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Tumor Protein D52 (TPD52) Inhibits Growth and Metastasis in Renal Cell Carcinoma Cells Through the PI3K/Akt Signaling Pathway

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Tumor protein D52 (TPD52) is a member of the TPD52-like protein family and plays different roles in various types of malignancies. However, its role in renal cell carcinoma (RCC) is still unclear. In this study, we investigated the role of TPD52 in RCC. The mechanism of TPD52 in RCC was also investigated. Our data demonstrated that the expression levels of TPD52 in both mRNA and protein were significantly decreased in RCC cells. Overexpression of TPD52 inhibited proliferation, migration, and invasion with decreased epithelial–mesenchymal transition (EMT) phenotype in RCC cells, as well as attenuated tumor growth in renal cancer xenografts. Mechanistically, overexpression of TPD52 significantly inhibited downregulated phosphorylation levels of PI3K and Akt in RCC cells. In conclusion, the present study demonstrated that TPD52 inhibited growth and metastasis of RCC, at least in part, by suppressing the PI3K/Akt signaling pathway. Therefore, these findings suggest that TPD52 may be a promising therapeutic target for the treatment of human RCC.

Key words: Renal cell carcinoma (RCC); Tumor protein D52 (TPD52); Proliferation; Invasion

INTRODUCTION

Renal cell carcinoma (RCC), accounting for approximately 3% of all malignancies, is the major cancer type in the kidneys. The incidence of RCC is increasing by a rate of approximately 2.5% each year[1,2]. About 30% of RCC patients have developed metastasis at the time of diagnosis[3]. Currently, the main therapy is a radical nephrectomy[4]. However, the 5-year survival rate in patients with advanced-stage RCC is poor (5%–10%) due to recurrence or distant metastasis[5]. Thus, there is an urgent need to further understand the molecular mechanisms underlying the process responsible for the development of RCC.

Tumor protein D52 (TPD52) is a member of the TPD52-like protein family, which are coiled-coil motif-bearing small hydrophilic polypeptides conserved from lower organisms to humans[6]. Previous studies showed that TPD52 plays a critical role in membrane trafficking[7–9]. Aside from the effects of TPD52 on membrane trafficking, a growing body of evidence suggests that TPD52 is also involved in the development and progression of malignancies. TPD52 was found to be highly expressed in several tumors such as ovarian, breast, oral squamous cell carcinoma, and melanoma[10–13]. However, several studies have revealed that the expression of TPD52 was decreased in liposarcoma, clear cell renal cell cancer, and lung cancer[14,15]. The dual roles of TPD52 could be attributed to organ-specific actions and different cellular contexts. Recently, TPD52 was found to be expressed in low amounts in RCC tissues[14]. However, its role in RCC is still unclear. In this study, we investigated the role of TPD52 in RCC. The mechanism of TPD52 in RCC was also investigated. Our results demonstrated that TPD52 inhibited growth and metastasis of RCC through downregulation of PI3K and Akt phosphorylation.

MATERIALS AND METHODS

Cell Culture

Human RCC cell lines (786-O, Caki-1, and UMRC-3) and immortalized proximal tubule epithelial cell line (HK-2) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin

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(Sigma-Aldrich, St. Louis, MO, USA) at 37°C in humidified air with 5% CO_2.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from RCC cells with TRIzol reagent (Invitrogen) according to the protocol. One microgram of total RNA was reverse transcribed to complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Invitrogen). qRT-PCR was performed using StepOne and StepOne Plus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The specific primers were as follows: TPD52, 5′-GAGGAAGGAGAAGATGTTGC-3′ (sense) and 5′-GCCGAATTCAAGACTTCTCC-3′ (antisense); β-actin, 5′-AAATCGTGCGTGACATCAAAGA-3′ (sense) and 5′-GGCCATCTCCTGCTCGAA-3′ (antisense). The PCR procedure was as follows: 94°C for 4 min; 94°C for 20 s, 58°C for 30 s, and 72°C for 20 s; 2 s for plate reading for 35 cycles; and melting curve from 65°C to 95°C. The relative expression was calculated by the comparative Ct method, and β-actin was used as the internal standard for all samples.

