

Over-Expression of miR-21 and Lower PTEN Levels in Wilms' Tumor with Aggressive Behavior

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Wilms' tumor (WT) is the most common pediatric kidney tumor. MiR-21 is one of the most frequently overexpressed microRNAs in solid tumors, while phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is the most highly mutated tumor suppressor gene. The aim of this study was to investigate the relationship between miR-21 and PTEN in WT. The expression levels of miR-21 and the PTEN protein were determined by qRT-PCR and Western blot analyses in WT specimens, respectively. In WT tissues, the miR-21 expression levels were significantly higher and the PTEN protein levels were significantly lower, compared to the adjacent non-tumorous renal tissues. The higher levels of miR-21 and lower levels of PTEN were correlated with age (> 24 months), late clinical stage, unfavorable histopathological type and lymphatic metastasis. A univariate linear regression analysis indicated a significant correlation between higher miR-21 levels and lower PTEN levels. Using the SK-NEP-1 WT cell line, we showed that the decreased expression levels of miR-21 promoted cell proliferation and invasion, but inhibited apoptosis. Importantly, lowered expression levels of miR-21 increased the expression levels of PTEN protein and decreased the expression levels of phosphoinositide 3-kinase (PI3K) and phosphorylated protein kinase B (p-AKT), each of which functions in the downstream signaling pathway. Dual luciferase-reporter assays indicated that PTEN mRNA was a direct target of miR-21. In conclusion, higher miR-21 levels and lower PTEN protein levels are predictive biomarkers for poor prognosis of WT patients. Over-expression of miR-21 promotes aggressive behavior of WT by targeting PTEN.

Keywords: miR-21; PTEN; SK-NEP-1 cell; target gene; Wilms' tumor

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Introduction

Wilms' tumor (WT), also known as nephroblastoma, is the most common pediatric tumor of the kidney. WT is usually diagnosed before the age 5 and accounts for approximately 0.1% of tumors in children under 15 years of age (Ehrlich and Shamberger 2012). The occurrence of WT involves the abnormal expression of multiple oncogenes and tumor suppressor genes and changes in the corresponding signal transduction pathways (Zhang et al. 2014). Therefore, extensive molecular investigation of the occurrence and development of tumors has become a key element of personalized treatments.

MicroRNAs (miRNAs) are short sequences of non-coding, single-stranded RNAs that modulate gene expres-

sion. They play important roles in cellular differentiation, proliferation, cell cycle control and cell apoptosis. MiRNAs have been shown to act as either oncogenes or tumor suppressors and are key molecules involved in cancer initiation and progression (Deng et al. 2008). MiR-21 has been confirmed to be overexpressed in almost all the solid tumors ever studied, accounting for 15-25% of the total miRNAs in tumor cells, and to play a role as a proto-oncogene (Krichevsky and Gabriely 2009). MiR-21 is able to negatively regulate multiple target genes, such as programmed cell death 4 (PDCD-4) (Asangani et al. 2008), reversion-inducing-cysteine-rich protein with kazal motifs (RECK) (Reis et al. 2012), tissue inhibitor of metalloproteinase-3 (TIMP-3) (Li et al. 2013a), tropomyosin 1 (TPM-1) (Zhu et al. 2007), and phosphatase and tensin homolog

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deleted from chromosome 10 (PTEN) (Meng et al. 2007) thereby playing an important role in promoting the proliferation and differentiation of certain tumor cells.

As the first tumor suppressor gene identified with phosphatase activity, genetic deletions or inactivated mutations of PTEN have been demonstrated to result in cancer susceptibility and progression (McCabe et al. 2016). PTEN also has been confirmed as a target gene of miR-21 in non-small cell lung cancer (Zhang et al. 2010), gastric cancer (Zhang et al. 2012), cervical cancer (Xu et al. 2015), and colorectal cancer (Xiong et al. 2013). Deletions or inactivated mutations of PTEN can mediate the promotional effect of miR-21 on the proliferation, invasion and metastasis of tumor cells (Selcuklu et al. 2009).

In-depth research on the mechanism of miR-21 and PTEN in a variety of adult tumors is ongoing. However, it remains to be investigated whether miR-21 could influence PTEN expression in WT. Based on various reports on miR-21 and PTEN expression in tumor tissues and cells (Selcuklu et al. 2009; Zhang et al. 2010; Zhang et al. 2012; Xiong et al. 2013; Xu et al. 2015), we hypothesized that PTEN is a latent target gene of miR-21 in WT. Therefore, the purpose of this study was to analyze the correlation between the expressions of miR-21 and PTEN and the clinicopathological parameters of WT, to elucidate the role of miR-21 in human SK-NEP-1 WT cell line and to further explore the targeting regulatory relationship between miR-21 and PTEN in WT.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of Shandong Provincial Hospital. The procedure of specimen collection and the whole procedure were harmless to the patients, and informed consent was obtained from the guardians of the child patients. The entire investigation conforms to the principles outlined in the Declaration of Helsinki.

Patients and tissue samples

From January, 2013 to June, 2016, WT tissues (41 cases) and paired homolateral adjacent non-tumorous renal tissues (≥ 2 cm away from the tumor, with pathological confirmation that no cancer cells were found in tangent lines, 20 cases) were obtained from 41 patients who underwent tumor nephrectomy at Shandong Provincial Hospital. Every nephrectomy specimen was immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA and protein extraction was performed.

