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ORIGINAL ARTICLE

Protein tyrosine phosphatase nonreceptor type 12 suppresses the proliferation of renal cell carcinoma by inhibiting the activity of the PI3K/mTOR pathway

Yong Rui Piao[1], Zhehu Jin[2]

[1]Department of Urology and [2]Department of Dermatology and Venereology, Affiliated Hospital of Yanbian University, Yanji, 133000, China

Summary

Purpose: *To determine the expression and functions of protein tyrosine phosphatase nonreceptor type 12 (PTPN12) in renal cell carcinoma (RCC).*

Methods: *All RCC tissue and corresponding normal kidney tissue from 116 RCC patients undergoing radical nephrectomy were examined. PTPN12 expression was detected by immunohistochemistry, and PTPN12 mRNA expression by real-time polymerase chain reaction (RT-PCR). PTPN12 expression was increased by stable transfection with a pcDEF3 vector containing full-length cDNA of PTPN12 and was decreased by RNAi in 4 RCC cell lines. Proliferative analysis of RCC cells was done using a WST-1 assay and animal xenograft study.*

Results: *PTPN12 expression in RCC tissue was significantly decreased compared with normal kidney tissue, and was overexpressed in larger tumors, metastasis, and high pathological grades.*

Conclusions: *PTPN12 expression decreases in human RCC, it is involved in progression and metastasis, and is an independent prognostic factor in RCC. Restoring PTPN12 activity could be a new therapeutic approach in advanced RCC.*

Key words: *carcinoma, clear-cell renal carcinoma, neoplasm metastasis, renal cell, tumor suppressor genes, tyrosine kinase inhibitor*

Introduction

RCC is the most frequent malignancy in adult kidneys and accounts for about 85-95% of kidney cancer cases [1]. Although localized clear cell renal carcinoma (CCRC) is considered a surgical disease, 40% of those who present with localized stage will experience metastatic disease [2]. No satisfactory treatment has been established for metastatic RCC because of its high resistance to conventional chemotherapy [3,4]. In addition, the response rates of immunochemical therapies, such as chemotherapy combined with interferon-α (INF-α) or with interleukin-2 (IL-2) were 2-39% [5,6]. However, strategies for metastatic RCC have developed dramatically in recent years, and drugs that inhibit mTOR signaling and vascular endothelial growth factor receptor tyrosine kinase inhibitors have become the mainstay for the management of this disease [7]. Although a number of genes has been characterised as potential therapeutic targets and outcome predictors for RCC [8,9], the molecular mechanisms in the development and progression of RCC remain disputable.

Protein phosphorylation and dephosphorylation are controlled by various protein kinases and phosphatases. The regulation of protein activity plays an important role in signaling transduction and tumorigenesis. Protein tyrosine phosphatases (PTPs) are inhibitors to tyrosine kinase signaling, although they are reported to play also a role in

Correspondence to: Zhehu Jin, MD. Department of Dermatology and Venereology, Affiliated Hospital of Yanbian University, 1327 Ju'Zi Street, Yanji, Jilin, 133000, China. Tel: +86 0433 2660075, Fax: +86 0433 2513610, E-mail: yrpiao@126.com
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cellular physiology and tumorigenesis [10,11]. PTPs usually serve as crucial proteins in tumor suppression [12]. Protein tyrosine phosphatase nonreceptor type 12 (PTPN12) gene is located in 7q11.23 and is a member of the PTP family [13]. Currently, decreased expression of PTPN12 has been found in various human malignancies (e.g. colon cancer, esophageal squamous cell cancer) [14,15]. PTPN12 is currently identified as a tumor suppressor gene and is associated with tumorigenesis in human breast cancer [16]. Another study analyzed the molecular mechanism of PTPN12 in breast cancer, and the results indicated that decreased expression of PTPN12 increased HER2/EGFR activity in breast cancer cells and the authors concluded that PTPN12 is a potential tumor suppressor gene of this cancer [17].

