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MicroRNA-1271 inhibits the progression of papillary thyroid carcinoma by targeting IRS1 and inactivating AKT pathway

Y. CHEN, S.-A. HAO, Y. JIANG, B. GAO, W.-G. TIAN, S. ZHANG, L.-J. GUO, L.-L. WANG, D.-L. LUO

Department of Breast, Thyroid Surgery, Research Institute of Surgery, Daping Hospital, Army Military Medical University, Chongqing, China

Yi Chen and *Shuai Hao* contributed equally to this work

Abstract. – OBJECTIVE: The important role of microRNA-1271 (miR-1271) has been identified in human diseases and cancers. However, the biological function of miR-1271 remains ambiguous in papillary thyroid carcinoma (PTC). Therefore, the specific role of miR-1271 was investigated in PTC.

PATIENTS AND METHODS: The expressions of miR-1271 and insulin receptor substrate 1 (IRS1) were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. The protein expression of the genes was measured by Western blot analysis. The function of miR-1271 was investigated using methyl thiazolyl tetrazolium (MTT) and transwell assays. The Dual-Luciferase assay was used to observe the relationship between miR-1271 and IRS1.

RESULTS: MiR-1271 was downregulated in PTC tissues. Moreover, overexpression of miR-1271 suppressed migration, invasion and proliferation of PTC cells. Furthermore, IRS1 was indicated as a direct target gene of miR-1271 and knockdown of IRS1 inhibited cell migration, invasion and proliferation in PTC. In addition, miR-1271 inhibited the progression of PTC by targeting IRS1. Besides that, miR-1271 blocked the epithelial-mesenchymal transition (EMT) and protein kinase B (AKT) pathway in PTC.

CONCLUSIONS: MiR-1271 inhibited the progression of PTC by targeting IRS1 and blocking EMT and AKT pathway.

Key Words:
MiR-1271, Papillary thyroid carcinoma, IRS1, AKT pathway.

Introduction

Papillary thyroid carcinoma (PTC) is the most common and minimally malignant. About half of thyroid cancers occur in children or young (pre-40) women[1]. Tumors of PTC are usually small and slow to develop, but metastasis can occur early. Moreover, the first lesion often found may be metastases[2]. Once diagnosed or highly suspected of thyroid cancer, patients often require early surgical treatment, which can make surgery easier and also inhibit cancer cell proliferation[3]. In addition, the prognosis of different thyroid cancers is very different. If the treatment is reasonable and timely, the life span is almost the same as that of normal people. However, for highly malignant undifferentiated thyroid cancer, the prognosis is extremely poor and patients usually die within six months[4]. Worst of all, the cause of thyroid cancer is not well understood, and may be related to dietary factors (high iodine or iodine deficiency diet), the history of irradiation exposure, increased estrogen secretion and genetic factors[5]. Therefore, it is urgent for us to understand the pathogenesis of PTC.

MicroRNAs (miRNAs) have recently received increasing attention due to specific functions. Previous studies[6] have shown that miRNAs regulate cell proliferation, cell death and tumorigenesis in many human cancers. Moreover, several miRNAs have been reported to exhibit different effects on the progression of PTC. For instance, miR-144-3p promoted tumor growth and metastasis of PTC by targeting paired box gene 8[7]. In contrast, Li et al[8] reported that miR-361-5p inhibited the progression of PTC by targeting ROCK1. In this work, we focused on the function of miR-1271 because it has conflicting roles in human cancers. Wang et al[9] found that miR-1271 promoted proliferation and invasion of non-small-cell lung cancer cells

Corresponding Author: Donglin Luo, MD; e-mail: luodl968@163.com

via targeting HOXA5. Xiang et al[10] suggested that miR-1271 inhibited gastric cancer cell proliferation, invasion and epithelial-mesenchymal transition (EMT) *via* targeting FOXQ1. These studies show that the role of miR-1271 depends on the type of cancer. Furthermore, the function of miR-1271 has not been confirmed in previous studies.

