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RESEARCH

Expression of Wip1 in Kidney Carcinoma and its Correlation with Tumor Metastasis and Clinical Significance

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Abstract This study aimed to analyze the expression, clinical significance of proto-oncogene in kidney carcinoma and the biological effect in its cell line by siRNA targeting wild-type p53-induced phosphatase 1 (Wip1). Immunohistochemistry and western blot were respectively used to analyze Wip1 protein expression in 78 cases of kidney cancer and normal tissues to study the relationship between Wip1 expression and clinical factors. Wip1 siRNA was transiently transfected into papillary kidney carcinoma cell by liposome-mediated method and was detected by Quantitative real-time RT-PCR (qRT-PCR) and western blot. MTT assay, cell apoptosis, cell migration and invasion were also conducted as to the influence of the down-regulated expression of Wip1 that might be found on ACHN cells biological effect. The level of Wip1 protein expression was found to be significantly higher in kidney cancer tissue than normal tissues ($P<0.05$). There were significant differences between Wip1 expression and lymph node metastasis, clinical stages and tumor differentiation ($P<0.05$). Meanwhile, Increased expression of Wip1 was significantly with poor overall survival time by Kaplan-Meier analysis ($P<0.05$). qRT-PCR and Western blot showed that ACHN cell transfected Wip1 siRNA had a lower relative expressive content than normal cell ($P<0.05$). MTT assay, cell apoptosis, cell cycles demonstrated that ACHN cell transfected Wip1 siRNA had a lower survival fraction, higher cell apoptosis, more percentage of the G0/G1 phases, significant decrease in migration and invasion, and higher P53 and P16 protein expression compared with ACHN cell untransfected Wip1 siRNA ($P<0.05$). Wip1 protein was increased in kidney carcinoma, specifically in T stages, lymph node metastasis, clinical stages and tumor differentiation. Wip1 may involved in the biological processes of kidney cancer cell proliferation, apoptosis, and migration and invasion by regulation P53 and P16 protein expression.

Keywords Wip1 · Kidney cancer · Cell proliferation · Cell apoptosis · Prognosis

Introduction

The incidence of kidney carcinoma varies substantially worldwide. The rates are generally high in Europe and North America while low in Asia and South America. Metastatic kidney carcinoma is a highly fatal disease, which accounts for about a third of the patients at initial presentation. Approximately 10 % to 28 % of kidney carcinoma develop a local recurrence or distant metastasis after curative nephrectomy. Metastatic kidney carcinoma is resistant to chemotherapy and radiotherapy but responds to tyrosine kinase inhibitors and interleukin-2-based immunotherapy [1]. kidney carcinoma is heterogeneous and comprises several histological cell types with different genetics, biology and behavior. The identification of the genes predisposing to inherited syndromes with kidney carcinoma has provided much of our knowledge of the molecular basis of early sporadic kidney carcinoma. Many of the oncogenes and tumor suppressor genes that are mutated leading to pathway dysregulation in kidney carcinoma remain

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to be elucidated. Global studies of copy number, gene sequencing, gene expression, miRNA expression and gene methylation in primary kidney carcinoma will lead towards this goal [2].

Thus, further studies will clarify the pathogenesis of kidney cancer and provide the molecular targets for effective treatment of kidney cancer. Recent studies have found that wild-type p53-induced phosphatase 1 gene demonstrated biological and genetic features similar to proto-oncogenes in some tumors, suggesting that Wip1 plays an important role in the development and progression of kidney cancer [3]. We studied Wip1 expression in kidney cancer tissue using immunohistochemistry and western blot, and performed a preliminary observation on its effect on the biological behavior of kidney cancer using Wip1 knockdown, in an attempt to look for new genetic targets for future clinical treatment.