Western Blotting

Proteins were extracted from RCC cells using cell lysis buffer, and protein concentration was determined using a Bradford protein assay (Takara Biotechnology, Dalian, P.R. China). Equal amounts of protein samples were loaded and isolated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto PVDF membranes (Millipore Corp., Billerica, MA, USA). After being blocked by 5% nonfat milk, the membranes were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-TPD52, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt, and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the film was incubated with horseradish peroxidase-labeled secondary antibody at room temperature for 1 h. The immune-reactive protein bands were visualized by the ECL kit (Pierce, Rockford, IL, USA). Densitometric analysis was performed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Construction of the pcDNA3.1-TPD52 Vector and Cell Transfection

The full-length TPD52 open reading frame was amplified from RCC cells by RT-PCR and cloned into the pcDNA3.1 expression vector to construct the pcDNA3.1-TPD52 recombinant expression vector. RCC cells were transfected with pcDNA3.1-TPD52 or pcDNA3.1 (empty vector) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Cell Proliferation Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was used to evaluate cell proliferation. Briefly, transfected Caki-1 cells at a density of 5×10^3 cells/well were seeded into 96-well plates and incubated for 24, 48, 72, or 96 h, respectively. Then 20 ml of the MTT solution (5 mg/ml; Sigma-Aldrich) was then added to each well at the indicated time. A total of 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added per well for 15 min to dissolve the formazan crystals. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Migration and Invasion Assays

Transwell chamber (Corning Costar Corp., Cambridge, MA, USA) assay was performed to assess cell migration. Cell invasion was determined with Matrigel (BD Biosciences, Bedford, MA, USA) coated on the upper surface of the Transwell chamber (Corning). Briefly, transfected Caki-1 cells were added to the upper chamber. The lower chamber of the Transwell was filled with 500 μl of DMEM containing 10% FBS as a chemoattractant. Twenty-four hours later, cells on the surface of the upper chamber were removed by scraping with a cotton swab. The migrated/invaded cells on the lower surface of the filter were fixed with 4% paraformaldehyde and stained with crystal violet. The number of migrated/invaded cells was counted in five randomly selected fields under a microscope.

Tumor Xenograft Growth Assay In Vivo

Animal experiments were carried out with the approval of the ethics committee of Huaihe Hospital of Henan University (P.R. China). Caki-1 cells (1×10^6 cells) transfected with pcDNA3.1-TPD52 or pcDNA3.1 diluted in 200 μl of PBS were subcutaneously injected into 5- to 6-week-old female Balb/C nude mice. The tumor volume was measured every 5 days and calculated using the formula: $V\ (mm^3) = width^2\ (mm^2) \times length\ (mm)/2$. About 20 days after inoculation, mice were anesthetized, and the tumors were weighed.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Comparisons between the two groups were performed using the Student's *t*-test and between multiple groups using ANOVA. A value of $p<0.05$ was considered

significant. All analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

TPD52 mRNA and Protein Expression in RCC Cell Lines

We first examined the mRNA expression of TPD52 in RCC cell lines by qRT-PCR analysis. The mRNA expression levels of TPD52 were decreased in the 786-O, Caki-1, and UMRC-3 cell lines relative to the immortalized proximal tubule epithelial cell line HK-2 (Fig. 1A). TPD52 protein level was also detected in RCC cell lines by Western blot analysis. Consistent with the qRT-PCR results, TPD52 protein expression was decreased in the RCC cell lines, compared to the HK-2 cells. In addition, the Caki-1 cell line displayed a lower expression level of TPD52 (Fig. 1B). Thus, we used Caki-1 cell line cells as a model to investigate the effect of TPD52 on RCC cell proliferation and invasion.