The clinicopathological parameters of each patient, including sex, age, clinical stage of tumor, histopathological type of tumor and lymphatic metastasis, were collected to analyze the correlation between the expression levels of miR-21 and PTEN protein. The exclusion criteria were the following: (1) patients who received chemotherapy or radiotherapy before surgery were excluded because these preoperative therapies may alter the characteristics of WT and possibly interfere with the histopathological diagnosis; (2) all stage IV cases were excluded because the quantity of patients with stage IV WT was small and not suitable for statistical analysis. Furthermore,

all these patients experienced metastasis, and as a result, they were almost all treated with preoperative therapies.

qRT-PCR to detect miR-21 expression in WT tissues

Total RNA from the tissue samples was extracted with TRIzol reagent (Thermo Fisher Scientific, USA). cDNA was synthesized by reverse transcription using TaqMan[®] MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Products were amplified by PCR using a TaqMan[®] Universal PCR Master Mix Kit (Thermo Fisher Scientific, USA). The relative levels of miR-21 in WT and in adjacent non-tumorous were calculated with the $2^{-\Delta\Delta\text{CT}}$ method, with U6 as an internal control. The primers of miR-21 and U6 are described in the section titled "qRT-PCR to detect mRNA in SK-NEP-1 WT cells".

Western blot analysis to detect PTEN protein expression in WT tissues

Whole-cell lysates of WT and adjacent non-tumorous tissues were prepared in RIPA lysis buffer. The protein concentration was measured with a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA). Cell lysates were electrophoresed using SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were immunoblotted overnight at 4°C with a primary antibody (PTEN 1: 500, β -actin 1: 5,000, Abcam, USA) in Tris-buffered saline containing 5% non-fat dry milk, and then incubated with a fluorescently labeled goat anti-mouse IgG antibody (1: 1,000, Abcam, USA) at room temperature for 1 h. The membranes were visualized using the Odyssey dual-color laser-based infrared imaging system, and Western blot bands were quantitatively analyzed with Odyssey 3.0 software.

Cell culture and transfection

The human SK-NEP-1 WT cell line was purchased from CAS Shanghai Life Sciences Cell Resource Center. MiR-21 inhibitor (5'-UCAACAUCAGUCUGAUAAAGCUA-3') and mismatched sequence negative control oligonucleotides (5'-CAGUACUUUUGU GUAGUACAA-3') were synthesized by Shanghai GenePharma Co., Ltd. MiR-21 inhibitor is a chemically synthesized, single stranded RNA molecule which is complementary to the nucleotide sequence of miR-21. MiR-21 inhibitor can be uptaken by cells with the aid of transfection reagent. The inhibitor can present specifically competitive combination to targeting endogenous miR-21 molecule following the complementary matching principle. This combination in turn decreases the levels of free and active miR-21 molecules, and prevents the binding of miR-21 to target its downstream mRNAs, thus inhibiting the effect of miR-21 on target genes and facilitating enhanced target gene expressions.

The SK-NEP-1 cell line was cultured in semi-adherent conditions in 84% Mc Coy's 5A medium (Modified, GIBCO, USA), 15% FBS (Qualified, Australia Origin) with 1% Penicillin-Streptomycin, and incubated in a humidity-saturated 50 mL/L CO_2 incubator at 37°C .

Twenty-four hours before transfection, SK-NEP-1 WT cells were cultured in 500 μL medium without antibiotics or FBS. When the cells grew to 50% confluence, 2×10^5 cells were collected and seeded into a 6-well plate. The cells were divided into IN (experiment) group, NC (negative control) group and MOCK (blank control) group. The grouping criteria were as follows:

IN group: SK-NEP-1 WT cells + transfection reagent + miR-21 inhibitor;

NC group: SK-NEP-1 WT cells + transfection reagent + miR-21 inhibitor negative control reagent;

MOCK group: SK-NEP-1 WT cells + transfection reagent + PBS.

Transient transfection was carried out using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions.

Cell proliferation assay

Cell proliferation assay was carried using CCK-8 (Dojindo, Japan). Twenty-four hours after transfection, 100 μ L cell suspension (at a density of 2×10^4 cells/well) of each group and 10 μ L of CCK-8 reagent were added into a 96-well plate. After culturing the cells for 24 h, 48 h and 72 h, the cell proliferation curve was determined by OD values (450 nm) and time.

Cell invasion assay

Cell invasion was measured using BD BioCoat™ Matrigel™ Invasion Chambers (BD BioScience, USA). Cells of the three groups were trypsinized and then seeded in the upper chambers with Mc Coy's 5A medium, at a density of 8×10^4 cells/well at 48 h after transfection. Meanwhile, 500 μ L of 15% FBS-Mc Coy's 5A medium was added to the lower chamber. After 24 h, the non-migrated cells were scraped from the upper chamber using a cotton swab. Migrated cells were fixed in 100% methanol for 30 min and stained with the 0.1% crystal violet for 20 min. The number of cells invading the matrigel was counted in three randomly selected fields under a phase-contrast microscope.

Apoptosis rate assay

The apoptosis rate was detected using the Annexin V-FITC apoptosis detection kit (Beyotime, Jiangsu, China). Cells were dual-stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. The early apoptosis rate in each group, which was identified as Annexin V (+)/PI (-), was analyzed by flow cytometry.

qRT-PCR to detect mRNA in SK-NEP-1 WT cells

Cells of all groups were harvested. The subsequent experimental procedure was the same as that in the section titled "qRT-PCR to detect miR-21 expression in WT tissues" above. The primer sequences of miR-21, PTEN and U6 were as follows: miR-21 F 5'-GCCAGGCATAGCTTATCAGACTG-3' and R 5'-CCACTG TCTAGCACGACACTAA-3'; PTEN F 5'-AAAGGGACGAAC TGGTGTAATG-3' and R 5'-TGGTCCTTACTTCCCATAGAA-3'; and U6 F 5'-GCTTCGGCAGCACATATACTAAAAT-3' and R 5'-CGCTTCACGAATTTGCGTGTTCAT-3'.