Materials and Methods

Main Reagents

Rabbit anti-human Wip1 monoclonal antibody (Epitomics Inc.). immunohistochemistry kit (Zhongshan Goldenbridge Biotechnology Co., Ltd.). Bovine serum albumin (BSA) and β-actin primary antibody were purchased from Sigma Chemical Company (St. Louis, MO, USA). Plenti6/V5-DEST Vector, lentiviral packaging mix, Opti-MEM, Lipofectamine 2000, and the SuperScript III Reverse Transcriptase (RT) kit were obtained from Invitrogen Corporation (Carlsbad, CA, USA). TaqDNA polymerase was purchased from Fermentas, Inc. (Waltham, MA, USA). The DyLight Fluor conjugated to goat anti-mouse IgG was obtained from LI-COR bioscience, Inc. (Lincoln, Nebraska, USA). An immunohistochemistry kit and the Annexin V-FITC/PI apoptosis detection kit were purchased from 4A Biotech Co. Ltd. (Beijing, China). Fetal bovine serum (FBS), cell-culture media, and supplementary materials were obtained from Gibco Co. (Grand Island, NY, USA).

Clinical Data

The samples for each patient included kidney cancer tissue and normal tissue. Normal tissues were collected from kidney epithelia at the surgical margin in the patients' esophagus after tumour resection. The samples were instantly taken after the surgery, one of which was immediately fixed in 4 % paraformaldehyde solution, and embedded in paraffin for immunohistochemistry, and the other stored in liquid nitrogen for Western blot detection. All patients selected in this study provided informed consent in advance. Of the 78 cases of kidney cancer: 27 of them with lesion ≤7 cm and 51 with lesion>7 cm. The included studies contained 47 cases of clear cell type, 21 cases of granule cell type, and 10 cases of papillary cell type. Meanwhile, 28 patients demonstrated no lymph node metastasis (N0), whereas 50 with identified lymph nodes involvement (N+). As for the clinic stages, 23 cases had I ~ II stages and 55 had a III ~ IV stages. The grades of differentiation were 29 with Grade I (well differentiated) and 49 with Grade II or III (moderately to poorly differentiated).

Cell Culture and Gene Transfection

In accordance with Wip1 gene sequence in the NCBI database and siRNA design principles, Wip1-specific siRNAs were designed and synthesized by Guangzhou RiboBio Co., Ltd., whose sequences were as follows: sense strand 5′-CCAAUG AAGAUGAGUUAUAdTdT3′, antisense strand 3′-dTdTGGUUACUUCUACUCAAUAU-5′, and target sequence CCAATGAAGATGAGTTATA. In addition, a negative siRNA control that shared no homology to siRNA-Wip1 genome sequence was designed and synthesized. The sense and antisense RNAs were synthesized in vitro and annealed to form double-stranded RNA. ACHN cells was maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum (Gibco BRL, Grand Island, NY) in a constant-temperature humidified incubator at 37 °C and in an environment containing 5 % CO_2 and 95 % air. Twenty-four hours before transfection, ACHN cells in logarithmic growth phase were seeded in 6-well plates at a concentration of 5×10^4 cells/well. When the cells reached a confluence of 30 % to 50 %, the cells were transfected with the mixture of siRNA and Lipofectimane 2000 at a concentration of 100 nmol/L. Next, the cells were cultured in serum-free Opti-MEM culture medium. The liposome group was cultured with Opti-MEM medium containing 5 μL of liposomes/well. The culture medium was discarded 6 h later and the cells were switched to RPMI-1640 medium containing 10 % FBS.

Immunohistochemistry

A 4 μm section was prepared from paraffin-embedded block and dehydrated, then incubated in 3 % hydrogen peroxide for 10 min to block endogenous peroxidase, followd by using trypsin for repair of 20 min; 10 % goat serum was introduced at room temperature for closure of 20 min, and Wip1 antibody (1: 100) was left in the wet box at 4 °C refrigerator for overnight. Then the secondary and third antibodies were dropped into the wet box at room temperature for incubation of 20 min, respectively; DAB staining was again visualized by the hematoxylin stain, and then came to normal dehydration with the coverslip sealed. Wip1 expression was determined based on the percentage of positive cells, combined with the staining intensity. The percentage of positive cells was divided

into four levels: 0 point: ≤ 5 % of positive cells, 1 point: 5 %~ 25 %, 2 points: 25 %~50 %, and 3 points: > 50 % of positive cells. The intensity of staining was clssfied as: 0 point: no staining, 1 point: weak staining (light yellow); 2 points: moderate staining (brown); and 3 points: strong staining (yellowish- brown). The final score of Wip1 expression was the product of the WIP1 expression rate and intensity, graded as 0 for negative, + for 1–3 points, ++ for 4–6 points, and +++ for 7–9 points. As for the negative control, the primary antibody was replaced with PBS.