Several studies also confirmed that PTPN12 is ubiquitously expressed in the cytoplasm and is reported to suppress various oncogenic tyrosine kinases and serves as an important regulator, showing proto-oncogene activities, to induce cell adhesion, migration and invasion [18-20]. In addition, decreased expression of PTPN12 could lead to the progression of human malignancies (eg, suppressing PTPN12 expression could prompt proliferation in colon cancer cells [21] and ovarian cancer cells) [13]. However, the effect of PTPN12 on the proliferation and prognosis of RCC remains unclear, and because advanced RCC has a poor prognosis, the expression of PTPN12 and its role in RCC should be well-elucidated.

In this study, we examined the expression of PTPN12 and analyzed its effect on the proliferation and prognosis in human RCC.

Methods

Patients and experimental samples

Our study included 116 patients with RCC who underwent radical nephrectomy at the Department of Urology, Affiliated Hospital of YanBian University Hospital. Histological cell type of all specimens was evaluated by an experienced pathologist, and all specimens in this study were confirmed to be conventional CCRC. The clinical tumor stages were classified according to the TNM classification system, and the nuclear grades were evaluated according to the Fuhrman grading system of malignant tumors. All RCC tissues and corresponding normal kidney tissues located as far as possible from the tumor were formalin-fixed, dehydrated, and paraffin-embedded. In addition, all tissue samples were also gathered immediately after surgical resection and kept in liquid nitrogen until carrying out mRNA and protein extraction. Our study was approved by the ethics committee of the Affiliated Hospital of YanBian University Hospital.

Cell culture

Four RCC cell lines, ACHN, NC 65, Caki-1 and Caki-2, were used in this study. All RCC cell lines were incubated using a complete medium consisting of RPMI-1640 medium (Gibco, Glasgow, Scotland, UK), 1% nonessential amino acids, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 100 µg/mL streptomycin or 100 units/mL penicillin. All RCC cell lines were cultured as a monolayer in a 10-cm plastic dish and incubated in a humidified atmosphere at 37°C containing 5% CO_2.

Immunohistochemistry

Four-µm paraffin slices of RCC and corresponding normal kidney were deparaffinized with xylene and then rehydrated with graded alcohol. Endogenous peroxidase activity was sufficiently inhibited with 0.3% hydrogen peroxide for 40 min, and all slices were also blocked with 10% rabbit serum for 30 min. Subsequently, slices were incubated with rabbit polyclonal antibody of PTPN12 (Abcam, Cambridge, MA) for 3 hrs. All slices were then washed three times with Tris-buffered saline and incubated with biotinylated anti-rabbit antibody (DAKO, Glostrup, Denmark) overnight. Determinations of antibody reaction were performed using a streptavidin-biotin complex, and the results of immunohistochemistry were analyzed with a light microscope. The immunostaining of PTPN12 was semiquantitatively evaluated for its positive intensity (negative: -; weak: +; moderate: ++; strong: +++).

Western blot

Protein expression was detected using Western blot analysis. The procedures were performed according to those described elsewhere [21], except that 0.2 mM Na_3VO_4, 50 mM NaF, 1 mM dithiothreitol and 5.7 µg/ml aprotinin were included in the lysis buffer. Protein concentration was examined by Bradford dye-binding protein assay (Bio-Rad, Richmond, CA, USA), then SDS polyacrylamide gel electrophoresis was carried out according to the manufacturer's instructions. Both rabbit polyclonal antibody of PTPN12 and anti-β-actin monoclonal antibody were purchased from Abcam, Cambridge, UK. All other antibodies were purchased from Cell Signaling Technology: PI3K p85 (19H8)/phospho-PI3K p85 (Tyr458) mAb; Akt (11E7)/phospho-Akt (Ser473) mAb; mTOR (7C10)/phospho-mTOR (Ser2481) mAb; p70 S6K (49D7)/phospho-p70 S6K (Thr421) Rabbit mAb, S6 Ribosomal Protein (5G10)/phospho-S6 Ribosomal Protein (Ser240) mAb. Finally, the immune complex was detected using an enhanced chemiluminiscence system (Amersham, Aylesbury, UK).