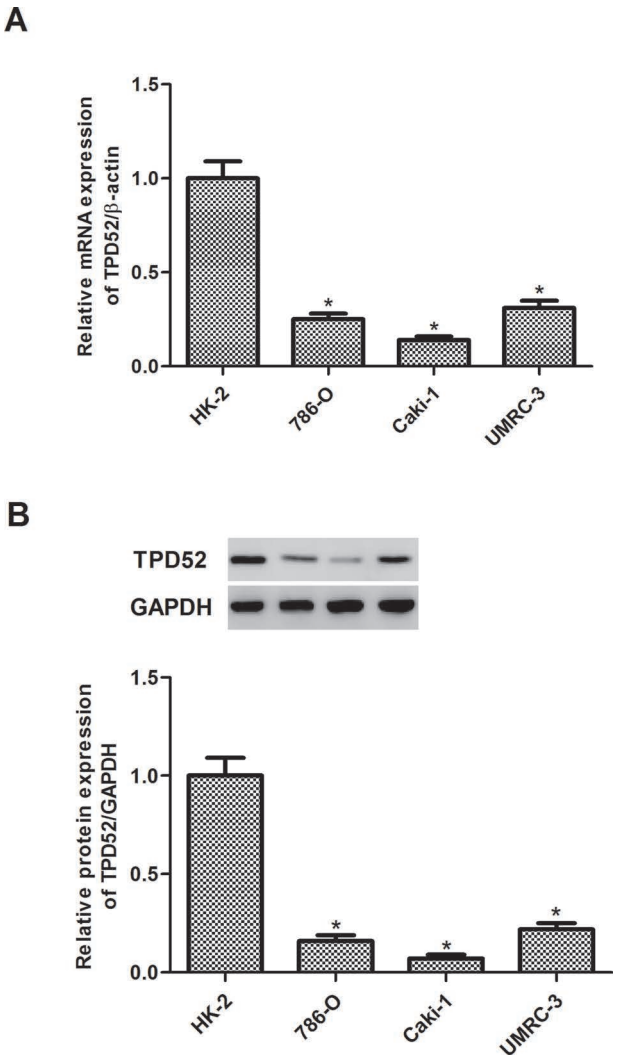

Figure 1. TPD52 mRNA and protein expression in RCC cell lines. (A) mRNA expression of TPD52 in RCC cell lines was detected by qRT-PCR analysis. (B) Protein expression of TPD52 in RCC cell lines was detected by Western blot analysis. Data are mean ± SD from three independent experiments. *$p < 0.05$ versus HK-2 cells.