Western blot analysis to detect protein expressions in SK-NEP-1 WT cells

At 48 h after transfection, the medium of all groups was removed, and RIPA cell lysis solution was added. The subsequent experimental procedure was the same as that in the "Western blot analysis to detect PTEN protein expression in WT tissues" section above except for the addition of primary antibody (PTEN 1: 500, PI3K 1: 500, p-AKT 1: 100, β -actin 1: 5,000, Abcam, USA).

Dual luciferase reporter gene assay detecting PTEN activity

Primers were designed according to bioinformatics software:

3'-untranslated Regions (UTR)-PTEN-wt (wild-type recombinant plasmid) F: 5'-CCACCAGAGCTCTTGTGCTGTGCAGCAG-3' and R: 5'-AAAACGGTGCTTATTTCTCTTACCGCGGTTCA GATGTCTGAAGAT-3'; 3'-UTR-PTEN-mt (mutant recombinant plasmid) F: 5'-AACCGCGGTAAGAGAAATAAGCACCGTTTT CCAAG-3' and R: 5'-AAAACGGTGCTTATTTCTCTTACCGC GGTTCAGATGTCTGAAGAT-3'. The SK-NEP-1 cells of the IN and NC groups were seeded in 24-well plates 24 h before transfection. The cells were then transiently transfected with wild-type (pGL3-3'-UTR-PTEN-wt) or mutant (pGL3-3'-UTR-PTEN-mt) reporter plasmid containing potential miR-21 binding sites. Groups were divided as follows:

IN-wt group: The SK-NEP-1 cells of IN group transfected with pGL3-3v-UTR-PTEN-wt;

IN-mt group: The SK-NEP-1 cells of IN group transfected with pGL3-3'-UTR-PTEN-mt;

NC-wt group: The SK-NEP-1 cells of NC group transfected with pGL3-3'-UTR-PTEN-wt; and

NC-mt group: The SK-NEP-1 cells of NC group transfected with pGL3-3'-UTR-PTEN-mt.

The firefly and renilla luciferase activities were determined with a Dual-luciferase assay (Promega, USA) 24 h after transfection.

Statistical analysis

Statistical analysis was conducted using SPSS 19.0 (IBM, USA). Bar graphs were drawn using GraphPad Prism 7 (GraphPad Software, Inc., USA). The expression of each gene was described by the mean value and its range of variability (lower limit, upper limit). The protein expression was described by the mean \pm the standard deviation ($\bar{x} \pm s$). The comparison of two sample means was carried out by the two independent samples t-test, and the comparison of multiple sample means was performed using an analysis of variance (ANOVA). The correlation of miR-21 and PTEN expressions and the clinicopathological parameters was tested by the χ^2 test. The interdependency of miR-21 and PTEN expressions was tested using the Pearson Correlation Coefficient. A difference was considered statistically significant when $P < 0.05$. All experiments were performed in triplicates.

Results

Relative miR-21 expression in WT tissues is significantly higher than that in adjacent non-tumorous tissues

The relative expression level ($2^{-\Delta\Delta Ct}$ method) of miR-21 in the WT and in the non-tumorous tissues was 8.448 (4.844, 14.734) and 1.000 (0.527, 1.899), respectively. Compared with the non-tumorous tissues, the expression level of miR-21 in WT tissues was significantly elevated (As $2^{-\Delta\Delta Ct}$ values were not normally distributed, ΔCt values were used for statistical analysis, $P < 0.01$, Fig. 1A). The median expression level of miR-21 in all 41 patients with WT was 8.658. We divided the patients into two groups according to their miR-21 expression levels using the median score as a cutoff: the high miR-21 expression group ($2^{-\Delta\Delta Ct} \geq 8.658$, $n = 21$) and the low miR-21 expression group ($2^{-\Delta\Delta Ct} < 8.658$, $n = 20$).

