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The Proliferation Marker Thymidine Kinase 1 Level is High in Normal Kidney Tubule Cells Compared to other Normal and Malignant Renal Cells

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Abstract The activity of the proliferation related enzyme thymidine kinase 1 (TK1) was reported to be 3-fold higher in extracts from normal kidney tissue as compare to renal carcinoma extracts [3]. To verify these unexpected results, determinations of the protein levels of TK1 in normal kidney and in samples from different types of renal cell carcinoma (RCC) were done with immunohistochemistry and Western blot analysis. Two anti-TK1 peptide antibodies reacting with different TK1 epitops were used. TK1 levels were high in tubule cells as compared to glomerulus cells and connective tissue cells, while an intermediary TK1 was observed in renal cell carcinoma (RCC) cells. Western blot analysis demonstrated high levels of TK1 in extract from normal kidney, and lower levels of TK1 in the RCC extracts. The specificity of TK1 staining was demonstrated in competition experiments with excess TK1 antigen. The high TK1 levels in normal kidney tubule cells suggest that they are in a form of activated G1-state. The relatively low TK1 level in RCC, representing TK1 expression in S-phase cells, is in accordance with the low overall proliferation rate of these tumors. These results suggest that cell cycle regulation of TK1 in normal tubule cells differ from that in other type of normal and malignant renal cells.

Keywords Kidney · Renal cell carcinoma · Thymidine kinase 1 · TK1 · Ki-67

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Introduction

It is well known that the activities of enzymes involved in the phosphorylation of deoxyribonucleotides are low in different types of differentiated cells, such as liver, kidney, brain, etc. For example, thymidine kinase 1 (TK1) expression in mammalian normal kidney tissue is significantly lower as compared to proliferating tissues such as spleen [1]. This is also the case with other proliferation related markers such as thymidylate synthase (TS) and Ki-67 [2].

Mizutani et al. [3] reported that the activity of TK1 in human normal kidney tissue was 4 times higher than in renal cell carcinoma (RCC), while TS activity was 5 times lower [4]. Parts of normal and malignant tissues were taken

from the same kidney and activities were determined in extracts from these samples. The high TK1 activity in normal kidney cells was unexpected since many earlier studies have shown that TK1 is only expressed in S phase and G2 cells and this enzyme is an established proliferation biomarker [5, 6 and references there in]. However, recent investigations [7, 8] show that tubule cells of normal mammalian kidney have high proliferative capacity. These cells are in an activated G1 phase of the cell cycle, and not in G0, and could be rapidly induced to divide as a result of "stress" stimuli. The expression of several cell cycle markers such as cycline D1 was also high [7] in tubule cells.

In this investigation detailed studies of the levels of TK1 in normal kidney tissues and in different types of RCC are presented, based on immuno chemical methods with two novel and highly specific anti-TK1 antibodies.

Material and Methods

Cultured Cells and Patient Samples

Human T cell lymphoma CCRF-CEM cells were used which were either wild type (CEMTK+) or cells selected for bromodeoxyuridine resistance leading to TK1 negative (CEMTK-) phenotype as described [9]. A total of 27 patients with RCC (5 women and 22 men; median age 61 years, range from 38 to 78) were included. The clinical history of the patients is summarized in Table 1. Informal consent was obtained and patients underwent radical nephrectomy and were prospectively evaluated at the Renmin Hospital of Wuhan University, China, from 2004–2006. The tumor stage and grading systems were used for the histo-pathological evaluation of the tumors (AJCC Cancer Staging Manual, 2002).

According to histological classification, 20 tumors were clear cell types, one renal granular cell type, two renal papillary cell carcinoma, and four had renal sarcomatoid cell. Seven of the tumours were of stage I, 18 were of stage III and two were of stage IV (Table 1). Five of the tumors were well differentiated (grade 1), 16 were moderately (grade 2), and six were poorly differentiated (grade 3). Fresh specimens were taken from all these patients. Five normal kidney specimens from the patients who underwent nephrectomy were taken from areas around the tumors, but well separated from the tumour area and identified by a pathologist as non-malignant. The lack of stage II patients is due to the fact that most of the patients already had advanced and metastatic disease when they arrived to the hospital. The patients with stage I were identified in regular health examinations. This study was done in accordance to the Helsinki Declaration, revised 1983.