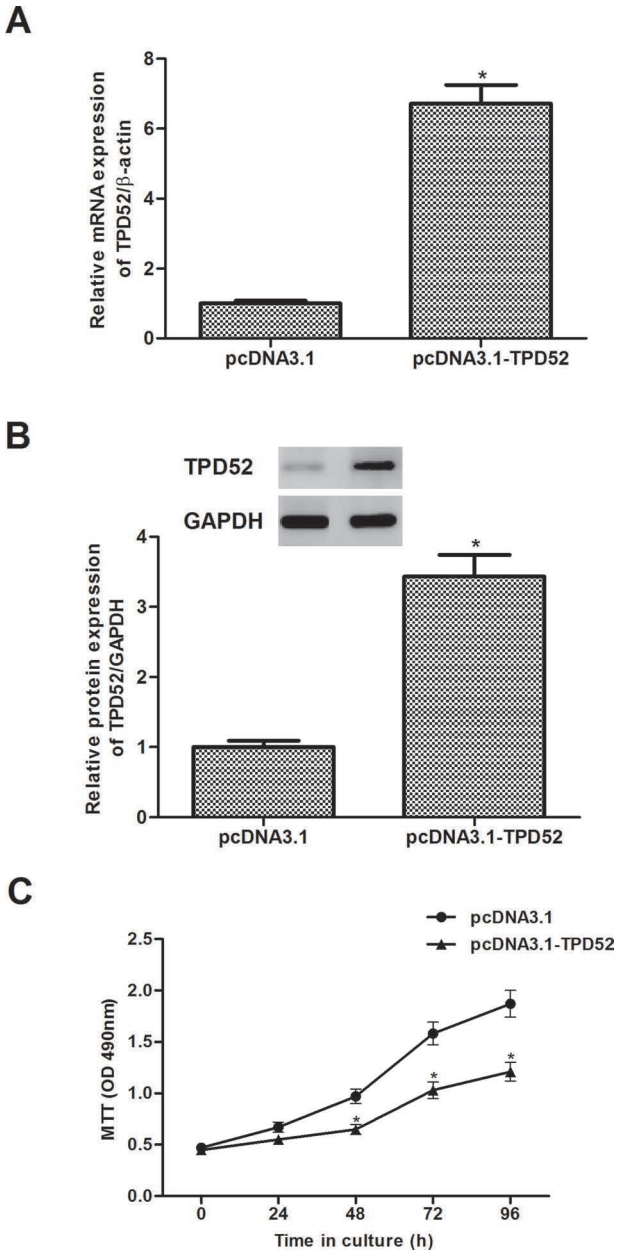

Figure 2. Effect of TPD52 on RCC cell proliferation. Caki-1 cells were transfected with pcDNA3.1-TPD52 or pcDNA3.1 (empty vector) for 24 h. (A) mRNA expression of TPD52 was detected by qRT-PCR analysis. (B) Protein expression of TPD52 was detected by Western blot analysis. (C) Cell proliferation was evaluated using the MTT assay. Data are mean ± SD from three independent experiments. *$p < 0.05$ versus pcDNA3.1 group.

Effect of TPD52 on RCC Cell Proliferation

We generated TPD52-overexpressing Caki-1 cells. Transfection efficiency was confirmed using qRT-PCR and Western blot analysis. Caki-1 cells that had been transfected with the TPD52 expression plasmid exhibited greatly increased TPD52 expression in both the mRNA and protein levels compared with the vector cell lines (Fig. 2A and B). Next, we performed the MTT assay to evaluate the effect of TPD52 on cell proliferation. We found that TPD52 overexpression dramatically inhibited the proliferation of Caki-1 cells (Fig. 2C), compared to the vector group.

Effect of TPD52 on RCC Cell Migration and Invasion

We performed a Transwell migration assay and invasion assay (using BD Matrigel-coated plates) to investigate the effects of TPD52 on RCC cell migration and invasion. Overexpression of TPD52 significantly inhibited the migration of Caki-1 cells (Fig. 3A). In addition, we observed that overexpression of TPD52 significantly inhibited the invasion of Caki-1 cells across the gelatin-coated membrane (Fig. 3B).

Effect of TPD52 on Epithelial–Mesenchymal Transition (EMT) Phenotype in RCC Cells

EMT was shown to play a critical role in cancer cell migration and invasion. Thus, we performed Western blot assay to test the effects of TPD52 on the expression of N-cadherin, vimentin, and E-cadherin at protein levels in Caki-1 cells. Overexpression of TPD52 markedly increased the expression of E-cadherin and decreased the