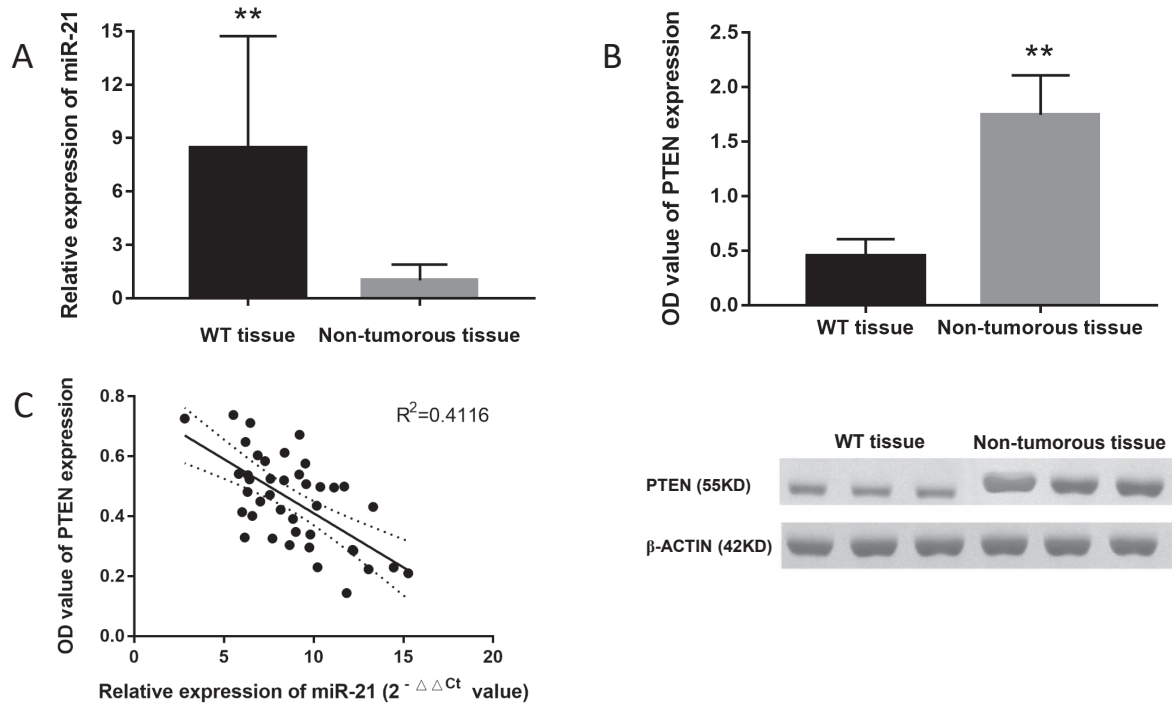


Fig. 1. Expression of miR-21 and PTEN protein in WT tissues.

(A) Relative miR-21 expression in 41 WT tissues and 20 adjacent non-tumorous renal tissues, $**P < 0.01$. (B) Relative PTEN expression in WT tissues and adjacent non-tumorous renal tissues. Top panel: the OD values of PTEN protein expression in 41 WT tissues and 20 adjacent non-tumorous renal tissues, $**P < 0.01$. Bottom panel: electrophorogram showing 3 typical sets of WT tissues and adjacent non-tumorous renal tissues. (C) Scatter plot and simple linear regression between miR-21 and PTEN expression in 41 WT tissues, $R^2 = 0.412$, $P < 0.01$.

PTEN protein expression in WT tissues is significantly lower than that in adjacent non-tumorous tissues

Fig. 1B shows the qualitative and quantitative analysis results of PTEN protein expression. Compared to the non-tumorous tissues (OD value = 1.744 ± 0.364), the WT tissues (OD value = 0.451 ± 0.151) expressed significantly lower levels of PTEN ($P < 0.01$). Similarly, in regards to miR-21, we divided the patients into two groups according to the expression levels of PTEN protein using the median score (0.471) as a cutoff: the high PTEN expression group (OD value ≥ 0.471 , $n = 21$) and the low PTEN expression group (OD value < 0.471 , $n = 20$).

A negative correlation between the expression levels of miR-21 and PTEN in WT tissues

The univariate linear regression showed that the correlation coefficient between miR-21 and PTEN had high statistical significance ($R^2 = 0.412$, $P < 0.01$), and a fitting linear equation was achieved: $y = -0.0361x + 0.771$ (independent variable x : miR-21 expression; dependent variable y : PTEN expression) (Fig. 1C).

Correlation between miR-21 and PTEN protein expression and clinicopathological parameters of WT

To analyze the Correlation between miR-21 and PTEN protein expression and clinicopathological parameters of the WT patients, we divided the high and low miR-21/

PTEN expression groups according to the median score as described above. Table 1 summarizes the correlation of miR-21 and PTEN protein expression with certain clinicopathological parameters including sex, age, clinical stage, histopathological tumor type and lymphatic metastasis. χ^2 test showed that the overexpression of miR-21 had a significant correlation with age (> 24 months), unfavorable histology (UH) tumor type and lymphatic metastasis; the low PTEN protein level was significantly associated with age (> 24 months), late clinical stage (stage III) and UH histopathological tumor type. No statistical correlation was found between the expression of miR-21 and PTEN protein and sex.

The proliferation of SK-NEP-1 WT cells is significantly inhibited after transfection with miR-21 inhibitor

Compared to the NC and MOCK groups (Fig. 2A), the OD value of the IN group of SK-NEP-1 WT cells significantly decreased after 48 h ($P < 0.01$) and 72 h ($P < 0.01$). The values of IN, NC, and MOCK groups after 48 h were 0.616 ± 0.052 , 1.069 ± 0.087 , and 0.989 ± 0.026 , respectively, and those after 72 h were 0.853 ± 0.101 , 1.708 ± 0.034 , and 1.820 ± 0.045 , respectively. No significant difference was found between the NC and MOCK groups ($P > 0.05$, Fig. 2A).

Table 1. Correlation of miR-21 and PTEN protein expression levels with clinicopathological parameters of WT patients.