Immunohistochemistry

Three micrometer thick tissue sections were prepared from formaline-fixed, paraffin-embedded specimens. The sections were deparaffinized and rehydrated. In order to unmask antigen activity in the tissues, sections were incubated with citric acid buffer (pH 6.0) for 30 min at +95° C in a water bath. Parts of the sections were also treated in a microwave oven adjusted to near boiling water. Immunohistochemical staining was carried out essential as described earlier [10]. Sections were incubated for 30 min using 3% H_2O_2 in order to block the endogenous peroxidase. Non-specific binding sites were blocked by incubation with normal sera (normal donkey serum for TK1 staining, Jackson Immuno-Research Lab, USA; normal horse serum for Ki-67 staining, Vector Lab. CA, USA). Slides were incubated with an anti-TK1 chicken IgY antibody, raised against a synthetic peptide, residue 161–183 of the human TK1 sequence [11], diluted in PBS to 100 μg/ml (SSTK Inc., Shenzhen, China), alternatively slides were reacted with anti-Ki-67 mAbs (Dako Lab. Denmark, dilution in PBS) at $+4^0$ C overnight, and rinsed in PBS. Biotinylated secondary antibodies (Donkey anti-chicken IgG antibody for TK1 staining, diluted in 1:400, Jackson Immuno-Research Lab, USA or anti-mouse IgG for Ki-67 staining, diluted in 1:200, Vector Lab. CA, USA) were applied for 40 min at room temperature. The ABC reagent (Vector Lab. CA, USA) in PBS, 0.1% Tween 20 was added and incubated for 40 min at room temperature. Diamino-benzidine was used as a chromogen and the slides were counterstained with haematoxyline. Staining without the primary TK1 antibody was performed in parallel and served as negative control.

The specificity of the anti-TK1 reaction was tested by means of a competition experiment with additions of a 300 fold molar excess of the peptide corresponding to the residue 161–183. PBS was used instead of peptide in the experiments as negative controls.

Evaluation of the Immunohistochemistry Experiments

TK1 and Ki-67 positive cells in the tissue sections were determined among at least 100 cells per microscopic fields of 10 fields at×200–400 magnification by two pathologists independently. Fields were accepted for evaluation if they did not contain areas of extensive necrosis, nonspecific background staining, or sectioning artifacts, The number of stained cells within the tumors was scored as follows: no staining (−), 1–19% (+), 20–49% (++) and 50%- (+++).

Preparation of Cytosolic Extracts

Human renal cell carcinoma tissues and normal kidney tissues were dissected and stored at -80^0 C. About 100 mg

Table 1 Characterization of patients with malignant kidney disease and normal individuals. The stage is based on the TNM notes (T= tumour size; *N*=metastasis in lymph node; M=metastasis spread in the body). Grade represents the degree of differentiation of the tumour. The TK1 and Ki-67 values corresponds to the number of cells stained in the tumour (see "Material and Methods")

Patient No.	Age	Sex	Pathological diagnosis	TNM	Stage	Grade	TK1	Ki-67
25	50	f	right renal clear cell carcinoma	T1aN0M0	I	1	+	+
29	65	m	left renal clear cell carcinoma	T1aN0M0	I	1	−	−
35	48	m	right renal clear cell carcinoma	T1bN0M0	I	2	−	+
36	50	f	left renal clear cell carcinoma	T1aN0M0	I	3	+++	++
39	68	m	left renal clear cell carcinoma	T3aN0M0	III	2	+	−
40	40	m	left renal clear cell carcinoma	T3aN0M0	III	1	+	+
41	53	m	left renal clear cell carcinoma	T1bN0M0	I	1	+++	+++
42	78	m	right renal papillary carcinoma	T3aN0M0	III	2	+++	+++
43	59	m	right renal clear cell carcinoma	T3bN0M1	III	2	+++	+
44	51	f	left renal clear cell carcinoma	T3aN0M0	III	2	−	−
45	46	m	right renal clear cell carcinoma	T3aN0M0	III	2	+++	+
46	65	m	right renal clear cell carcinoma	T3aN0M0	III	2	−	−
47	59	m	right renal clear cell carcinoma	T3bN0M1	III	2	+++	+
48	71	m	right renal clear cell carcinoma	T3aN0M0	III	2	−	+
49	42	f	left renal clear cell carcinoma	T1aN0M0	I	1	−	−
50	68	m	left renal clear cell carcinoma	T3aN0M0	III	2	+	−
51	61	m	left renal clear cell carcinoma	T3aN0M0	III	2	+	−
52	61	m	left renal clear cell carcinoma	T3aN0M0	III	2	+	−
53	61	m	left renal clear cell carcinoma	T3aN0M0	III	2	+	−
54	72	m	left renal sarcomatoid cell carcinoma	T3aN0M0	III	3	+++	++
72	72	m	right renal sarcomatoid cell carcinoma	T4N1M0	IV	3	+++	+++
55	38	f	right renal papillary carcinoma	T4N0M1	IV	2	−	+
56	71	m	left renal sarcomatoid cell carcinoma	T1aN0M0	I	3	−	+++
73	72	m	left renal sarcomatoid cell carcinoma	T3aN0M0	III	3	+++	++
57	52	m	left renal grandular cell carcinoma	T3aN0M0	III	2	−	−
58	68	m	left renal clear cell carcinoma	T3aN0M0	III	2	+	−
59	72	m	left renal clear cell carcinoma	T3aN0M0	III	3	++	++
normal tissue							+++	−
normal tissue							+++	−
normal tissue							+++	−
normal tissue							+++	−
normal tissue							+++	−