As a member of Insulin receptor substrates (IRSs) family, overexpression of insulin receptor substrate 1 (IRS1) was found to promote mammary tumorigenesis and metastasis[11]. IRS1 as an oncogene had been identified in many human cancers, such as endometrial cancer[12], breast cancer[13] and colorectal carcinoma[14]. It had been reported[15] that IRS1 activated the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, which was critical for the development and progression of human cancers. The AKT pathway also has a major impact on the development of PTC. For example, miR-34a targeted GAS1 to promote cell proliferation and inhibit apoptosis in PTC *via* the PI3K/AKT pathway[16]. Therefore, the interaction between IRS1 and AKT pathway was investigated in PTC.

In this work, the alternation of miR-1271 expression was detected in PTC. Moreover, the effect of miR-1271 on migration, invasion and proliferation of PTC cells was explored by the regulatory mechanism involved in IRS1. Furthermore, the effect of miR-1271 on EMT and AKT pathway was examined in PTC.

Patients and Methods

Clinical Tissues

Forty-four human PTC tissues and adjacent normal tissues were acquired from the Daping Hospital, Army Military Medical University. None of the PTC patients received treatment before surgery. These PTC patients provided informed consents. The tissues were frozen in liquid nitrogen and then stored in a -80°C refrigerator. This study was approved by the Institutional Ethics Committee of Daping Hospital, Army Military Medical University.

Cell Lines Culture

The TPC-1, K1, BCPAP cell lines and the human thyroid epithelial cell line Nthyori3-1 were used for this experiment. These cell lines were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were then seeded in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The growth condition included 5% CO_2 and 37°C.

Cell Transfection

The miR-1271 mimics or inhibitor and negative control (NC) were obtained from RiboBio (Guangzhou, China). The IRS1 overexpression plasmid and IRS1 siRNA were purchased from GenePharma (Shanghai, China). They were then transferred to TPC-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively, based on the manufacturers' protocols.

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

Total ribonucleic acids (RNA) in non-small-cell lung cancer (NSCLC) were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The synthesis of complementary deoxyribonucleic acid (cDNA) was performed using PrimeScript reverse transcription (RT) Master Mix (TaKaRa, Otsu, Shiga, Japan). We performed quantitative Real Time-Polymerase Chain Reaction using the SYBR PrimeScript miRNA qRT-PCR Kit (TaKaRa, Otsu, Shiga, Japan) on the ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA). The forward primer for miR-1271 was 5'-CTTGGCACCTAGCAAGCACTCA-3', and the reverse primer was 5'-TATGGTTGTTCTCCTCTCTGTCTC-3'. The internal control for miR-1271 was GAPDH (forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'). The primers for IRS1 were 5'-CAACTGGACATCACAGCAGAA-3 (forward) and 5'-ACTGAAATGGATGCATCGTACC-3 (reverse). The internal control was U6 (forward, 5'-CTCGCTTCGGCAGCACA-3'; reverse, 5'-AACGCTTCACGAATTTGCGT-3') and their expressions were calculated using the $2^{-\Delta\Delta ct}$ method.

Methyl Thiazolyl Tetrazolium (MTT) Assay

TPC-1 cells transfected with miR-1271 mimics/inhibitor or negative control (NC) were incubated at a density of 4×10^3 cells in 96-well plates (Corning, Lowell, MA, USA). At 24, 48, 72 and 96 h after transfection, transfected TPC-1 cells were incubated for 4 h at 37°C in a serum-free medium containing 1 mg/mL MTT. After re-

moving the MTT solution, they were dissolved in 100 μL of dimethyl sulfoxide. Finally, the absorbance was read at 490 nm on a microplate spectrophotometer (Thermo Labsystems, Vantaa, Finland).

