PTEN and p27^{Kip1} are not downregulated in the majority of renal cell carcinomas - implications for Akt activation

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Received July 13, 2007; Accepted October 8, 2007

Abstract. Activation of the PKB/Akt pathway is supposed to substantially contribute to the pathogenesis and progression of malignant disease. The present study aimed at determining the occurrence of an impaired PTEN and p27^{Kip1} expression alone or in combination in a renal cell carcinoma to further clarify the role of Akt-pathway-associated proteins for the development and/or progression of this malignant disease. By using tissue microarray analysis, tissue samples from renal cell cancers and the corresponding benign tissue samples were investigated for expression of the PTEN, pAkt and p27^{Kip1} protein by immunohistochemistry. Additionally, a Western blot and RT-PCR analysis was performed to verify the results obtained from the immunohistochemical approach and to further clarify the mechanisms underlying the regulation of both proteins in renal cell cancer. Western blot analysis revealed an overexpression of PTEN and p27^{Kip1} in renal cell cancer samples and a significantly elevated expression of both proteins when compared with the corresponding benign tissue ($p<0.0001$ and $p<0.0005$). The latter finding was confirmed by real-time RT-PCR ($p<0.05$ and $p<0.01$) and immunohistochemistry ($p<0.001$ and $p<0.0001$). PTEN and p27^{Kip1} expression were positively correlated with each other both in the tumour and benign tissue ($p<0.001$ and $p<0.0001$). We concluded that a strong expression of PTEN in renal cell cancer did not block the PI3K-mediated phosphorylation of Akt in the tumour specimens analysed. Furthermore, Akt activation may not result in a decreased p27^{Kip1}, the latter being retained and overexpressed in the majority of renal cell cancers when compared with the corresponding benign renal parenchyma.

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Key words: renal cell cancer, Akt, PTEN, RT-PCR, p27^{Kip1}

Introduction

Renal cell carcinoma (RCC), the most frequently occurring solid renal lesion, comprises of different histological subtypes, each revealing specific histopathologic and genetic characteristics (1). Remaining asymptomatic during the early course of the disease, up to 50% of renal cancers are detected incidentally during imaging for the evaluation of a variety of non-specific symptom complexes (2).

For renal cell cancer, no diagnostic marker for routine clinical use is currently available and, in addition, the predictive value of none of the formerly investigated biomarkers (3) is sufficient enough to allow the initiation of a therapeutic strategy adjusted to the biological potential of an individual tumour.

The treatment of metastatic renal cell cancer represents an additional clinical challenge since currently available systemic treatment options show restricted efficacy. Recently, molecular-targeted therapeutic approaches were established, for example either by an antibody-based inhibition of proteins that are highly expressed in renal cell cancer or by an application of tyrosinkinase inhibitors (4,5). However, a molecular-based therapy can be considered to have a higher efficacy when it is initiated on a rational basis and therefore targeted against genetic alterations that have been proven to participate in the development and/or progression of renal cell cancer. Therefore, a sufficient and detailed knowledge of the various, so far, identified cell signalling cascades is urgently needed to have a better understanding of the cellular processes responsible either for the retention of a physiological or, when deteriorated, the development of a malignant cellular phenotype.

The Akt signalling cascade regulates crucial checkpoints of angiogenesis, cell survival and apoptosis (6). The deregulation of this pathway has been implicated to play an important role in the pathogenesis and progression of human malignant disease (7-9). Whereas Akt pathway alterations occur in several human malignancies, only very limited data, mostly addressing the occurrence of an altered PTEN 'phosphatase and tensin homolog deleted on chromosome ten' tumour suppressor gene (10,11) have been reported for renal cell cancer up to now (12-15).

As a common mechanism, the activation of Akt, one of the most important regulatory proteins within the PKB/Akt pathway, has been attributed to an inactivated PTEN, the latter inhibiting Akt phosphorylation via PI3 kinase (16). The activated Akt subsequently modulates a variety of downstream target proteins including mTOR that regulate, among others, apoptosis and cellular proliferation. Substantial antiproliferative activity is attributed to the $p27^{Kip1}$ protein, another downstream target of Akt and one of the most important cell cycle regulators at the G1 checkpoint of the cell cycle. The $p27^{Kip1}$ protein regulates cell cycle progression from the G1 to S phase by its inhibitory interaction with the cyclin E/cdk2 complex. In addition to alterations at the PTEN locus, posttranslational inactivation or down-regulation of $p27^{Kip1}$ as a result of Akt activation was reported as substantially contributing to the development and progression of malignant disease (17). Consequently, for a variety of human malignancies including renal cancer a decreased $p27^{Kip1}$ expression was identified as a biological variable of independent prognostic importance (18,19).