RT- and real-time polymerase chain reaction

Total RNA was extracted from RCC and normal

kidney tissues with the Quick PrepmRNA purification kit (GE Healthcare, Buckinghamshire, UK). First-strand cDNA was synthetized using a synthesis kit (Amersham Biosciences, Little Chalfont, UK) for reverse transcription according to the manufacturer's instructions. RT-PCR was performed according to the manufacturer's instructions, and the PCR products were assessed using agarose gel electrophoresis. Moreover, RT-PCR was also carried out using LC FastStart DNA Master

SYBR Green I (Roche, Indianapolis, USA), and the PCR products were quantified with Light Cycler (Roche, Indianapolis, USA). The primer sequences of the human PTPN12 were designed using Primer Premier 5.0. The primer sequences were sense: 5'- AATACTGCAGCCACCGGAA-3', and antisense: 5'-CAACACTGGCTTTGGATG-3'. In addition, GAPDH was used as an internal control, and the primer sequences were sense: 5'-TCAAGAAGGTGGTGAAGCAG-3', and antisense: 5'-GTGGAGGAGTGGGTGTC-3'.

RNAi and transfection

siRNA oligonucleotide sequences for PTPN12 were designed using siDirect software. RCC cells were seeded in a culture dish and cultured with complete medium (not containing antibiotics) until the cell confluence reached 60%, then RCC cells were transiently transfected with siRNA oligonucleotides with lipofectamine 2000 (Invitrogen, CA, USA). After continuous incubation for 2 days, the expression of PTPN12 in RCC cells was detected by Western blot. Moreover, the human cDNA coding sequence of PTPN12 was also cloned using RT-PCR from normal kidney tissue as a substrate, and the PCR products were subcloned into a pcDEF3 vector. Then 4 RCC cell lines were stably transfected with pcDEF3 vector containing full-length cDNA of PTPN12 by lipofectamine 2000. Finally, the monoclonal RCC cell lines were selected with G418, and the expression PTPN12 was detected by Western blot.

Proliferation analysis of RCC cells

WST-1 assay was used to investigate the effect of PTPN12 on the proliferative ability of RCC cells. 5×10^3 RCC cells were harvested and seeded into a 96-well microtiter plate. After continuous incubation for 24, 48, and 72 hrs, 10 µl WST-1 (Roche, Penzberg, Germany) were added into each well after incubation, which was continued for another 3 hrs. Finally, the absorbance of each well, representing RCC cell count, was detected by microculture plate reader (Immunoreader, Tokyo, Japan) at 450 nm.

Animal xenograft experiment

Forty BALB/C nude mice (3-5 weeks old) were randomly divided into the following two groups: (1) PTPN12 vector group and (2) a control group receiving no treatment. A total of 6×10^8 RCC cells were injected into the back region of each nude mouse, and then all mice were fed for 5 weeks. The volume of each RCC xenograft was measured once a week. Five weeks later, all mice were sacrificed under deep anesthesia, and the final volume of each RCC xenograft was recorded.

Statistics

All statistical calculations in our study were performed with SPSS version 16.0. All calculations were carried out in triplicate, and the results were presented as mean±standard deviation. Statistical analysis was carried out by Student's t-test, and the x^2 test was also used to investigate the correlation between PTPN12 expression and the clinical characteristics. Survival curves were plotted by Kaplan-Meier analysis and a p value of 0.05 or less was considered statistically significant.

Results

Clinical characteristics of RCC patients

Data from 68 male and 485 female patients were obtained for this study. The patient age ranged from 42 to 83 years (median 62), and the tumor size from 1.2 to 18.4 cm (median 3.9).

From 116 RCC patients, 65 (56%) had stage I disease, 25 (21.6%) stage II, 15 (12.9%) stage III, and 11 (9.5%) stage IV. Fifty-two (44.8%) patients had Fuhrman grade 1 cancer, 36 (31%) grade 2, and 28 (24.1%) grade 3. Ninety-five (81.9%) patients had localized RCC, and 21 (18.1%) had lymph node or distant metastasis. Malignancy was an incidental finding on routine examination in 63 (54.3%) patients. Presenting symptoms included flank pain (17 patients; 44.7%), hematuria (9 patients; 7.8%) and palpable mass (5 patients; 4.3%). Laboratory examination revealed elevated sedimentation rate in 29 (25%) patients, while anemia, erythrocytosis, and thrombocytopenia were found in 5, 7, and 6 patients, respectively. Eighteen patients (15.5%) had one or more concurrent diseases, including urolithiasis, diabetes mellitus, and angina pectoris at diagnosis. Three (2.6%) patients had been treated with radical nephrectomy on the other side.