Transwell Assay

The transwell chambers (8-μm pore size membranes) were used for cell migration and invasion assays. The lower chamber was added with 10% FBS and incubated with 5% CO_2 at 37°C. The upper surface with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for cell invasion. At the same time, cell migration assay was performed without Matrigel. Next, 2×10^3 TPC-1 cells with miR-1271 mimic or inhibitor were cultured in the upper chamber with the serum-free medium. After 48 hours, the migrated or invaded cells were fixed with methanol and stained with crystal violet. Finally, we calculated the number of removed cells using a microscope.

Dual-Luciferase Assay

The 3'-Untranslated Region (3'-UTR) of wild or mutant type IRS1 was inserted into plasmid complementary deoxyribonucleic acid3.1 (pcDNA3.1) plasmid vector (Promega, Madison, WI, USA) for Luciferase reporter assay. Next, the pcDNA3.1 vector and miR-1271 mimic were transfected into TPC-1 cells. After 48 h, Luciferase activity was measured by a Dual-Luciferase assay system (Promega, Madison, WI, USA).

Western Blot Analysis

The protein samples were obtained using a radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) lysis buffer. The protein was separated by 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After that, the protein was incubated with 5% skim milk at room temperature. Next, we incubated the membranes with EMT markers (E-cadherin, N-cadherin, Vimentin), AKT pathway markers (AKT, p-AKT), IRS1 and GAPDH antibodies overnight at 4°C. After washing, they were incubated with the corresponding secondary antibody (Abcam, Cambridge, MA, USA) for 2 h at room temperature. Finally, the protein expression levels were measured by electrochemiluminescence (ECL; Pierce, Waltham, MA, USA).

Statistical Analysis

Data were shown as mean ± SD (Standard Deviation). Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 6 (La Jolla, CA, USA) was employed to analyze these data. The correlation between miR-1271 expression and clinic-pathological characteristics of PTC patients was calculated by the Chi-squared test. Comparisons between groups were performed using One-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). Survival curves were drawn by the Kaplan-Meier analysis, and the log-rank test was used to compare survival differences. A significant difference was defined at $p<0.05$.

Results

MiR-1271 Was Downregulated in PTC Tissues

First, the expression level of miR-1271 in PTC tissues was examined by the qRT-PCR assay. The expression of miR-1271 was significantly reduced in PTC tissues compared to normal tissues (Figure 1A). Moreover, abnormal miR-1271 expression was closely associated with lymph nodes metastasis ($p=0.032$) and TNM stage ($p=0.011$, Table I). Furthermore, PTC patients with low expression of miR-1271 had shorter overall survival ($p=0.018$, Figure 1B). These results suggested that miR-1271 might be involved in tumorigenesis of PTC.

MiR-1271 Suppressed the Migration, Invasion and Proliferation of PTC Cells

Next, the expression of miR-1271 was observed in TPC-1, K1, BCPAP and Nthyori3-1 cell lines. Similarly, miR-1271 was downregulated in TPC-1, K1 and BCPAP cell lines compared to Nthyori3-1 cells (Figure 2A). To investigate the function of miR-1271 in PTC, miR-1271 mimics or inhibitor was transfected into TPC-1 cells. We found that miR-1271 mimics significantly enhanced the expression level of miR-1271. In contrast, miR-1271 inhibitor reduced the expression of miR-1271 in TPC-1 cells (Figure 2B). Functionally, the proliferation of TPC-1 cells was markedly suppressed by overexpression of miR-1271 (Figure 2C). Inversely, knockdown of miR-1271 promoted cell proliferation in PTC (Figure 2D). In addition, cell migration was suppressed by upregulation of miR-1271 and promoted by