The recently reported prognostic significance of both PTEN and $p27^{Kip1}$ inactivation seems to implicate the involvement of an impaired regulation/expression of these proteins in the development of an aggressive phenotype, possibly due to a molecular enhancement sequence. However, the synergism between the latter alterations that appear to promote tumour growth and progression remains poorly understood.

For clinical application, prognostically significant alteration patterns of Akt signalling parameters may identify the invasive molecular phenotype of renal cell cancer and help to determine the most effective therapeutic strategy for an individual patient (10). Furthermore, the disruption of Akt signalling has been shown to inhibit cell proliferation and has therefore been discussed as an attractive and effective therapeutic approach for RCC (20).

Therefore, the present investigation aimed at determining the occurrence of the essential Akt signalling parameters PTEN and $p27^{Kip1}$ in renal cell cancer both on the RNA (real time RT-PCR) and protein level (Western blot analysis/ immunohistochemistry) and to possibly evolve a sequence of molecular events responsible for tumour progression. Additionally, the phosphorylation status of Akt was analyzed to investigate the impact of the PTEN status on the activation of the Akt/PKB pathway.

Materials and methods

Patients. Paraffin and, in parallel, fresh frozen tissue specimens from 22 patients (#1-22) (17 males/5 females) subjected to radical nephrectomy for renal cell cancer between 2/2003 and 9/2004 at Tuebingen University Hospital were collected from the primary tumours (clear cell carcinomas, 20 cases; papillary tumours, 2 cases) and the corresponding benign renal parenchyma. The age range was 43-78 (mean 62.8±9.8, median 63.0) years. According to the TNM classification system, the tumour stages and histological differentiations of the 20 clear cell carcinomas were: T1, 15 cases; T2, 1 case; T3, 4 cases; G1, 4 cases; G2, 14 cases; G3, 2 cases.

At the time of surgery, one patient revealed bone and another patient bone in combination with lung metastases. Tissue samples from all 22 patients were investigated by immunohistochemistry. The tissue samples from patients #1-10 were subjected to Western blot and RT-PCR analysis. Whereas tumour staging was performed according to the 2002 UICC TNM classification system (21), histological grading was determined according to Fuhrman (22) and the histological subtyping according to the WHO classification (23).

Immunohistochemistry. Tissue specimens were formalin-fixed, dehydrated and paraffin-embedded. A tissue microarray was constructed containing 2 cores from each tissue, resulting in a total of 86 (no normal kidney tissue was available from patient #1) cores. The tissue areas used for tissue microarray were selected by primary evaluation of haematoxylin and eosin-stained slides and the slides were constructed as described previously (24). PTEN and $p27^{Kip1}$ were immunohistochemically detected by commercially available antibodies (PTEN: dilution 1:200, clone 26H9, monoclonal mouse, Cell Signaling, Beverly, USA; $p27^{Kip1}$: dilution 1:150, clone SX53G8, monoclonal mouse, Dako Cytomation). After 12 h of incubation at 4˚C the sections were incubated with a biotinylated anti-mouse IgG secondary antibody (Vectastain Elite ABC Kit, Vector) for 60 min. The DAB system (SK-4100, Vector) was used for visualization according to the manufacturer's instructions. Sections were briefly rinsed in tap water, counterstained with Meyer's haematoxylin and mounted. For the negative control, the primary antibody was replaced by a non-immune serum. Tissue from a well-differentiated grade I breast carcinoma served as a positive control. Tissue micro-array slides were thoroughly evaluated by two independent investigators (histology) who were unaware of the origin of each tissue core. The staining reaction was classified according to a semi-quantitative IHC reference scale ranging from 0-3+ depending on the intensity of the parameter expression. The percentage of positively-stained tumour cells and the rating of the staining intensity resulted in a score from 0-300 as previously described (25). Staining was demonstrated as a percentage of the maximum. JMP (SAS Inc.) software was used for statistical analyses, implementing one-way ANOVA analyses of variance.