PTPN12 expression in RCC

PTPN12 protein expression in human normal kidney and RCC tissues was detected by immunohistochemistry. The results indicated that the expression of PTPN12 in RCC was significantly decreased compared with that in normal kidney tissue (Figure 1). PTPN12 expression was detected in 80/116 (69%) patients with RCC, of which 23.3% had median and strong PTPN12 expres-

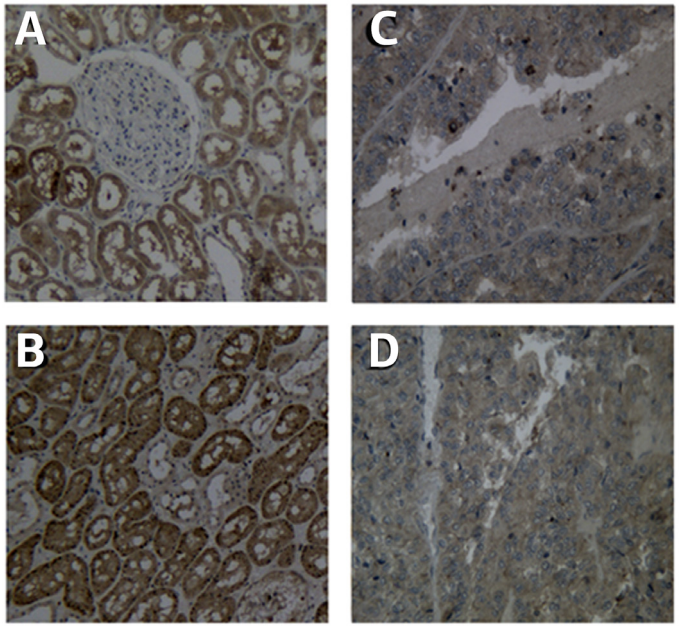

Figure 1. PTPN12 is low-expressed in RCC tissues. Immunohistochemical analysis of two paired renal tissue samples, indicating high PTPN12 expression in normal kidney tissue obtained from a stage T2N0M0 patient **(A)** and a stage T1N0M0 patient **(B),** and low PTPN12 expression in the corresponding RCC tissues of a stage T2N0M0 patient **(C)** and a stage T1N0M0 patient **(D).**

Table 1. Characteristics of renal cell carcinoma patients and protein tyrosine phosphatase nonreceptor type 12 expression detected by immunohistochemistry and real-time polymerase chain reaction

	N	*PTPN12 mRNA (mean±SD)*	*p value*	*PTPN12*				*p value*
				-	+	++	+++	
Renal cell carcinoma	116	2.15±0.26		36	53	18	9	
Normal kidney tissue	116	5.42±0.65	<0.05	5	10	59	42	<0.05
Sex								
Male	68	2.16±0.29		20	33	10	5	
Female	48	2.13±0.28	>0.05	16	20	8	4	>0.05
Age (years)								
<60	59	2.18±0.22		19	27	9	4	
≥60	57	2.12±0.26	>0.05	17	26	9	5	>0.05
Tumor size (cm)								
<7	65	2.85±0.31		17	26	15	7	
≥7	51	1.26±0.19	<0.05	19	27	3	2	<0.05
Histologic grade								
G1	52	2.97±0.32		11	23	11	7	
G2	36	1.67±0.18		13	16	5	2	
G3	28	1.26±0.14	<0.05	12	14	2	0	<0.05
Tumor stage								
I	65	2.85±0.31		17	26	15	7	
II	25	1.54±0.18		9	13	2	1	
III	15	1.16±0.14		4	9	1	1	
IV	11	0.77±0.11	<0.05	6	6	0	0	<0.05
Local or metastasized								
Local	95	2.39±0.29		27	42	17	9	
Metastasized	21	1.07±0.17	<0.05	9	11	1	0	<0.05