Clinicopathologic Parameters	n (%)	miR-21		χ^2	P	PTEN		χ^2	P
		High	Low			High	Low		
Sex				1.172	0.279			0.158	0.691
Male	24 (58.5)	14	10			11	13		
Female	17 (41.5)	7	10			10	7		
Age (months)				4.193	0.041			8.789	0.003
> 24	19 (46.3)	13	6			5	14		
≤ 24	22 (53.7)	8	14			16	6		
Stage				3.840	0.05			4.668	0.031
I + II	29 (70.7)	12	17			18	11		
III	12 (29.3)	9	3			3	9		
Histopathological type				8.497	0.004			6.034	0.014
Favorable (FH)	28 (68.3)	10	18			18	10		
Unfavorable (UH)	13 (31.7)	11	2			3	10		
Lymphatic metastasis				4.385	0.036			0.666	0.414
Yes	10 (24.4)	8	2			4	6		
No	31 (75.6)	13	18			17	14		

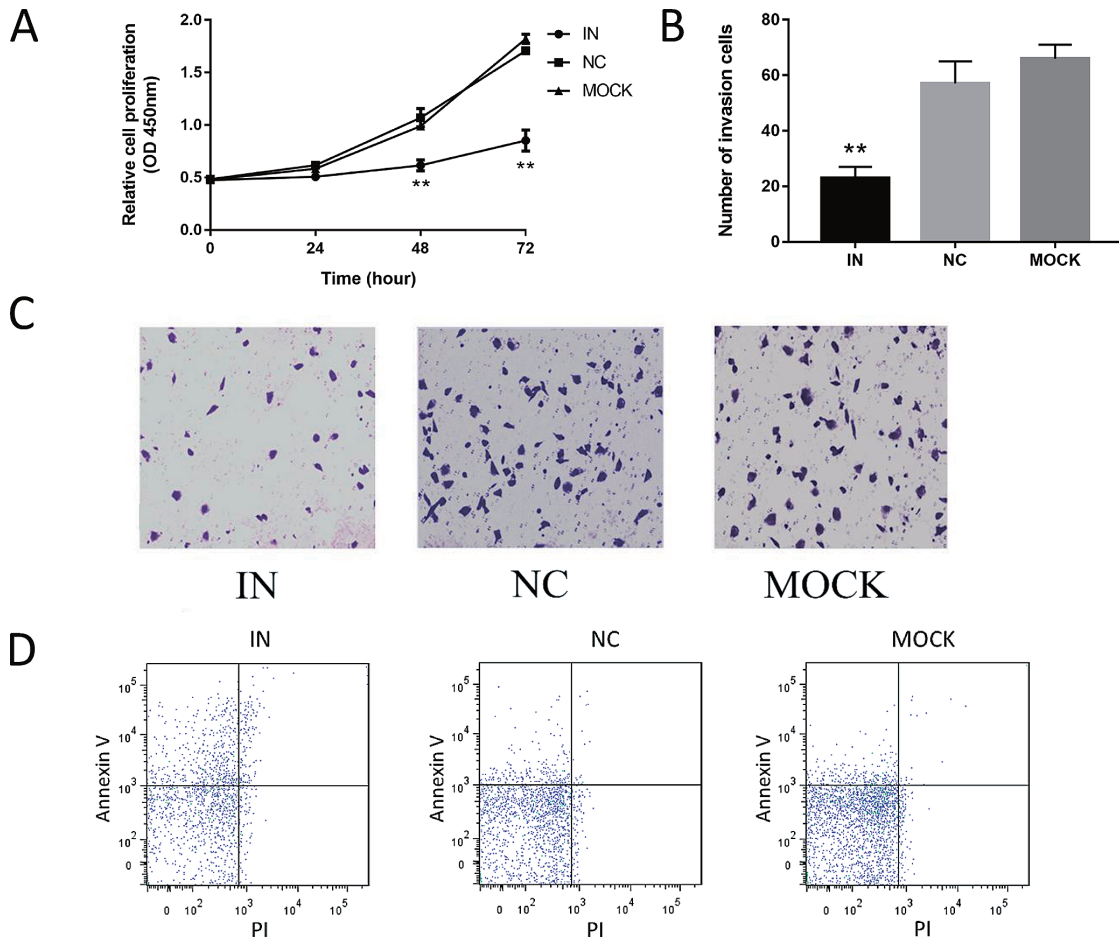


Fig. 2. The biological behavior of SK-NEP-1 WT cells after transfection with miR-21 inhibitor.

(A) Cell growth curve showing cell proliferation after 24, 48 and 72 h (** $P < 0.01$). (B) and (C) Transwell assay showing cell invasion ($P = 0.337$) (D) Flow cytometry analysis showing the early apoptosis rate (** $P < 0.01$).