Preparation of whole cell lysates. Lysates were prepared by ultrasound sonifier using 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). Frozen samples (300-500 mg of tissue) were cut into small pieces and rubbed through a cell strainer (40 μm mesh) to obtain single cell suspensions. Cells were collected in ice-cold PBS, pelleted by centrifugation and weighed. After re-suspension in an appropriate volume of lysis buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and protease inhibitor cocktail), cells were incubated on ice for up to 60 min. Following the addition of NP 40 (0.5%) nuclei were pelleted by centrifugation (1 min, 14,000 rpm, 4˚C). For the preparation of the nuclear proteins the pellet was re-suspended in lysis buffer B (20 mM HEPES, 0.4 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol and protease

Table I. Sequences of the PCR primers used in this study.

Gene	Forward primer	Reverse primer	Amplicon size (bp)
18s rRNA	5'-CGG AGA GGG AGC CTG AGA A-3'	5'-CGC CAG CTC GAT CCC AAG A-3'	275
GAPDH	5'-TCA ACA GCG ACA CCC ACT CC-3'	5'-TGA GGT CCA CCA CCC TGT TG -3'	189
ATP S6	5'-CAG TGA TTA TAG GCT TTC GCT CTA A-3'	5'-CAG GGC TAT TGG TTG AAT GAG TA-3'	156
$p27^{Kip1}$	5'- CAG ACG GTT CCC CAA ATG C-3'	5'-TTC TGA GGC CAG GCT TCT TG -3'	190
PTEN	5'-GTC TGA GTC GCC TGT CAC CA-3'	5'-TTG GAG GCA GTA GAA GGG GAG-3'	73

inhibitor cocktail) and incubated on ice for 60 min, vortexing every 15 min. Cell debris was eliminated by centrifugation (10 min, 14,000 rpm, 4˚C). Total protein concentration was determined by BioRad protein assay.

Western blot analysis. Protein (100 μg) of each sample was loaded for SDS-PAGE on 12% gels, and electroblotted onto nitrocellulose membranes (Optitran BA-S 83, Schleicher and Schüll). 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 primary antibodies, diluted in blocking buffer at 4˚C overnight and with secondary antibodies for 1 h at room temperature. Antibodies used were anti-$p27^{Kip1}$, anti-p-Akt (BD Transduction Laboratories), anti-PTEN (Cell Signalling) and anti-actin (SIGMA). Primary antibodies were used at dilutions recommended by the supplier. Horseradish peroxidase-conjugated secondary antibodies were diluted 1:2000 to 1:4000 and ECL (Amersham Biosciences) was used for immunodetection.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA and proteins were isolated from the same samples in a common isolation step using Trizol reagent (Invitrogen, Karlsruhe, Germany). All RNA samples were subjected to treatment with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany). Consecutively, 1 μg of every RNA sample was reverse-transcribed with 100 U of Superscript II RNase H-Reverse (Invitrogen), using 500 ng oligo $(dT)_{15}$ primer (Boehringer) according to the manufacturer's instructions. Quantitative real-time RT-PCR including primer-design and control of the specificity and efficiency of the primer pairs was done as previously described (26). The sequences of the PCR primers used in this study are listed in Table I. For the evaluation of all PCR runs, Cycle Threshold values (CTs) obtained for the different amplicons were processed to mean normalized expression (MNE) as previously described (27).

Results

Immunohistochemistry. In the benign renal tissue, the expression of PTEN was detected in tubular and glomerular structures with a preferably nuclear and additional weak intracytoplasmatic staining reaction (Fig. 1A). In the malignant tissue, the nuclear staining was in general more intense than in the benign cells. Occasionally, a cytoplasmatic staining, significantly less intense than in the benign tissue samples, was observed (Fig. 1B). When compared with the benign parenchyma, the PTEN expression was significantly elevated within the tumour tissue specimens investigated ($p<0.001$). Although the tumour tissue obtained from patient #7 exhibited a decreased PTEN expression, the mean staining of tumours classified as >T1 and T1 disease was 71.5 and 58.0% ($p=0.086$), respectively.