tissue was rinsed in washing buffer (Tris-HCl 0.1 M, NaCl 0.07 M, EDTA 0.005 M, pH 7.5) and homogenized in 1 ml lysis buffer (Tris-HCL 10 mM pH 7.5, sucrose 250 mM, KCl 100 mM, MgCl 5 mM, NaF 3.8 mM, NP-40 0.5%, PMSF 0.1 mM) at $+4^0$ C. The homogenates were centrifuged for 40 min at 48,000×g at $+4^0$ C and then the supernatant was stored in small aliquots at -80^0 C. Protein concentration was determined by the Protein Assay reagent method (Pierce Biotechnology, US).

The CEM TK1 positive and TK1 negative cells were grown in DMEM medium including glutamine, 10% calf serum and antibiotics [9]. Exponentially growing CEM cells were washed (Tris-HCl 0.1 M, NaCl 0.07 M, EDTA 0.005 M, pH 7.5) and lysed in 1 ml lysing buffer (Tris-HCL 10 mM pH 7.5, sucrose 250 mM, KCl 100 mM, MgCl 5 mM, NaF 3.8 mM, NP-40 0.5%, PMSF 0.1 mM) at $+4^0$ C.

Native Page and Western-Blotting

Native page was performed at $+4^0$ C using the BioRad Mini-Protein instrument with a non-gradient gel (10% resolving, 8% stacking; both without detergents, W/V) according to the manual. Fifty five μg proteins from renal cell carcinoma and normal kidney extracts and 20 ug CEM TK1+ and CEM TK1- cell extracts were applied in each well of the native gel. Electrophoresis was performed with

the following running condition: 40 V constant 1 h and then 80 V constant 4 h. The separated proteins in the gels were transferred to PVDF membranes using BioRad semi-dry slot device. The TK1 polypeptide was identified with an anti-TK1 chicken IgY antibody raised against a peptide (residue 195–225, GQPAG PDNKE NCPVP GKPGE AVAAR KLFAPQ as described in ref 12, and they were obtained from SSTK Inc., China, (www.biosstk.com)). A competition experiment was performed to test the specificity of the immuno-reaction in the Western blot analysis by adding a 300 fold molar excess of the peptide antigen over the primary TK1 antibody.

Statistical Analysis

Chi-square test were used to calculate TK1 and Ki67 expressions in relation to tumor stage and tumor grade. A p-value less than 0.05 was considered as significant.

Results

Immunohistochemistry Studies of TK1 and Ki-67 Expression in Normal Renal Cells and in Renal Cell Carcinoma Samples

TK1 immuno-reactive material was found in the cytoplasm in a granular pattern (Fig. 1), while Ki-67 staining was observed in the nuclei (Fig. 2). Surprisingly, strong TK1 positive staining was found in all types of normal kidney tubule cells. In contrast no clearcut TK1 staining was observed in glomerulus cells and in connective tissue cells (Table 1, Fig. 1). The TK1 positive staining in tubule cells was scored as (+++) in kidneys from five healthy persons, while these cells were negative for Ki-67 expression (Table 1, Fig. 2a).