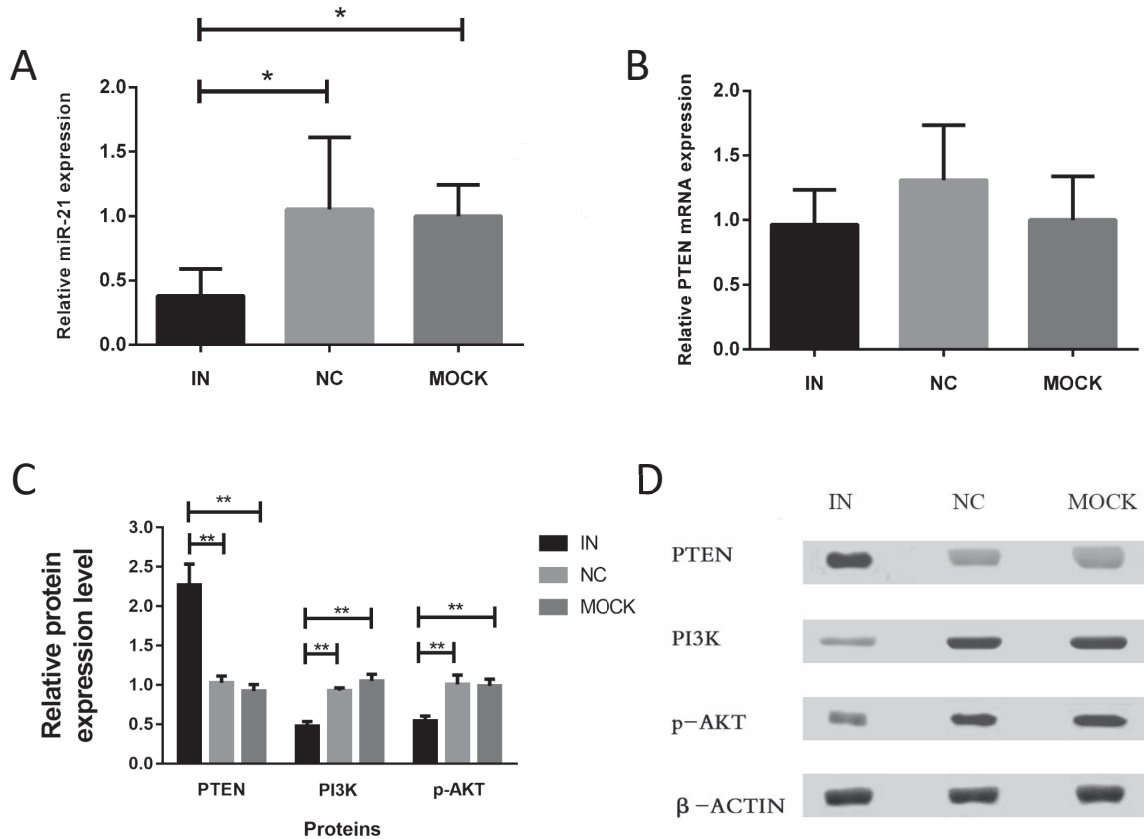


Fig. 3. Expressions of miR-21 and PTEN/PI3K/p-AKT in SK-NEP-1 WT cells after transfection with miR-21 inhibitor. (A) Relative miR-21 expression level in SK-NEP-1 cells after transfection. * P (IN vs. NC) = 0.033, * P (IN vs. MOCK) = 0.041, P (NC vs. MOCK) = 1.000. (B) Relative PTEN mRNA expression level in SK-NEP-1 cells after transfection. P (IN vs. NC) = 0.250, P (IN vs. MOCK) = 1.000, P (NC vs. MOCK) = 0.347. (C) Relative qualitative expression of PTEN/PI3K/p-AKT proteins in SK-NEP-1 cells after transfection. ** P < 0.01. (D) The Western blots of PTEN/PI3K/p-AKT protein (typical one blot of each group).

The invasion of SK-NEP-1 WT cells is significantly inhibited after transfection with miR-21 inhibitor

Transwell invasion assay showed that the number of invading cells in the IN group (23 ± 4.04) significantly decreased as compared to the NC (57 ± 8.00) and MOCK groups (66 ± 5.00) (P < 0.01). No statistical difference was found between the NC and MOCK groups (P = 0.337, Fig. 2B, C).

The apoptosis of SK-NEP-1 WT cells is promoted after transfection with miR-21 inhibitor

Flow cytometry showed that after transfected with miR-21 inhibitor for 48 h, the early apoptotic rate (%) of the IN group (21.37 ± 1.68) was significantly higher than the NC (3.21 ± 0.55) and MOCK groups (3.96 ± 0.36) (P < 0.01, Fig. 2D). No significant difference in the apoptotic rate was detected between the NC and MOCK groups (P > 0.05).

Expression of miR-21 in SK-NEP-1 WT cells after transfection with miR-21 inhibitor

Taking miR-21 expression of MOCK group as a reference, the relative expression level ($2^{-\Delta\Delta Ct}$ method) of miR-

21 in the IN, NC, and MOCK groups was 0.383 (0.247, 0.592), 1.077 (0.678, 1.711) and 1.000 (0.858, 1.166), respectively. Compared to the MOCK group and the NC group, the expression level of miR-21 in the IN group significantly decreased after transfection with miR-21 inhibitor (ΔCt value, P < 0.05, Fig. 3A).

PTEN mRNA and protein expression in SK-NEP-1 WT cells after transfection with miR-21 inhibitor

In SK-NEP-1 WT cells, the mRNA expression level of PTEN showed no statistical differences among the IN, NC and MOCK groups (P > 0.05, Fig. 3B). However, the protein expression level of PTEN in the IN group increased significantly (P < 0.05) while presenting no apparent differences between the other two control groups (P > 0.05, Fig. 3C, D).

Expression of proteins in the downstream PTEN/PI3K/AKT signaling pathway in SK-NEP-1 WT cells after transfection with miR-21 inhibitor

After transfected with miR-21 inhibitor, the relative expression levels of PTEN/PI3K/p-AKT proteins in three study groups are shown in Table 2. The relative expression

Table 2. The relative expression of PTEN/PI3K/p-AKT proteins in SK-NEP-1 cells after transfection with miR-21 inhibitor

	Relative protein expression in the different groups			<i>P</i> (IN vs. NC)	<i>P</i> (IN vs. MOCK)
	IN	NC	MOCK		
PTEN	2.266 ± 0.267	1.027 ± 0.085	0.923 ± 0.083	< 0.001	< 0.001
PI3K	0.477 ± 0.058	0.928 ± 0.034	1.051 ± 0.084	< 0.001	< 0.001
p-AKT	0.545 ± 0.061	1.008 ± 0.117	0.988 ± 0.084	0.002	0.003