Quantitative Real-Time RT-PCR

Total RNA was extracted from the kidney cancer ACHN cell using TRIzol reagent (Invitrogen; Life Technologies, Gaithersburg, Maryland) the manufacturer's protocol. Five hundred nanograms of total RNA was reverse transcribed using TakaRa Reverse Transcriptase Reagents (TakaRa). Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI Prizm 7300 according to the standard protocol of SYBR Premix ExTaq perfect real time system (TakaRa). Primers for Wip1 and β-actin as a reference for normalisation were as follows: Wip1 sense 5′- GTTCGTAGCAATGCCTTCTC A, antisense 5′- CACTTTCTTGGGCTTTCATTTG; β-actin sense 5′- ATCGTCCACCGCAAATGCTTCTA, antisense 5′- AGCCATGCCAAT CTCATC TTGTT. Thermal cycling conditions were 95 °C for 1 min, 95 °C for 15 s and 40 cycles at 60°Cfor 1 min. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method in SDS 1.3 software.

Western Blot

All operations were completed on the ice. At 4 °C, 12,000 r/min centrifugation lasted for 20 min, and then the supernatant was taken for backup at −20 °C. After the detection of protein concentration with BCA Protein Assay Kit, each hole was given a sample amount of 50ug for SDS-PAGE electrophoresis. Regulator power for ice bath was transferred to nitrocellulose membrane, followed by closure for 2 h with 5 % skim milk. Subsequent to Anti-one overnight incubation at 4 °C (Wip1 1:1000, β-actin 1:3000). The latter was from Sigma Chemical Company, St. Louis, MO, USA in 5 % nonfat dry milk for 1 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit fluorescent secondary antibody (IRDye800, 1:20,000 dilution; the DyLight Fluor conjugated to goat anti-rabbit IgG was obtained from LI-COR Bioscience, Inc., Lincoln, Nebraska, USA) in the dark for 1 h at room temperature. The blots were then scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience). Western blot data were quantified by normalizing the signal intensity of each sample to that of β-actin.

MTT Assay

Cell viability was determined by using the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were plated into 96-well culture plates at an optimal density of 5×10^3 cells/ml in 200 μl of culture medium per well. After 24–96 h of culture, 20 μl of 5 mg/ml MTT was added to each well and incubated at 37ºC for 4 h. The medium was then gently aspirated and 150 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad) at 570 nm.

Flow Cytometry Assay

Cell apoptosis: An Annexin V-FITC-flow cytometry assay was used to detect the apoptosis rate in the cells after Wip1 siRNA transfection. Cells were seeded into 60-mm dishes for 48 h and grown to approximately 70–75 % confluence. After quick detachment from the plate, cells were collected, washed with ice-cold PBS, and resuspended at a cell density of 1×10^6/mL in a binding buffer from the Annexin V-FITC apoptosis detection kit (4A Biotech Co. Ltd, Beijing, China) and then stained with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI, 20 μg/ml). The cells were then incubated in the dark at 25ºC for 15 min before 10,000 cells were analyzed by a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and Cellquest software (Becton-Dickinson) for apoptosis rate determination. The data were evaluated using the ModFit software program (Verity Software House, Topsham, ME, USA).

Invasion and Migration Assays

The Costar Transwell 8 μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences Franklin Lakes, NJ) for invasion assay according to the manufacturer's instruction. Invasion Chambers (BD China, Shanghai, China) at 12×10^5 cells per chamber. The membrane in the chamber was coated with Matrigel (BD China). Medium supplemented with 10 % FBS was used in the lower chamber. Migration assays were performed in the same manner excluding the Matrigel. After 16 h, non-invading cells, and media were removed with a cotton swab. Cells on the lower surface of the membrane were fixed with polyoxymethylene (Sigma) and stained with 0.1 % crystal violet (Sigma) for 0.5 h. Stained cells were counted under a microscope in 4 randomly selected fields, and the average was used to indicate cell migration and invasion.

