained enhance the understanding of mTOR activation in cellular growth and regulation. This study suggests that elevated levels of activated p-mTOR are not merely a result of protein overexpression, but rather of an increased mTOR phosphorylation. This may lead to improved rapamycin-based targeted therapies with a potentially improved clinical response in patients with advanced RCC.

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