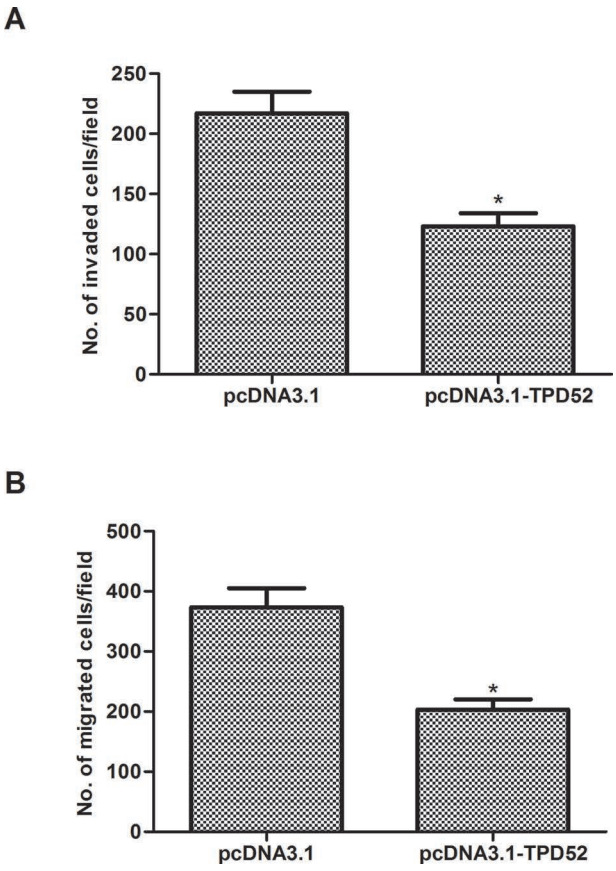

Figure 3. Effect of TPD52 on RCC cell migration and invasion. Caki-1 cells were transfected with pcDNA3.1-TPD52 or pcDNA3.1 (empty vector) for 24 h. (A) Cell migration was evaluated using the Transwell migration assay. (B) Cell invasion was determined by the Matrigel invasion assay. Data are mean ± SD from three independent experiments. *$p < 0.05$ versus pcDNA3.1 group.

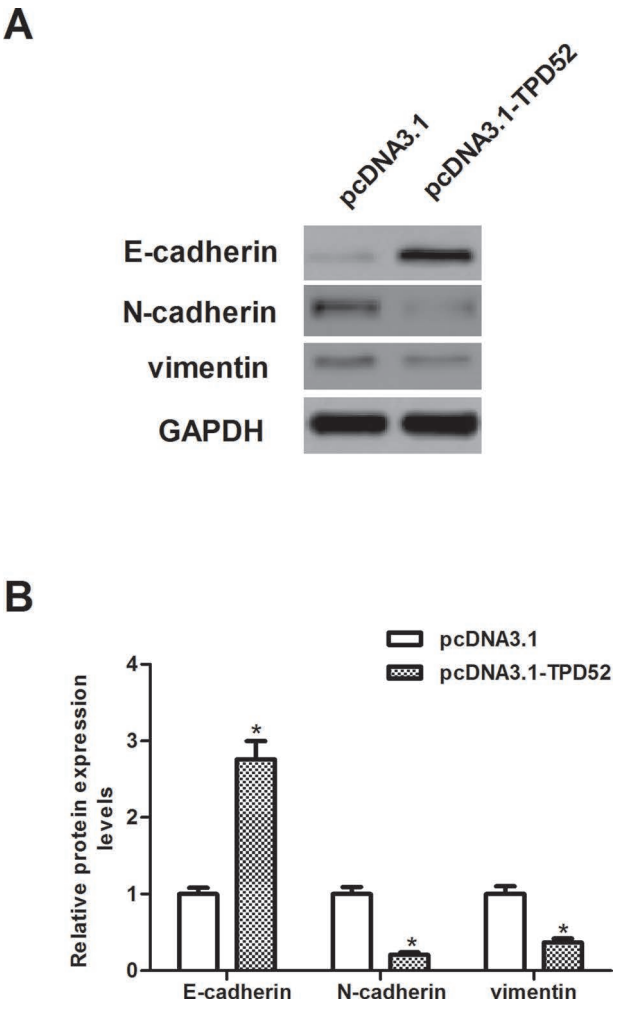

Figure 4. Effect of TPD52 on epithelial–mesenchymal transition (EMT) phenotype in RCC cells. Caki-1 cells were transfected with pcDNA3.1-TPD52 or pcDNA3.1 (empty vector) for 24 h. (A) The protein expression levels of N-cadherin, vimentin, and E-cadherin were evaluated using Western blot analysis. (B) Quantification of Western blot data. Data are mean ± SD from three independent experiments. *$p < 0.05$ versus pcDNA3.1 group.

expression of N-cadherin and vimentin in Caki-1 cells, compared to the vector group (Fig. 4).