Statistical Analyses

All statistical analyses were performed using SPSS16.0 software. For the clinicopathologic features, *P* values were calculated using the χ^2 test. Wip1 mRNA expression was shown in mean ± SD. Student *t*-test was used to analyze the difference between groups. A 5 % or lower *P-value* was considered to be statistically significant.

Results

Wip1 Protein Expression in Kidney Cancer and Normal Tissue

In normal kidney tissues, Wip1 staining was negative or weak. In kidney cancer tissues, Wip1 staining ranged from light yellow to brown. Statistically, Wip1 was expressed in 67.9 % (53/78) of kidney cancer tissues, which was higher than the 28.2 % (22/78) in normal tissues. The difference was statistically significant ($P<0.05$, Table 1, Fig. 1). While Western blot showed that the relative expression of Wip1 protein presented volume between cancer lesion and adjacent normal tissue were 0.673±0.069 and 0.251±0.026, showing the difference with statistical significance ($P<0.05$). The expression of Wip1 was correlated with T stages, clinical stages, lymph node metastasis and pathological differentiation ($P<0.05$, Table 2).

Wip1 Expression and Prognosis

Survival analysis was performed in all the patients and follow-up data were collected. All patient follow-ups ended in 2012 after a revisit time of 60 months. Among all cases, 15 were still alive at this time and 63 were dead. Patients were divided into two groups according to Wip1 expression level. There were 53 individuals with positive levels of Wip1 expression, among whom 6 were still alive and 47 were dead. The survival rate was 11.3 %. There were 25 individuals with negative levels of Wip1 expression, among whom 9 were still alive and 16 were dead. The survival rate was 36.0 %. Patients with positive levels of Wip1 expression had significantly lower 5-year survival rates than those with low levels of Wip1 expression group ($P<0.05$).

Table 1 Expressions of Wip1 in kidney cancer tissue and in normal kidney tissue

Groups	Case	Expression of Wip1 protein					
		−	+	++	+++	χ^2	*P*
Normal tissue	78	56	9	7	6	25.125	0.000
Cancer tissue	78	25	17	19	17		

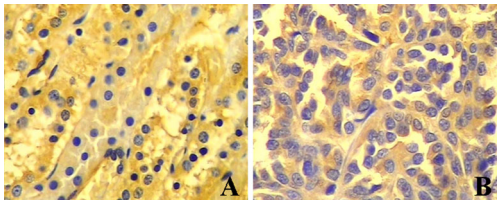

Fig. 1 Expressions of Wip1 protein in kidney carcinoma and normal tissue. **a** normal tissue (SP×400); **b** kidney carcinoma (SP×400)

Expression and Identification of Wip1 siRNA in ACHN Cell

Wip1 siRNA was transiently transfected into kidney carcinoma cell by liposome-mediated method and was detected by qRT-PCR and western blot. qRT-PCR data showed that the Wip1 mRNA expression level was 0.758±0.082 in empty vector-transfected cells. In contrast, the amount of Wip1 mRNA in the Wip1 siRNA -transfected cell lines was 0.213±0.021. The difference was statistically significant ($P<0.05$, Fig. 2a). Furthermore, western blot analysis showed that the control cells had approximately equal amounts of immunoreactive protein (0.767 ±0.075). In contrast, the amount of Wip1 protein in the Wip1 siRNA-transfected cell lines was 0.113±0.010. The difference was statistically significant ($P<0.05$, Fig. 2b).