Reactivity for $p27^{Kip1}$ in the benign tissue samples was predominantly presented as a nuclear staining, with partly weak cytoplasmatic staining reaction (Fig. 1C). Reactivity for $p27^{Kip1}$ in the malignant tissue samples was homogeneously distributed and exclusively detected in the nuclei (Fig. 1D). In the malignant tissue, the expression was significantly increased when compared with the benign renal parenchyma ($p<0.0001$). Although advanced stage tumours of patient #3, 7 and 18 (T3 G2) exhibited no elevated protein $p27^{Kip1}$ expression, the mean staining scores of all tumour samples investigated were comparable for tumours classified as >T1 (66.0%) and T1 (68.5%). Notably, both papillary tumours demonstrated an only weak expression of $p27^{Kip1}$. The vast majority of renal cancers investigated exhibited a retained expression for PTEN and $p27^{Kip1}$ that was much more intense when compared with the tissue samples from the benign renal parenchyma.

Western blot analysis. To verify the unexpected observation of a significantly elevated $p27^{Kip1}$ protein expression and the detection of remarkable levels of the PTEN protein in most of the renal cell cancer specimens investigated when compared with the benign renal parenchyma, a Western blot analysis was conducted. Whole cell extracts were prepared from 10 tumour specimens as well as one corresponding normal tissue sample (patient #2). Eight of the RCC samples (patient #3-10) and the corresponding benign tissue samples from each patient were re-analysed by Western immunoblotting. The overexpression of both PTEN and $p27^{Kip1}$ in the RCC samples re-analysed was confirmed (Fig. 2, above). Expression levels of the latter proteins were always lower in the corresponding benign tissue specimens as determined by densitometric analysis (PTEN: mean 0.89 and 0.26, for tumour and benign tissue, respectively, $p<0.0001$ and $p27^{Kip1}$: 1.03 and 0.22, $p<0.0005$). With the increasing tumour stage (patient #3, T3 G3; patient #7, T2 G2) there was a slight decrease of the $p27^{Kip1}$ protein expression that remained, however, at a higher level when compared with the corresponding benign tissue samples (densitometric mean values 0.64 in the case of >T1