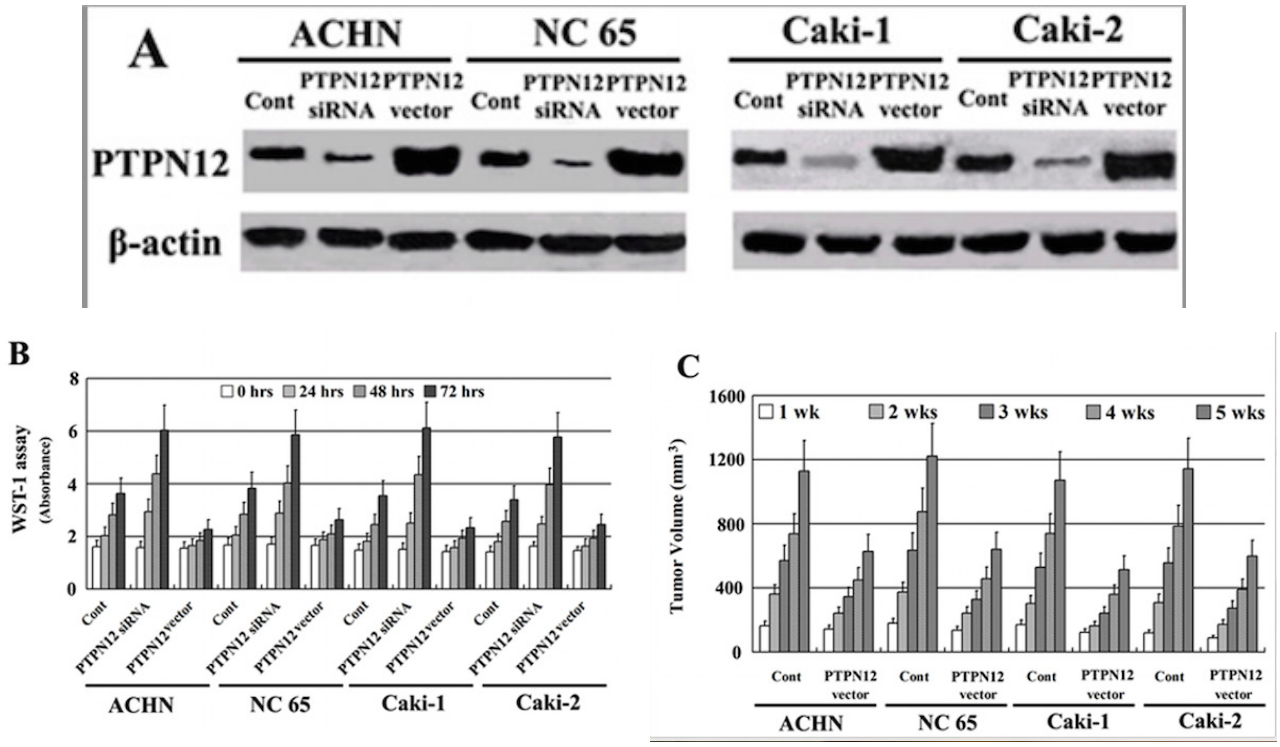

Figure 2. The effect of protein tyrosine phosphatase non-receptor type 12 (PTPN12) on the proliferation of renal cell carcinoma (RCC). pcDEF3 vector of PTPN12 was stably transfected into RCC cell lines, and PTPN12 expression was also decreased using RNAi. All transfections were confirmed by Western blot analysis **(A)**. The proliferation of RCC cells in vitro was investigated by WST-1 assay **(B)** and animal xenograft with BALB/C nude mice **(C)**. All experiments were performed in triplicate, and the error bar represents standard deviation.

sion. However, PTPN12 expression was detected in 111/116 (95.7%) patients in normal tissue (p=0.012). In addition, 87.1% of normal renal tissue showed median and strong PTPN12 expression. Regarding the correlation between PTPN12 expression and clinical characteristics, PTPN12 expression was negatively associated with tumor size, pathological grade, tumor stage, and tumor metastasis by x^2 analysis. No other variable (eg, sex, age) indicated a significant correlation with PTPN12 expression (Table 1). PTPN12 expression in normal kidney and RCC tissues was also confirmed by RT-PCR, and the relative level of PTPN12 expression was presented with correction to the expression of GAPDH. These findings demonstrated that decreased PTPN12 protein expression may be involved in the tumorigenesis of human RCC.