TPD52 Inhibits the Activation of PI3K/Akt Signaling Pathway in RCC Cells

To explore the mechanisms by which TPD52 inhibits RCC proliferation and invasion, we examined the effect of TPD52 on the expression of certain molecules involved in the PI3K/Akt signaling pathway by Western blot. Compared to the vector group, overexpression of TPD52 obviously decreased the levels of PI3K and Akt phosphorylation in Caki-1 cells (Fig. 5A). Furthermore, we investigated the effects of an Akt inhibitor (wortmannin) on invasion and cadherin and vimentin expressions in Caki-1 cells. Because administration of 100 nM wortmannin had no significant effect on cell viability, 100 nM wortmannin was chosen for additional experiments (Fig. 5C). Results of the invasion assay indicated

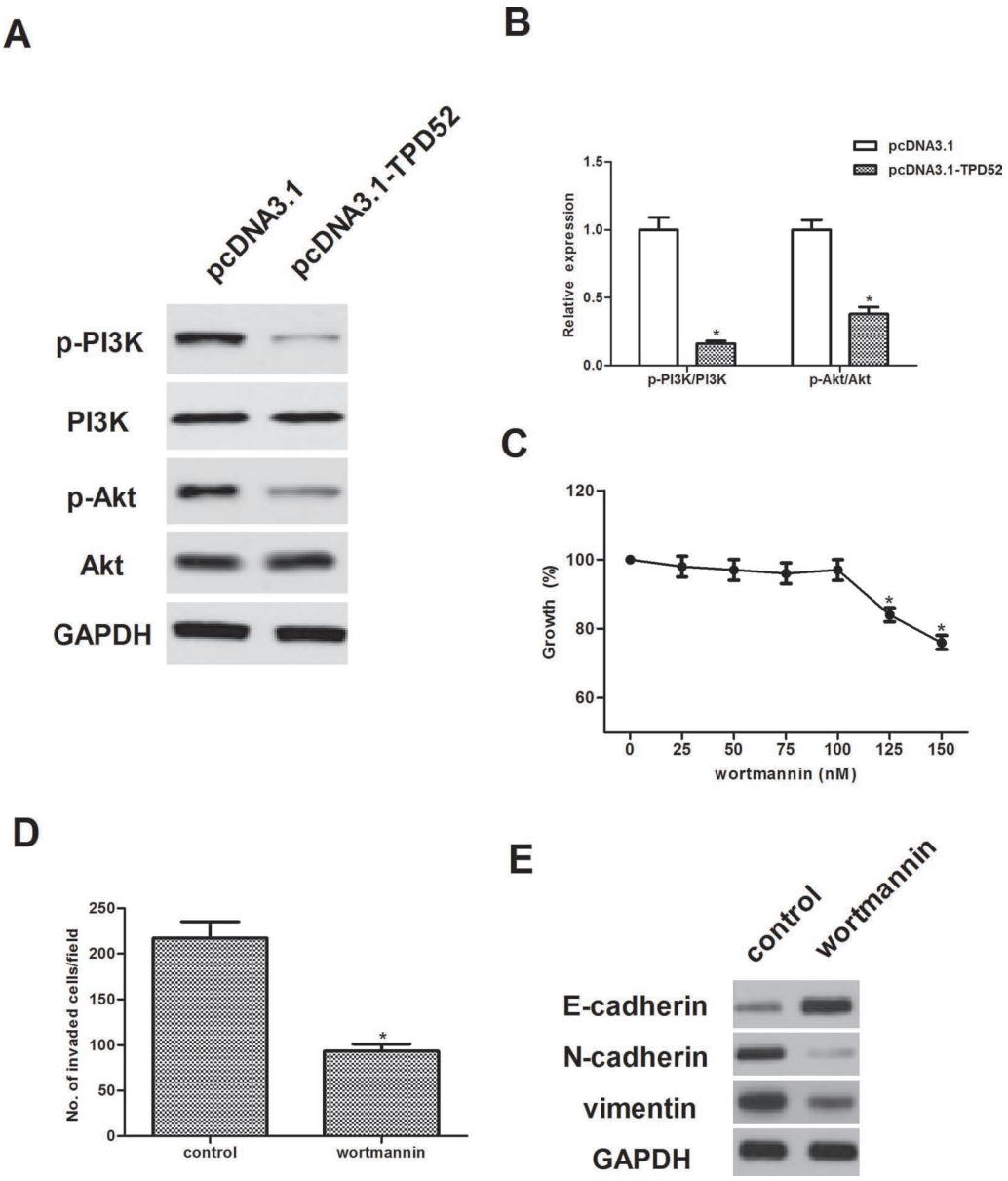

Figure 5. TPD52 inhibits the activation of the PI3K/Akt signaling pathway in RCC cells. Caki-1 cells were transfected with pcDNA3.1-TPD52 or pcDNA3.1 (empty vector) for 24 h. (A) The protein expression levels of p-PI3K, PI3K, p-Akt, and Akt were evaluated using Western blot. (B) Quantification of Western blot data. *$p<0.05$ versus pcDNA3.1 group. (C) Caki-1 cells were incubated with various concentrations of wortmannin, and cell growth was determined after 24 h using MTT assay. Caki-1 cells were treated with wortmannin (100 nM) for 24 h. (D) Wortmannin inhibited the invasion of Caki-1 cells. (E) EMT phenotypic changes were detected via Western blot analysis. Data are mean ± SD from three independent experiments. *$p<0.05$ versus control group.

that wortmannin significantly suppressed cell invasion (Fig. 5D). In addition, wortmannin obviously upregulated the expression of E-cadherin and downregulated the expression levels of N-cadherin and vimentin in Caki-1 cells (Fig. 5E).

TPD52 Reduces Xenografted Tumor Growth In Vivo

To examine the role of TPD52 on RCC growth in vivo, the xenografted tumor in nude mice was employed. Overexpression of TPD52 significantly suppressed tumor volume of Balb/C nude mice compared to the vector group (Fig. 6A). In addition, we observed a significant decrease in tumor weight in Caki-1 cells transfected with pcDNA3.1-TPD52 (Fig. 6B).

DISCUSSION

It has been reported that TPD52 was expressed in low amounts in RCC tissues, but the function and mechanism of TPD52 involved in RCC remain unclear. The current study demonstrated that the expression levels of TPD52 in both mRNA and protein were significantly decreased in RCC cells. Overexpression of TPD52 inhibited proliferation, migration, and invasion with decreased EMT phenotype in RCC cells and attenuated tumor growth in renal cancer xenografts. Mechanistically, overexpression of TPD52 significantly downregulated the phosphorylation levels of PI3K and Akt in RCC cells.