Four types of RCC were studied and several were TK1 positive (Table 1, Fig. 1 a), but the intensity of the staining was lower in RCC as compared to normal tubule cells (Fig. 1a). The number of TK1 positive RCC ranged from no staining (−) in six clear cell samples and in one each of a papillary, a sarcomatoid and a grandular cell samples, to plus one (+) in eight clear samples; plus three (+++) in five clear cell samples, in three sarcomatoid cell samples and in one papillary cell sample (Table 1). These renal carcinoma cells were in many cases also Ki-67 positive (Table 1, Fig. 2b), to about the same extent as they were TK1 positive.

TK1 and Ki-67 Expression in Relation to Tumor Stage and Grade

In this immuno-histochemical investigation, both TK1 and Ki-67 correlated to stage and grade (Table 2). No difference

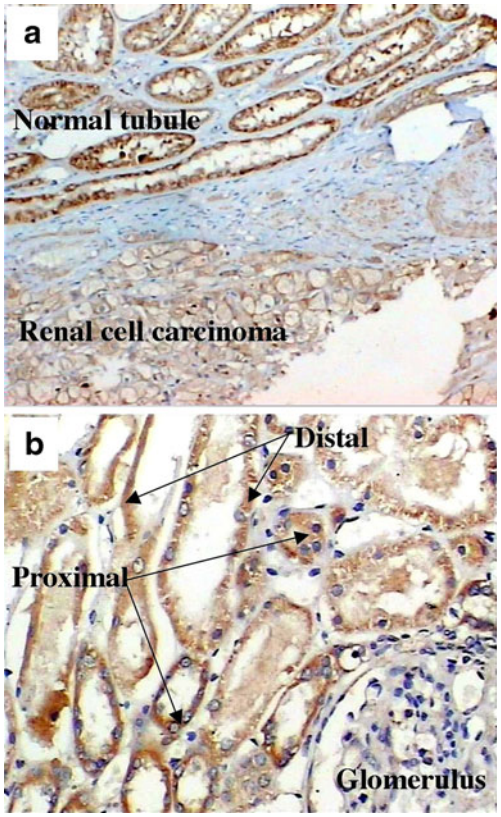

Fig. 1 TK1 expression in the boundary of normal and tumor tissues. **a** High TK1 staining of the normal kidney tubule cells as compared to low TK1 staining of renal clear carcinoma; **b** High TK1 staining in distal, proximal tubule cells, and no or low TK1 staining of glomerulus cells. Magnification×200

in the number of TK1 positive cells (66.7%) and the number of Ki-67 positive cells (59.3%,) was found (Table 1). Neither was there significant difference between TK1 and Ki-67 staining as related to different tumor grades (Table 2). However, there was a tendency (p=0.083) of higher number of TK1 positivity in patients of stage III (pos/neg=14/4, 77.8%), as compared to positivity for Ki-67 (pos/neg=9/9, 50.0%) (Table 2). Thus, TK1 is apparently as useful as Ki-67 for determination of proliferation in renal cell carcinoma.

Levels of TK1 Protein in Normal Kidney and in Renal Carcinoma Extracts

In order to confirm the identity of the high TK1 protein level in normal kidney tubule cells (see Fig. 1) a Western blot experiments were performed with samples from normal kidney and RCC. The TK1 band, migrating at the known position of human TK1 tetramer [12], was stained significantly stronger in extracts from normal kidney tissue, as compared to extracts from renal cell carcinoma samples (Fig. 3). To exclude false positive immuno-reactivity,

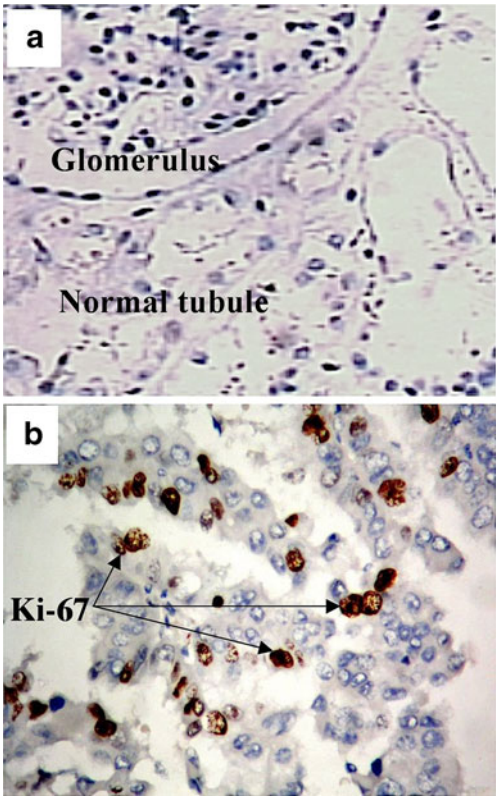

Fig. 2 Ki-67 staining of normal kidney cells **a** and of renal clear carcinoma cells **b**. Magnification×400. While Ki-67 staining was found in the renal clear carcinoma cells (brown color), no KI-67 staining was found in the normal kidney tissue