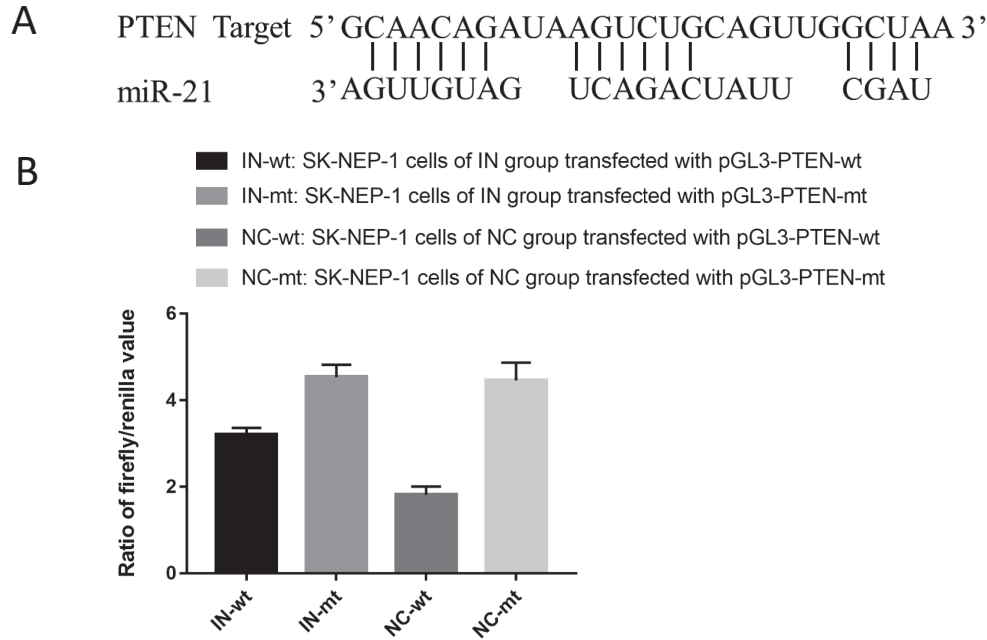


Fig. 4. Changes in relative luciferase activity after transfection with miR-21 inhibitor.

(A) Complementarity between miR-21 and the 3'-UTR of PTEN. (B) Dual luciferase activity of PTEN 3'-UTR after transfection with miR-21 inhibitor. P (IN-wt vs. NC-wt) = 0.002; P (IN-wt vs. IN-mt) = 0.002; and P (IN-mt vs. NC-mt) = 1.000.

of the proteins is qualitatively shown in Fig. 3C, and the Western blot of these proteins is shown in Fig. 3D. Results demonstrated that after the inhibition of miR-21 expression, the protein expression level of PTEN increased, while the expression levels of downstream signaling pathway proteins PI3K and p-AKT decreased, with statistically significant differences.

Changes in relative luciferase activity (3'-UTR-PTEN activity) after transfection with miR-21 inhibitor

As predicted by miRanda and TargetScan, there was complementarity between miR-21 and the 3'-UTR of PTEN (Fig. 4A). In the IN-wt group, relative luciferase activity was significantly higher than that in the NC-wt group ($P = 0.002$). Meanwhile, the relative luciferase activity of the IN-wt group was significantly lower than that of the IN-mt group ($P = 0.002$). The relative luciferase activity between the IN-mt and NC-mt group showed no statistical differences ($P = 1.000$, Fig. 4B). These results indicated that miR-21 directly targeted the 3'-UTR region of PTEN and repressed its expression.

Discussion

The occurrence and development of WT is a complex multi-step process involving multiple factors, stages and genes. During WT progression, the activation of oncogenes and deletion or inactivation of tumor suppressor genes coexisted (Gadd et al. 2012). Moreover, *in vivo* and *in vitro*, studies on numerous tumors have confirmed that miR-21 is one of the representative oncogenes among miRNAs (Si et al. 2007). MiR-21 is highly expressed in almost all reported solid tumors (Krichevsky and Gabriely 2009), and plays a very important role in the occurrence and development of tumors. Additionally, miR-21 is closely related to the growth, proliferation, apoptosis, cell cycle, invasion and metastasis of tumor cells (Ma et al. 2011).

Meng et al. (2006, 2007) first demonstrated that miR-21 can regulate the tumor suppressor gene PTEN in bile duct carcinoma and hepatocellular carcinoma cells and promote the invasion and migration of tumor cells, which showed that PTEN is a latent target gene of miR-21. Later PTEN was proved to be a target gene of miR-21 in other tumors (Zhang et al. 2010; Li et al. 2013b; Liu et al. 2013; Xiong et al. 2013; Mabuchi et al. 2015; Chen et al. 2016).

Our study determined a significantly negative correlation between the expression of miR-21 and the PTEN protein in WT tissues. The high miR-21 level had a significant correlation with age (> 24 months), UH tumor type and lymphatic metastasis; the low PTEN protein level was significantly associated with age (> 24 months), late clinical stage and UH tumor type. These results suggest that high miR-21 level and low PTEN protein level may be valuable predictive biomarkers for tumor progression and poor prognosis of WT patients. Furthermore, our study using the SK-NEP-1 WT cell line confirmed that miR-21 promoted cell growth of WT by repressing the target gene of PTEN.