Decreased PTPN12 expression enhances the proliferation of RCC cells

Expression pcDEF3 vector containing full-length cDNA for PTPN12 was stably transfected into RCC cell lines (ACHN, NC 65, Caki-1, and Caki-2), and PTPN12 expression was also down-regulated using RNAi. After transfection, PTPN12 protein expression was confirmed by Western blot analysis. PTPN12 expression was significantly increased ($p<0.05$) by the PTPN12 vector insert and decreased significantly by RNAi in all RCC cell lines, respectively ($p<0.05$) (Figure 2A). The role of PTPN12 in the proliferation of RCC cells *in vitro* was detected by WST-1 assay. The results indicated that RCC cells with high level of PTPN12 expression exhibited significantly decreased proliferative activity than untreated RCC cells ($p<0.05$). However, RCC cells with low level of PTPN12 expression had higher proliferative activity ($p<0.05$) (Figure 2B). The effect of PTPN12 on the proliferation of RCC cells in vivo was also investigated by the animal xenograft study with BALB/C nude mice, and the same results were observed (Figure 2C). These findings suggest that decreased PTPN12 activity may be involved in proliferation in human RCC.

PTPN12 suppresses PI3K/mTOR activity in RCC cells

To clarify how the PI3K/mTOR pathway is associated with PTPN12-induced antiproliferation in RCC cells, the phosphorylation of the PI3K/mTOR pathway was assessed. PTPN12 expression was increased through transfection with a pcDEF3

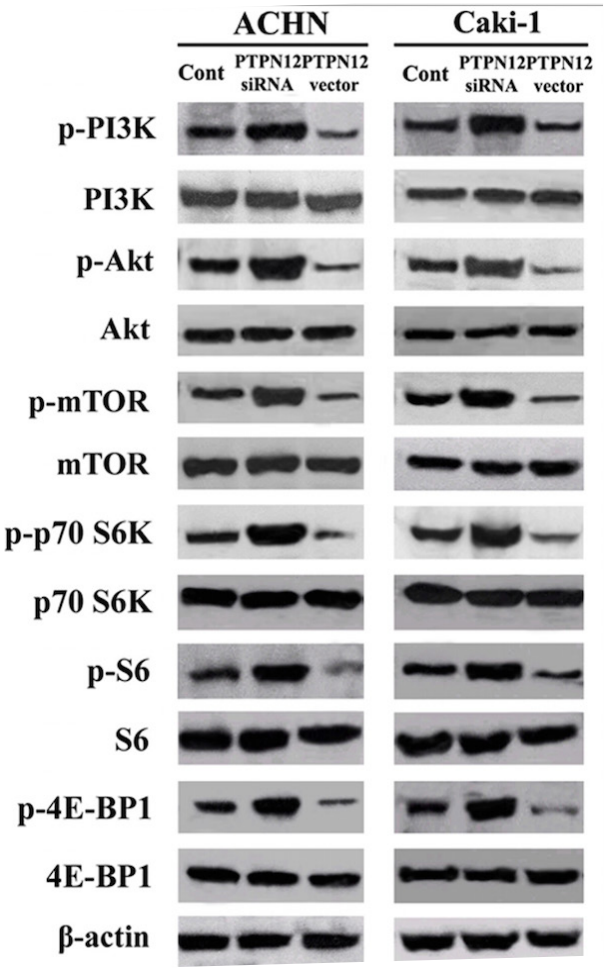

Figure 3. PTPN12 suppresses the activity of the P13K/mTOR pathway as shown by Western blot. In two renal cell carcinoma lines, although protein tyrosine phosphatase non-receptor type 12 (PTPN12) did not affect the total protein expression of P13K/Akt and mTOR/p70 S6K/S6/4E-BP1, the increased expression of PTPN12 suppressed the phosphorylation of components in the P13/mTOR pathway. However, it decreased the expression of PTPN12 and enhanced the phosphorylation of the P13K/mTOR pathway.