sections of normal kidney tissues were stained with TK1 antibody in the absence (Fig. 4a) or presence (Fig. 4b) of an excess of peptide antigen. No staining in the section was observed in the presence of the peptide. Similar results were obtained in a competition experiments in the Western blot analysis (Fig. 3).

The TK1 antibody used in the Western blot experiments were produced against a C-terminal epitop (residue 195–225) different from the one used in the immunohistochemistry experiments (residue 161–183). The results shown

Table 2 Number of patient samples that are TK1 and Ki-67 positive in relation to stage and grade of the renal cell tumours

	TK1	TK1	Ki-67	Ki-67	Chi-square
Stage	Neg	Pos	Neg	Pos	*p*-value
I	4	3	2	5	0.280
III	4	14	9	9	0.083
IV	1	1	0	2	0.083
Grade	Neg	Pos	Neg	Pos	
1	2	3	2	3	1.000
2	6	10	9	7	0.288
3	1	5	0	6	0.231

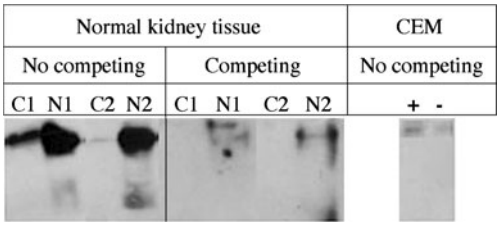

Fig. 3 Two independent Western blot experiments with normal kidney tissue (N1, N2) and renal clear cell carcinoma (C1) and papillary renal cell carcinoma (C2) extracts. In the competition experiment, peptide used for the production of the TK1 antibody was added. Cell extracts from CEM TK1+ and CEM TK1- cells run as control of the migration rate of TK1 polypeptide in the gel

here and elsewhere [11, 12] demonstrate that both antibodies are specific for TK1 protein and give similar results in Western blotting and immunohistochemistry studies.

Discussion

In this study higher TK1 protein levels were found in tubular cells of the normal kidney, as compared to other normal kidney cells (glomerulus, connective tissue) and to

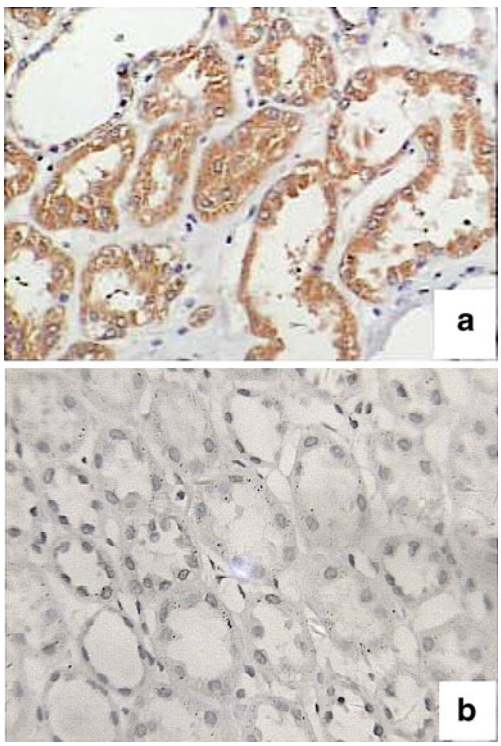

Fig. 4 Immunohistochemical staining with TK1 antibody in the absence **a** or presence **b** of the peptide used for production of the antibody (active site epitope) (competition experiment). The staining of TK1 is shown by the brown color in the cytoplasma of the tubular cells (see figure **a**). In the presence of the peptide no TK1 staining was found (Figure **b**)

renal cell carcinoma cells. These results were confirmed by Western blot experiments. The differences were observed by using two different types of anti-TK1 antibodies, one generated against an active site epitope (residues 161–183) in the immuno-histochemical study and another, specific for the C-terminal region (residue 195–225) [11, 12] used in the Western blot experiments. Thus, the present study supports the observation that TK1 is expressed at higher levels in normal kidney cells as compare to renal cell carcinoma cells [3].