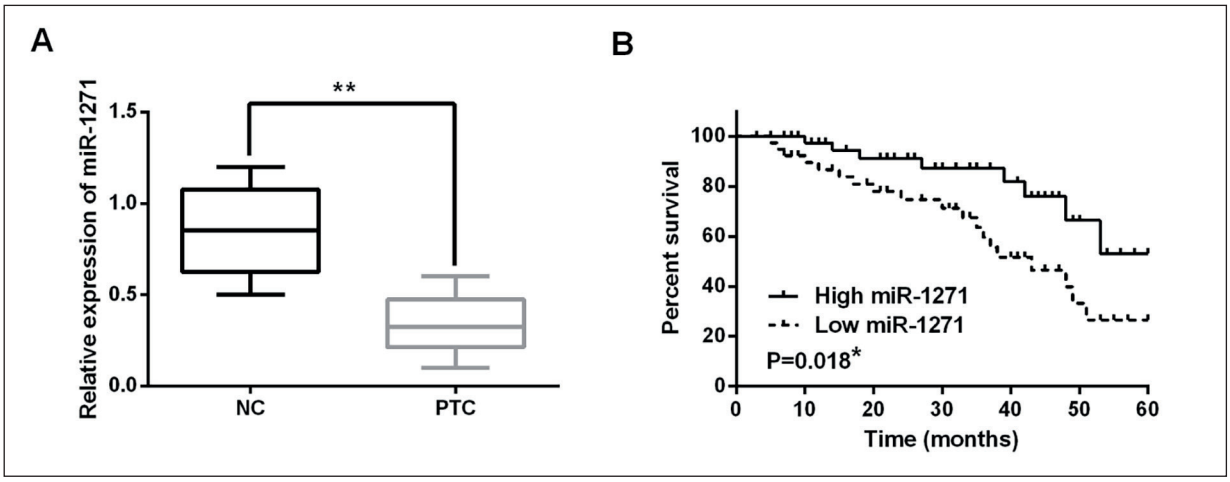

Figure 1. MiR-1271 was downregulated in PTC tissues. **A,** The expressions of miR-1271 in PTC tissues. **B,** Low miR-1271 expression was correlated with shorter overall survival of PTC patients. *p<0.05, **p<0.01.

downregulation of miR-1271 in TPC-1 cells (Figure 2E). Similarly, miR-1271 mimics inhibited invasion of TPC-1 cells, and miR-1271 inhibitor enhanced the invasive ability of TPC-1 cells (Figure 2F). In summary, miR-495 functioned as a tumor inhibitor in the progression of PTC.

IRS1 Was a Direct Target Gene of MiR-1271

Furthermore, IRS1 was selected as a target gene of miR-1271. TargetScan database (http://www.targetscan.org/) shows that miR-1271 has bind sites with the 3'-UTR of IRS1 (Figure 3A). To confirm this prediction, a Luciferase reporter was performed. MiR-1271 mimics was found to significantly reduce the Luciferase activity of Wt-IRS1. However, the Luciferase activity of Mut-IRS1 was not affected by miR-1271 mimics (Figure 3B). Moreover, a negative correlation between IRS1 and miR-1271 expression was identified in PTC tissues (p<0.0001, R^2=0.6528; Figure 3C). The expression of IRS1 regulated by miR-1271 mimics or inhibitor was then examined in TPC-1 cells to further confirm this corre-

Table I. Relationship between miR-1271 expression and their clinic-pathological characteristics of PTC patients.

Characteristics	Cases	miR-1271		*p*-value
		High	Low	
Age (years)				0.056
≥ 60	20	9	11	
< 60	24	9	15	
Gender				0.121
Male	16	6	10	
Female	28	12	16	
Tumor size (mm)				0.229
≤ 2	18	6	12	
> 2	26	12	14	
Lymph nodes metastasis				0.032*
Yes	30	12	18	
No	14	6	8	
TNM stage				0.011*
I-II	34	13	21	
III-IV	10	5	5	

Statistical analyses were performed by the χ^2-test. *p < 0.05 was considered significant.