vector containing full-length cDNA for PTPN12, and PTPN12 expression was also downregulated using RNAi. In two RCC cell lines, although PTPN12 activity did not affect the protein expression of PI3K/Akt and mTOR/p70 S6K/S6/4E-BP1, increased expression of PTPN12 downregulated significantly the phosphorylation of PI3K/Akt, mTOR/p70 S6K/S6/4E-BP1 as shown by Western blot. In contrast, decreased expression of PTPN12 upregulated significantly the phosphorylation of PI3K/mTOR (Figure 3). These findings suggest that the PI3K/mTOR pathway could be negatively regulated by PTPN12 and plays an important role in PTPN12-induced antiproliferation in RCC cells.

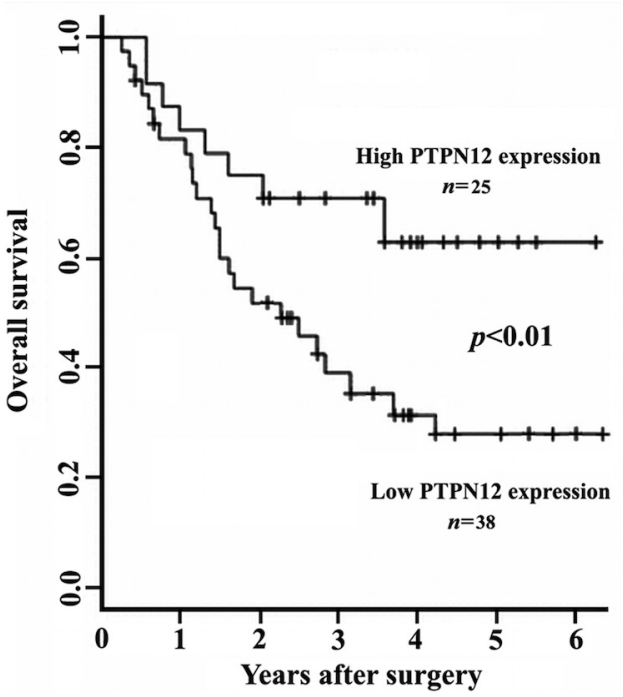

Figure 4. Kaplan-Meier analysis was performed to analyse the correlation of protein tyrosine phosphatase non receptor type 12 (PTPN12) expression and survival of patients with renal cell carcinoma (RCC). Decreased expression of PTPN12 was associated with poor prognosis in patients with RCC, and PTPN12 maybe an independent marker for predicting prognosis of human RCC.

Prognostic significance of PTPN12 expression in RCC

Because a correlation between PTPN12 expression, clinical stage, and histologic grade in RCC was shown, we analyzed whether PTPN12 could be regarded as a prognostic factor in human RCC. Kaplan-Meier analysis was performed to calculate the association of PTPN12 expression and survival in all RCC patients. The results indicated that overall survival time was significantly different between the high PTPN12 expression group and the low PTPN12 expression group (Figure 4). After 6.5 years of follow up, 17/27 (63%) patients who exhibited high PTPN12 expression on immunohistochemistry (++ and +++) were alive and disease- free, compared with only 24/89 (27%) patients in whom immunohistochemical staining demonstrated low PTPN12 expression (- and +) (p=0.03). These findings indicated that PTPN12 expression may be an independent factor in predicting prognosis of human RCC.

Discussion

RCC is the most common malignancy of the kidney and is insensitive to chemotherapy. No agents are available to control advanced RCC, and a novel molecular mechanism and therapeutic target for treating patients with RCC is still desperately needed. In recent years, PTPN12 has been regarded as a tumor suppressor gene in tumorigenesis. In a study, PTPN12 expression was significantly downregulated in hepatocellular carcinoma (HCC) tissue than in the normal liver tissue, and was an independent predictor of shortened cancer-specific and recurrence-free survival for patients with HCC [22]. In addition, loss of PTPN12 activity will cause cell transformation in mammary epithelial cells and aberrant acinar morphogenesis in breast cancer [17]. Decreased expression of PTPN12 was also observed in breast cancer and was associated with tumorigenesis and poor prognosis [16]. Although several studies have indicated that PTPN12 is an important gene in human tumors, the role of PTPN12 in human RCC remains unclear; the effect of PTPN12 activity on the progression and prognosis of RCC needs to be further elucidated. To our knowledge, this is the first study concerning the functions of PTPN12 in human RCC. In this study, we detected the expression of PTPN12 in human RCC, and our results demonstrated that PTPN12 expression is significantly suppressed in RCC tissues compared with that in normal kidney tissues, as shown by immunohistochemistry and RT-PCR. Moreover, PTPN12 expression was significantly related to tumor size, pathological grade, clinical stage, and metastasis.