Table 2 Relation between Wip1 expression and clinic characteristics in kidney cancer tissue

Groups	Case	Expression of Wip1 protein			
		—	+~+++	χ^2	*P*
Sex					
Male	54	18	36	0.132	0.716
Female	24	7	17		
Age (year)					
≤50	30	9	21	0.094	0.759
>50	48	16	32		
Tumor length (cm)					
≤7	27	8	19	0.111	0.739
>7	51	17	34		
Pathological types					
Clear cell type	47	16	31	0.768	0.681
Granule cell type	21	7	14		
Papillary cell type	10	2	8		
Lymph node metastasis					
N0	28	14	14	6.461	0.011
N+	50	11	39		
Clinic stages					
I ~ II	23	12	11	6.056	0.014
III ~ IV	55	13	42		
Histological grade					
I	29	16	13	4.282	0.039
II ~ III	49	19	40		

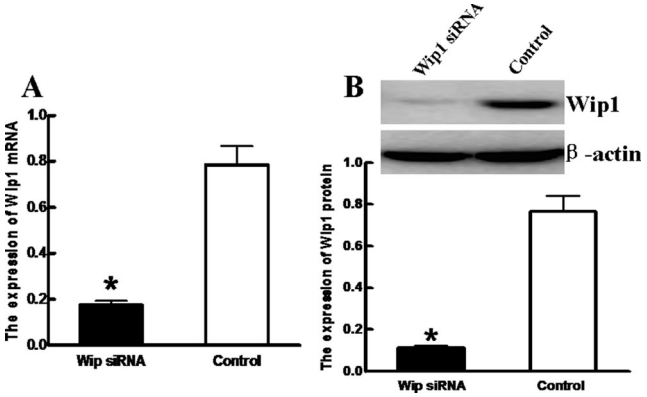

Fig. 2 Expression and identification of the Wip1 gene. **a** qRT-PCT, **b** Western Blot. *$p<0.05$ compared to the control cell

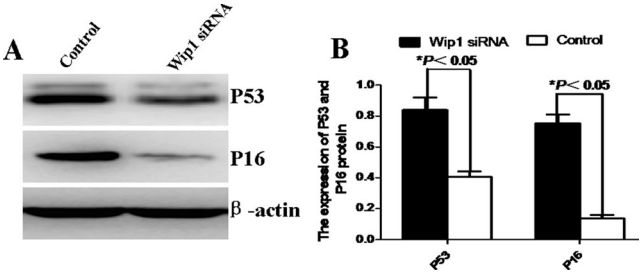

Fig. 3 The effects of Wip1 siRNA on P53 and P16. *$p<0.05$ compared to the control cell

Effects of Wip1 siRNA on Kidney Cancer ACHN Cell

Next, we assessed the effect of Wip1 siRNA on the regulation of kidney cancer cell viability. MTT assay showed that relative proliferative capacity of the Wip1 siRNA cell relative grew slower at 24, 48, 72 h and 96 h compared with the parental cell. The difference was statistically significant ($P<0.05$). Meanwhile, there was a relative large increase in the early and late apoptosis rate was 16.9±1.5 % in Wip1 siRNA cell compared to control cells (6.5±0.6 %). The difference was statistically significant ($P<0.05$). Furthermore, the ACHN cells transfected with Wip1 siRNA or empty vector were transferred to transwell chambers or Matrigel-coated transwell chambers to evaluate the effect of Wip1 siRNA on cell invasion potential. Introduction of Wip1 siRNA clearly led to a significant decrease in ACHN cell migration and invasion (117.0±16.0 and 68.0±9.0, respectively) compared with the cells transfected with empty vector (147.0±19.0 and 96.0± 14.0, respectively). The difference was statistically significant ($P<0.05$). To further identify the mechanisms by which Wip1 siRNA inhibited kidney cancer cell proliferation, promoted cell apoptosis and decreased migration and invasion, we analyzed the expression level of P53 and P16 protein due to their critical roles in cell proliferation, cell apoptosis, and migration and invasion. Western blot analysis revealed that Wip1 siRNA significantly upregulated P53 and P16 protein expression in ACHN cell transfected with Wip1 siRNA (0.839±0.081, 0.751±0.060, respectively) compared to control cell (0.406± 0.038, 0.137±0.022, respectively). The difference was respectively statistically significant ($P<0.05$, Fig. 3).

Discussion

Wip1 is a serine/threonine protein phosphatase whose encoding sequence is located in human chromosome 17q22/ q24 region. Human Wip1 protein has a molecular weight of about 61kD and consists of 605 amino acids. In recent years, studies have shown that Wip1 is highly expressed in breast cancer [4], neuroblastoma [5], and medulloblastoma [6] and exercises a negative feedback regulation on p53 through the p38MAPK/p53 signaling pathway, leading to decreased expression of p53 and p53 mutation, suggesting a close association between Wip1 and prognosis [7].