The clear renal cell carcinoma account for 90–95% of all renal carcinomas [13], and the growth rate of this tumor is about 0,5 to 1 cm/year, with a doubling time of about 650 days [13]. Thus, clear cell carcinomas are slow growing tumors. This fact is in accordance with our finding that 74% of the clear cell carcinoma samples showed low TK1 levels, while among the other types of renal carcinomas 43% had low TK1 levels.

TK1 is part of the salvage pathway of deoxynucleotide metabolism, and thus closely linked to cell proliferation. TK1 activity was suggested to be a good proliferation biomarker already 1960 and serum TK1 activity determinations have been used both for prognosis and monitoring the effect of tumor therapy since 1980´s [5, 14 and references there in]. More recently immuno-histochemical screening of malignancy with TK1 antibodies has also been performed [6, 15, 16]. In the present study we observed that TK1 unexpectedly was highly expressed in normal non-proliferating kidney tubule cells. In accordance with this finding, recent results of Vogetseder et al [7] clearly demonstrated that the bulk of the proximal tubule cells, especially section S3 of rat tubule have high proliferative capacity. Upon mitotic stimulation (e. g. by exposure to lead acetate) the proximal tubule cells can enter the division cycle. This study also showed that about 30% of the bulk proximal tubule cells were in G1 stage of the cell cycle (cyclin D1 positive cells), i.e. already in an activated G1 stage ready to re-enter into the S-phase when needed. This is a confirmation of a previous study using thymidine analog incorporation, demonstrating that new cells in the proximal tubule arise from differentiated normally slow-cycling cells [8]. In a recent study on normal epithelial cells we found that the expression of TK1 started to increase in G1, probably just after the restriction point [11], and not at the boarder of G1/S, as has been stated before. The results from the present study indicate that tubule cells are in a proliferation active state, which likely corresponds to late G1 with high level of TK1 [11, 17, 18].

Vogetseder et al [7] argue that the exceptional proliferation potential of the tubule S3 cells is a mechanism for replacement of injured tubule cells in the kidney. However, other types of the normal kidney cells did not show this proliferation capacity, not even the epithelial cells of section S1 and S2 of the proximal tubule. Here we describe that the DNA precursor enzyme TK1 is high in almost all tubule cells, and it may be an important factor in the potential of these cells to rapidly repair renal injuries.

Studies of TK1 expression in tubule cells may also help to define a new type of cell cycle phase, pre-S-phase arrested differentiated cells.

The origin for clear cell carcinoma is most probably the proximal renal tubule epithelium, although other type of tissues has been discussed [19]. Loss of chromosomes 3p and 17p and VHL and p53 mutations are furthermore associated with clear cell carcinoma development. These changes might be involved in the apparent down regulation of TK1 expression observed in RCC, as compared to normal tubule cells.

The status of the TK1 in the tubule cells with regard to enzyme activity is not known, since there seems to be a discrepancy between the high protein levels and the relatively low TK1 activity observed in extracts of normal and malignant renal specimens [1–3]. However, there are to our knowledge no TK1 activity measurements reported with isolated renal tubule cells and at present we do not know if this TK1 protein is fully active. In the case when tubule cells contain high levels of active TK1 it could lead to increased dTTP pool which is a potentially mutagenic situation, as has been demonstrated by Chang C-F, et al [20]. Thus, besides the mutation of VHL and p53, there could also be a connection between high TK1 expression in the tubule cells and the development of renal cell carcinoma, through unbalanced nucleotide pools.

The high staining level of TK1 in normal tubule kidney cells may be a concern in the use of TK1 as a proliferation marker in patients with renal cell carcinoma. However, since the morphology of normal tubule cells and renal carcinoma cells is clearly different, it should not be a major problem to use TK1 as a proliferation marker in renal malignancies. Recently staining of human normal renal tissue has been performed with TK1 antibodies of unknown epitope specificity as part of the human protein atlas project (www.humanproteinatlas.com). In this case no over-expression of TK1 in normal tubular cells was reported. The reason1 for the discrepancy between their results and those described here is not known.

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a stock owner and scientific officer of Arocell AB, Uppsala, Sweden, which produce one of the TK1 antibody used in this study. N Wang owns stocks in Arocell AB. This investigation was supported by TK1 kits by SSTK Inc., Shenzhen, China.

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