These findings demonstrated that decreased PTPN12 expression may be involved in the tumorigenesis of human RCC. We also further investigated the effect of PTPN12 on the proliferative ability of RCC cells and found that decreased expression of PTPN12 could significantly enhance the proliferation of RCC cells *in vitro*. Similar results were also observed in an animal xenograft study with BALB/C nude mice. In this study, the association between PTPN12 expression and survival in human RCC was also investigated by Kaplan- Meier analysis. The results suggested that decreased expression of PTPN12 could be a valuable marker for prediction of poor prognosis of patients with RCC and that PTPN12 could be used as an independent prognosticator and guide the follow up schedule in patients with RCC. These results suggest that PTPN12 plays a key role in the progression and prognosis of human RCC.

Although massive efforts have been carried out to understand the increasing prevalence of RCC cases, the mechanism of tumor progression and metastasis remains obscure. PTPN12 is an important gene in regulating cell adhesion, motility, and invasion by suppressing multiple oncogenic tyrosine kinases [18]. It is well known that promoter hypermethylation is an important molecular mechanism in gene silencing of tumor suppressor genes; one study reported that hypermethylation in the promoter CpG region is frequently found and associated with decreased expression of PTPN12, so hypermethylation is a potential regulator of PTPN12 expression [16]. On the other hand, the molecular mechanism of PTPN12 in malignancy is also unclear. A recent study indicated that PTPN12 could regulate Rho GTPase activity and suppress epithelial cell motility in colon cancer [14]. Another study also suggested that PTPN12 suppresses cell motility through the regulation of PI3K/Akt activity in ovarian cancer cells [13]. PI3K phosphorylates primarily phosphatidylinositol-4, 5- bisphosphate, which could generate phosphatidylinositol-3, 4, 5-trisphosphate. Akt is a target protein of PI3K and phosphorylates downstream mediators such as Bad, FOXO, MDM2, GSK3, and GSK3β to enhance cell survival and proliferation [23]. Activation of the PI3K-Akt pathway also increases the phosphorylation of mTOR, and p-mTOR increases the phosphorylation of p70 S6K/S6, 4E-BP1/eIF-4E and facilitates cell proliferation [24]. A study reported that p-mTOR and p-S6 are also strongly co-expressed in RCC [25]. Furthermore, the activity of PI3K-Akt-mTOR pathway in RCC is significantly higher than that in normal kidney tissue [26]. Recently, the inhibitor of mTOR activity has emerged as a new targeted agent and treatment strategy for advanced RCC [27,28]. Although the expression of PTPN12 and its functions in RCC have been analyzed in this study, the underlying molecular mechanism of PTPN12 in RCC remains unknown and should be further investigated. Our results suggest that, although PTPN12 did not alter the protein expression of components in PI3K/mTOR pathways, PTPN12 decreased the phosphorylation of PI3K/Akt, mTOR/p70S6K/S6/4E-BP1 in RCC cells. These findings suggest that the PI3K/mTOR pathway is a potential target of PTPN12 and plays an important role in the PTPN12- induced proliferation of RCC cells.

In summary, our study indicated that PTPN12 expression is decreased in human RCC and may cause progression and metastasis of this disease. Moreover, it is an intriguing possibility that RCC

patients with decreased expression of PTPN12 may be vulnerable to development and metastasis of RCC. PTPN12 maybe an independent predictive factor for the RCC prognosis. Our study also suggested that restoring PTPN12 expression may prove to be a novel therapeutic strategy for patients with advanced RCC.

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