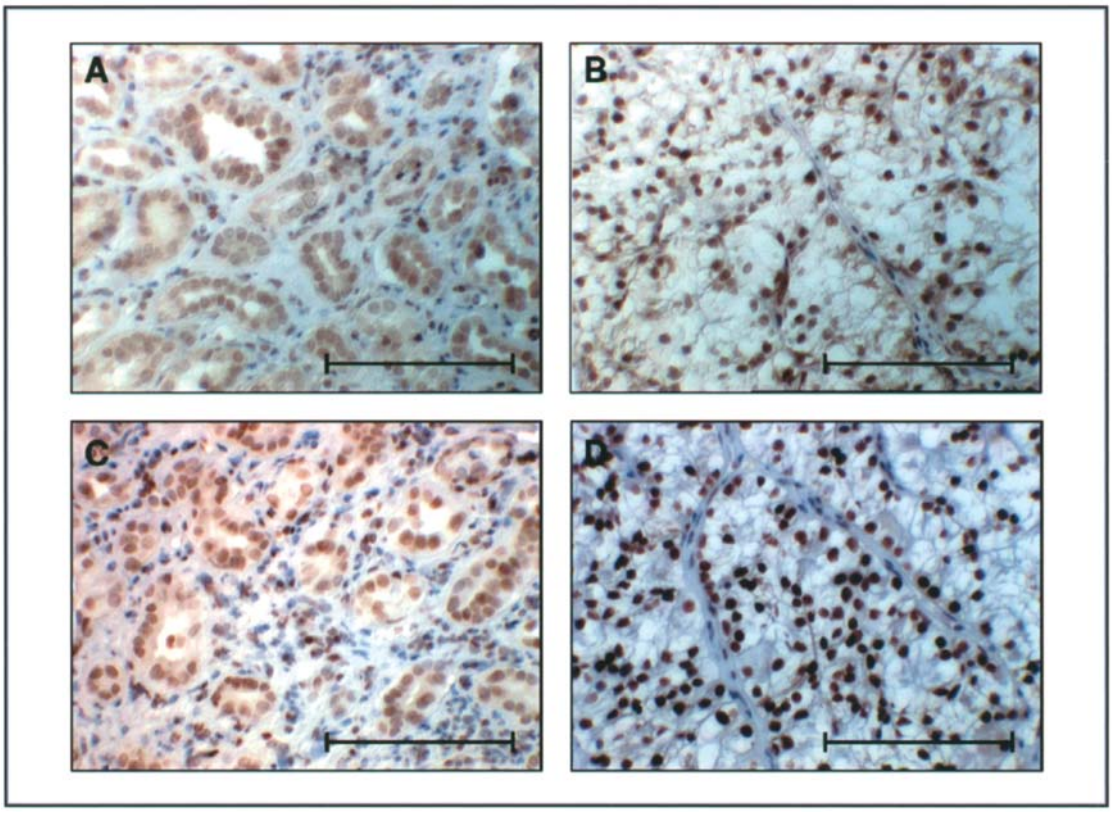

Figure 1. Immunohistochemical staining for PTEN (A and B) and $p27^{Kip1}$ (C and D) in benign renal parenchyma and clear cell renal carcinoma, respectively (160x magnification, Bar, 100 μm).

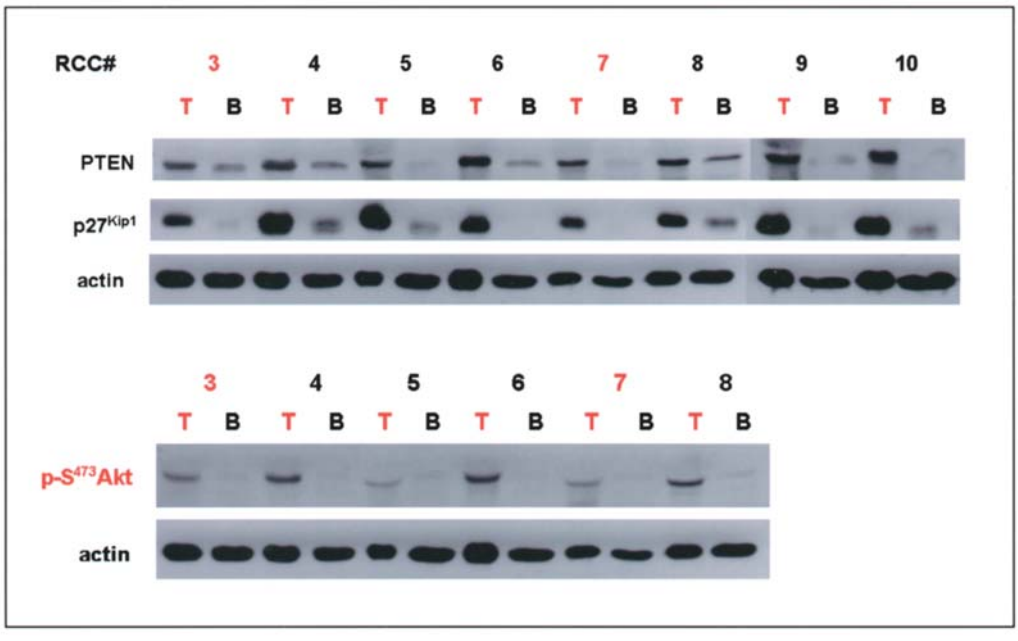

Figure 2. Expression of PTEN, $p27^{Kip1}$ (above) and phospho-Ser473 Akt (below) in total cell lysates of renal cell carcinoma (T) and the corresponding benign renal parenchyma (B). RCC#: 3, T3 G3; 7, T2 G2; 4,5 and 10, T1 G2; 6,8 and 9, T1 G1.

and 1.16 for T1). An analysis of sample #3-8 revealed higher amounts of phosphorylated Akt in all tumours versus normal tissue specimens (Fig. 2, lower), especially in the tumours from patients #4-6 and 8 that were classified as T1 disease.