Transcript profiling studies of miRNA expression levels in tumor samples and cancer cell lines have revealed that miR-21 is over-expressed in almost all solid tumor cell lines or tissues (Selcuklu et al. 2009). Some functional studies conducted with miR-21 have also strongly suggested that miR-21 demonstrates its oncogenic activity by promoting the proliferation, growth and invasion of tumor cells. Löffler et al. (2007) reported that ectopical overexpression of miR-21 in myeloma cells in the absence of IL-6 significantly reduced their apoptosis levels. Asangani et al. (2008) first reported that miR-21 induces invasion, intravasation and metastasis in 10 colorectal cell lines via reducing the PDCD-4 protein. Zhang et al. (2010) reported that in non-small cell lung cancer (NSCLC) cells, miR-21 promoted tumor growth and invasion through inhibiting the expression of the tumor suppressor gene PTEN at the post-transcriptional level. Our study demonstrated that the lower expression of miR-21 can significantly promote growth of SK-NEP-1 WT cells, including inhibiting proliferation and invasion but increasing apoptosis. This strongly suggests that miR-21 has some oncogenic activity in WT cells.

The oncogenic activity of miR-21 on biological behavior of tumor cells is mainly by regulating downstream target genes (Xu et al. 2014). Downstream target genes of miR-21 that have been experimentally confirmed include PDCD4, RECK, TIMP-3, and TPM1 which are involved in NSCLC, gastric cancer, cervical cancer, and colorectal cancer. PTEN was the first tumor suppressor gene identified with phosphatase activity (Salmena et al. 2008). PTEN plays a role in suppressing tumors through a variety of pathways, and it particularly suppresses multiple oncogenic signaling pathways (McCabe et al. 2016), such as the PI3K/AKT signaling pathway (Chalhoub and Baker 2008) and the FKBP12-rapamycin associated protein (FRAP)/mammalian target of rapamycin signaling pathway (mTOR) signaling pathway (Zhang et al. 2013). Our study determined that in WT tissues, miR-21 expression had a negative correlation with PTEN protein expression, while in SK-NEP-1 WT cells, miR-21 inhibition caused an increase in PTEN protein level, but the change in PTEN mRNA level was not obvious, which confirmed that miR-21 silences the expression of PTEN at post-transcriptional level. MiR-21 inhibition caused an increase in the fluorescence activity of wild-type 3'-UTR-PTEN, while there was no effect on the

fluorescence activity of mutant 3'-UTR-PTEN, verifying that PTEN is one of the direct target genes of miR-21 in WT cells.

PTEN, as a downstream target gene of miR-21 and a main inhibitor of the PI3K/AKT signaling pathway, can specifically dephosphorylate the 3' phosphoric acid of phosphatidylinositol 3,4,5-trisphosphate (PIP3), leading to the inhibition of the PI3K/AKT cell signal transduction pathway (McCabe et al. 2016). PI3K/AKT is an important tumor-promoting pathway that can promote the growth of tumor cells, increase glucose metabolism and enhance cell viability (Porta et al. 2014). Genetic mutations or deletions of PTEN have been identified in a variety of malignant tumors, including WT (Song et al. 2012). In this research, we studied the correlation between miR-21 and PTEN in WT cells and further confirmed the inhibitory effect of PTEN on the PI3K/AKT signaling pathway in WT cells. In addition, the inhibition of miR-21 expression caused the high expression of PTEN and decreased the PI3K and p-AKT protein expression levels, which was consistent with the research results of the PI3K/AKT signaling pathway in other tumors.

As for miR-21, located in the upstream of PTEN, the mechanism of its negative regulation of PTEN is still not clear. Currently, most studies on the regulation mechanisms of miR-21 in tumor cells mainly focus on the binding sites of miR-21 and the 3'-UTR of downstream target genes, which may be one of the important mechanisms by which miR-21 represses PTEN expression at the post-transcriptional level (Zhang et al. 2010; Tavano et al. 2012). Our study also obtained consistent results in SK-NEP-1 WT cells, showing that the inhibition of miR-21 expression can directly lead to an increase in the protein expression of PTEN via binding to the 3'-UTR-PTEN.

Although a large number of studies, including our study, indicate that in most solid tumors, miR-21 promotes the proliferation and metastasis of tumor cells by targeted inhibition of PTEN, a few investigators have reported conflicting results (Xiong et al. 2013). Hatley et al. (2010) reported that PTEN is not a target gene of miR-21 in NSCLC. Cheng et al. (2005) reported that inhibiting miR-21 in HeLa cells could significantly accelerate cell growth. Folini et al. (2010) found that the knockdown of miR-21 expression in prostate cancer cells had no effect on proliferation, invasion ability or the sensitivity to radiotherapy and chemotherapy in prostate cancer cells and did not regulate the expression of the tumor suppressors PTEN and PDCD-4. These findings indicated that the oncogene properties of miR-21 may have some tissue or cell specificity, and the targeting regulatory relationship between miR-21 and PTEN was not in a simple one-to-one correspondence model. How the complex regulatory signaling network involving miR-21 and PTEN leads to the occurrence and development of WT and its relevant mechanisms is yet to be fully elucidated.

In conclusion, our results have shown that (1) the

higher levels of miR-21 and lower levels of PTEN have a possible correlation with certain clinicopathological parameters of WT, including age (> 24 months), late clinical stage, unfavorable histopathological type and lymphatic metastasis; (2) miR-21 affects biological behaviors, including cell proliferation and invasion promotion but apoptosis inhibition, through directly targeting and negatively regulating PTEN at the post-transcriptional level in SK-NEP-1 WT cells. This novel miR-21/PTEN axis may provide new insights into the mechanisms underlying WT, and miR-21 may become a valuable predictive biomarker for tumor progression and poor prognosis and potential therapeutic candidate for the treatment of WT in the future.

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Conflict of Interest

The authors declare no conflict of interest.

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