This study quantified for the first time Wip1 expression in kidney tissue and normal kidney tissue using immunohistochemistry and western blot. Our results showed that Wip1 protein levels were significantly higher in kidney tissue than in normal tissue. Our results are consistent with a previous research finding that Wip1 expression is significantly higher in glioblastoma multiforme [8], ependymoma [9], thyroid papillary carcinoma [10] than in normal tissue. The univariate analysis in the present study also showed that Wip1 protein levels were correlated with lymph node metastasis, clinical stage and pathological differentiation. This result agrees with a previous research finding that Wip1 protein levels are not correlated with age, sex, and tumor size of patients with thyroid papillary cancer, but runs counter to the research finding that Wip1 protein levels are not correlated with lymph node metastasis and pathological staging [10]. Recent studies indicate that when DNA damage occurs, ATM causes Chk2 phosphorylation to prevent tumorigenesis. Wip1 combines with Chk2 to dephosphorylate and inactivate Chk2, leading to tumorigenesis. In vivo and in vitro experiments confirmed that Wip1 could also contribute to tumorigenesis by inhibiting Chk1 dephosphorylation and inactivation [11]. Further studies demonstrated that Wip1 high expression disrupted the homeostasis maintained by the p38MAPK-p53-Wip1 pathway, caused downstream Wnt-p53 inactivation through p38 MAPK dephosphorylation, and promoted the development of malignant breast cancer in humans by reducing p16 protein levels [10].

Currently an effective treatment modality for kidney cancer is surgical lesion resection, accompanied by chemotherapy and/or radiotherapy before and after surgery. However, the survival rate with this strategy is not very satisfactory [12]. Therefore, more attention should be directed at the importance of early detection of kidney cancer, and the choice of treatment strategies should be carefully weighed for individual

patients. To preserve organs and their functions and handling relevant coexisting situations, clinicians should focus on the most effective treatment method so as to reduce failure and improve survival and quality of life. Traditional treatment of kidney cancer and prognosis rely mainly on TNM classification [13]. This system is subjective and not informative for early kidney cancer, and offers limited information about disease severity, prognosis, and response to treatment, while early detection of kidney cancer is the most effective way to improve survival [14]. This study showed in survival analysis that Wip1 expression-positive patients had a significantly lower 5-year overall survival rate than patients without Wip1 expression. Therefore, the combination of TNM classification system and Wip1 expression scores may provide some valuable information for clinicians in the choice of treatment options, prognosis judgment and prediction of disease severity.

Further in vitro experiments demonstrated for the first time that kidney cancer cells with high Wip1 expression had significantly weakened proliferation and migration and invasion, significantly increased apoptosis, and markedly increased P53 and P16 protein levels [15]. A Chinese team reported the use of siRNA silencing of high Wip1 expression in U251 glioblastoma cell line to inhibit tumor cell proliferation [8]. Baxter et al. [16] knocked down highly expressed Wip1 in medulloblastoma D283 cells with RNA interference technique, which increased p53 expression and induced tumor cell apoptosis. A Chinese study also found that sRNAi knockdown of highly expressed Wip1 in medulloblastoma D283 cells increased P53 expression and induced tumor cell apoptosis [5]. A study suggests that this phenomenon may be related to the extensive roles of Wip1 in NF-κB, Notch, and Wnt signaling pathways, which play an important role in proliferation, apoptosis, cell cycle, angiogenesis and epithelial-mesenchymal transition [16, 17].

The results of this study showed that Wip1 protein and mRNA were both increased in kidney cancer and that the increase was correlated with lymph node metastasis, clinical stage and poor prognosis of patients with kidney cancer. Wip1 is involved in a number of biological processes including cell proliferation, apoptosis, migration and invasion in kidney cancer. However, carcinogenesis is a complex and integrated process [18, 19], and further research is warranted to gain more insight into the signaling mechanism whereby Wip1 is involved in the process.

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