Real-time reverse transcription-polymerase chain reaction. The stability of the expression of the 3 candidate housekeeping genes was evaluated by comparing the CT values in normal and in tumour tissue. Only ATP S 6 was stably expressed in

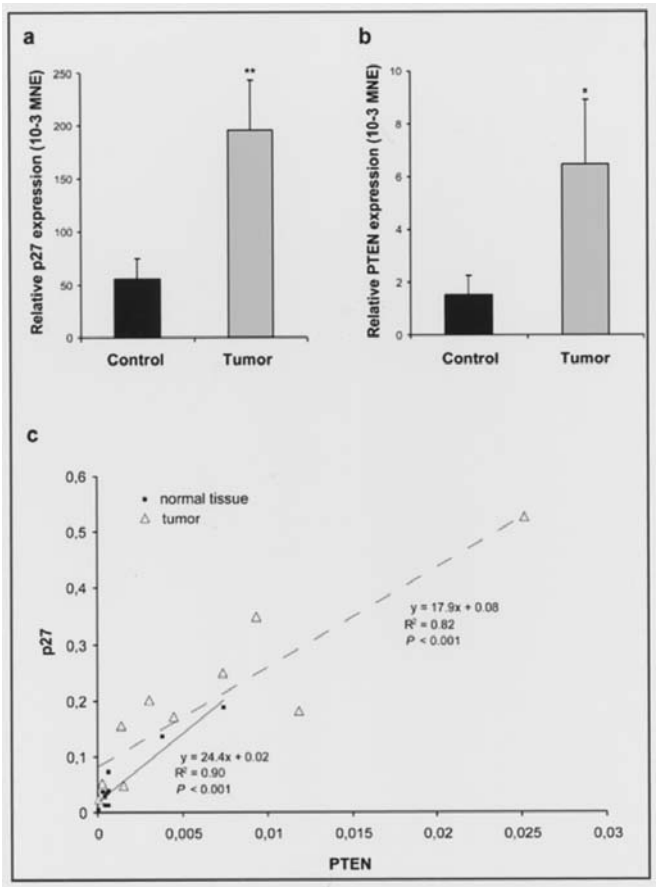

Figure 3. Relative expressions of $p27^{Kip1}$ (A) and PTEN (B) mRNA in control and tumour tissue, respectively. Within tumour and benign tissue a significant correlation between PTEN and $p27^{Kip1}$ mRNA expression was observed (C).

the tumour and benign renal tissue. In contrast, GAPDH and 18s rRNA showed a significant decrease of the CT in tumour tissue, indicating that in kidney cancer both of these conventional housekeeping genes are expressed at significantly higher levels. Therefore, ATP S 6 served as a housekeeping gene in the present investigation.

In the tumour tissue, $p27^{Kip1}$ and PTEN mRNA expression was 3.51/-4.23 fold and herewith significantly up-regulated compared to the benign control tissue (Fig. 3 A and B; $^{**}p<0.01$ and $^{*}p<0.05$). $P27^{Kip1}$ and PTEN are significantly positively correlated with each other in benign tissue (Fig. 3 C; R2=0.90, F1,10=72.4, p<0.0001) as well as in the tumour tissue samples analyzed (R2=0.81, F1,10=35.2, p<0.001).

Discussion

In contrast to former investigations that have reported a decreased expression of the PTEN tumour-suppressor protein in a substantial number of renal cell cancer specimens as a result of alterations at the PTEN gene locus (12,13), PTEN protein levels were significantly elevated in RCC tissue in the present study when compared with benign renal parenchyma. The positive correlation between PTEN mRNA and protein levels in the tumours investigated confirms the results obtained on the protein level and indicates that increased PTEN levels result from a transcriptional activation of the gene than from a posttranslational modification. A transcriptional regulation of PTEN was accordingly observed by Ma *et al* (28) in cell lines cultivated from primary hepatocellular carcinomas. Even in advanced stage renal cancers (≥T2), no considerable reduction of PTEN levels were noted (rather an increase was visible). This observation is contradictory to the previously reported negative impact of PTEN loss on the clinical prognosis of RCC patients (29,30) on the one hand and a reported PTEN loss in advanced stage RCC as a molecular late stage event on the other (10).

According to the current hypothesis, the activation of Akt via PI3 kinase is mainly triggered by an inactivated PTEN gene. However, the result reported herein rather supports the assumption that in the majority of renal cell cancer specimens PTEN does not substantially contribute to the control of Akt pathway activation.