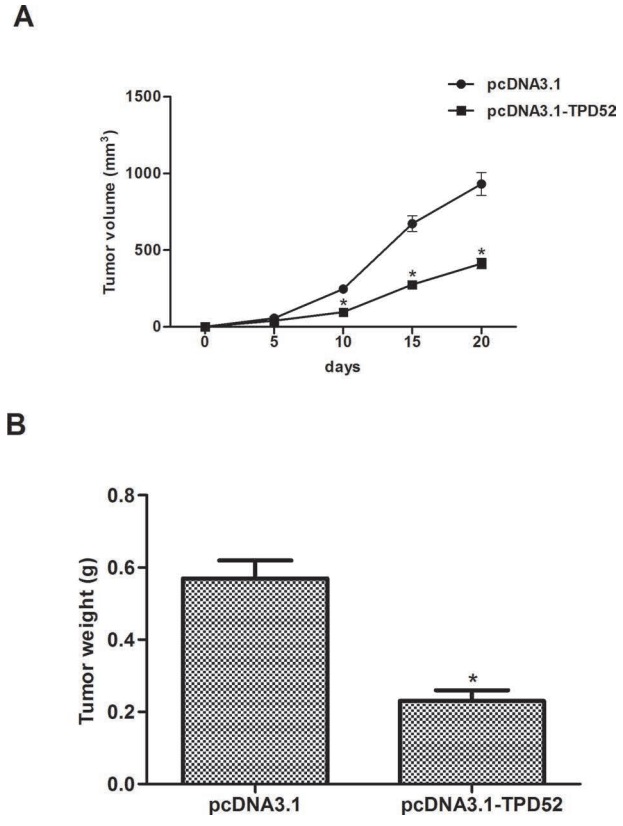

Figure 6. TPD52 reduces xenografted tumor growth in vivo. Caki-1 cells (1×10^6 cells) transfected with pcDNA3.1-TPD52 or pcDNA3.1 diluted in 200 µl of PBS were subcutaneously injected into 5- to 6-week-old female Balb/C nude mice. (A) Tumor volume was measured every 5 days. (B) After 20 days, mice were anesthetized, and the tumors were weighed. *$p<0.05$ versus pcDNA3.1 group.

TPD52 has been reported to be associated with various cancers. A previous study by Wang et al. showed that TPD52 expression was significantly decreased in hepatocellular carcinoma tissues and cell lines[16]. In contrast, TPD52 was found to be overexpressed in prostate cancer, and overexpression of TPD52 promoted proliferation and invasion in prostate cancer cells[17]. In this study, we found that the expression levels of TPD52 in both mRNA and protein were significantly decreased in RCC cells. Furthermore, overexpression of TPD52 obviously suppressed the proliferation, migration, and invasion of RCC cells, as well as attenuated tumor growth in renal cancer xenografts. These findings suggest that TPD52 is a tumor suppressor in the development of RCC.

EMT plays an important role in RCC metastasis[18]. It is well known that increased motility and invasion are positively associated with EMT, which is characterized by a loss of cell adhesion, repression of epithelial marker (E-cadherin) expression, and induction of mesenchymal markers[19]. In this study, we found that overexpression of TPD52 markedly upregulated the expression of E-cadherin and downregulated the expression of N-cadherin and vimentin in RCC cells. These results suggest that TPD52 inhibits the EMT phenotype, consequently affecting cell migration and invasion in vitro.

A number of studies have demonstrated that the PI3K/Akt signaling pathway is involved in a diverse number of cellular functions including proliferation, cell cycle progression, and invasion[20–22]. The PI3K/Akt pathway is modestly mutated but highly activated in RCC[23]. Thus, it is reasonable to assume that reduced Akt phosphorylation is related to reduced cell proliferation and invasion. For example, one study demonstrated that two PI3K inhibitors (LY294002 and wortmannin) greatly decreased Akt activation and GSK-3 phosphorylation and inhibited the proliferation of RCC cells, as well as attenuated tumor growth in vivo in nude mice[24]. The mechanisms of regulation of TPD52 expression in RCC have not yet been investigated. The current study demonstrated that overexpression of TPD52 obviously downregulated the protein expression levels of p-PI3K and p-Akt in Caki-1 cells. In human prostate cancer, TPD52 expression has been linked to the protein kinase B/Akt signaling pathway[25]. These data suggest that TPD52 inhibits growth and metastasis of RCC, at least in part, through suppressing the PI3K/Akt signaling pathway.

In conclusion, the present study demonstrated that TPD52 inhibited growth and metastasis of RCC, at least

in part, by suppressing the PI3K/Akt signaling pathway. Therefore, these findings suggest that TPD52 may be a promising therapeutic target for the treatment of human RCC.

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