In the renal cell cancer tissue investigated herein, $p27^{Kip1}$ protein was commonly overexpressed and strictly localized within the cell nuclei. Similar to PTEN, the correlation between mRNA and protein levels in tumour tissue indicates the increased expression levels in RCC which result from a transcriptional activation. This is in clear contrast to findings reported by Ciaparrone *et al* (31) in colon cancer, for example. Whereas Osipov *et al* (15) reported that $p27^{Kip1}$ protein levels decrease with increasing tumour size, we observed a slight increase of the $p27^{Kip1}$ expression in the advanced stage of the disease. Notably, the papillary tumours exhibited an extremely reduced $p27^{Kip1}$ expression, possibly indicating that Akt signalling alterations differ between histological subgroups of renal cancer.

According to the available literature, decreased $p27^{Kip1}$ protein levels were reported mainly in terms of their prognostic value as suggested by Slingerland *et al* (17), for example, to induce a growth advantage for 27^{Kip1}-depleted cell clones. In contrast, as reported by Hedberg *et al* (19), 76% of renal cell cancer specimens presented a staining reaction for $p27^{Kip1}$ that was similar to that observed in tissue samples from benign renal parenchyma. In contrast to our observation of a retained or even increased $p27^{Kip1}$ protein expression in the majority of renal cell cancers, Chiarle *et al* (32) reported high expression levels in most breast cancers investigated.

The inactivation by phosphorylation (33) and translocation into the cytosol [seen in breast cancer (34)], followed by proteolytic degradation, could serve as a possible explanation for the higher total amount of $p27^{Kip1}$ as detected by Western blot analysis, possibly due to an intermittent intracytoplasmatic accumulation. However, a substantial cytoplasmatic localization of $p27^{Kip1}$ could not be detected by immunohistochemistry in the present study. Therefore, the higher amount of the nuclear proliferation inhibitor $p27^{Kip1}$ in RCC tissue samples may suggest a more complex regulation of $p27^{Kip1}$ signalling than assumed thus far.

According to observations in ovarian cancer (35) a correlation between the $p27^{Kip1}$ and PTEN protein expression was not found. In contrast, the positive correlation between the expressions of both parameters as detected at the mRNA level in the present study seems to suggest a relatively close coherence between regulatory processes that affect the two parameters in renal cell cancer.

In summary, PTEN activity is assumed to be associated with the ability to antagonize signalling through the PI3K pathway (36), thus blocking the activation of Akt. Therefore, we additionally studied the phosphorylation of the Akt protein at serine 473 in exemplary samples by Western blot analysis and as a result detected activated Akt in all the tumour specimens versus the benign tissue samples. Thus, a strong expression of PTEN in tumour tissue clearly did not block the PI3K-mediated phosphorylation of Akt in the tumour specimens analysed. Furthermore, Akt activation appears not to lead stringently to a decrease of $p27^{Kip1}$, the latter being retained and overexpressed in the majority of renal cell cancers investigated herein when compared with corresponding benign renal parenchyma.

Addressing former immunohistochemical studies, the morphology of clear cell carcinoma cytoplasm may result in a misinterpretation of results. Therefore, a standardized immunohistochemical staining and interpretation modus is urgently required for future investigations (37). Horstmann *et al* (38) pointed out further parameter modulations in renal cell cancer that were not considered in the present study such as local gradients within the tumour showing a superior impact on expression data within the tumour margin near the invasion front. A sampling bias in big tumour volumes where the sample has been harvested more from the centre may affect the results, especially when a correlation to T stage is carried out.

Further investigations will aim at obtaining an exact Akt parameter status in renal cell cancers of different tumour stages and histological differentiations to determine clinically reliable diagnostic and prognostic marker profiles as well as molecular targets for therapeutic purposes.

Acknowledgements

The study is supported by a grant from the Federal Ministry of Education and Research (Fö. 01KS9602) and the Interdisciplinary Center of Clinical Research Tuebingen (IZKF). A.S.M. is partly supported by the Fortune program (grant F 1235097) and P.S. is supported by the Fortune program (grant F 1481010).

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