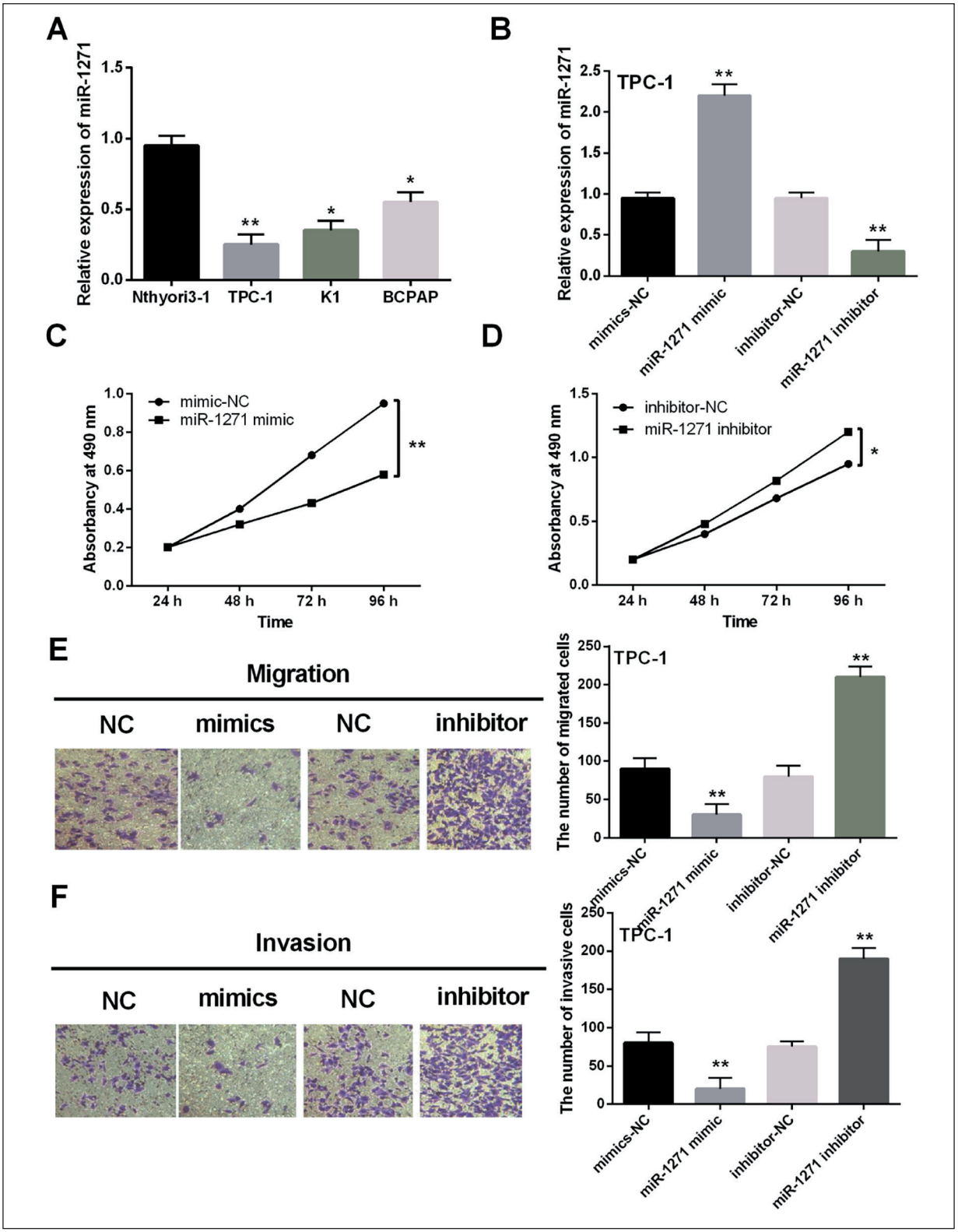

Figure 2. MiR-1271 suppressed migration, invasion and proliferation of PTC cells. **A,** The expression of miR-1271 in TPC-1, K1, BCPAP and Nthyori3-1 cell lines. **B,** The expression of miR-1271 was examined in TPC-1 cells with miR-1271 mimics or inhibitor. **C, D,** Cell proliferation was measured in cells containing miR-1271 mimics or inhibitor. **E, F,** Cell migration and invasion analysis in cells containing miR-1271 mimics or inhibitor (magnification: 40×) *$p<0.05$, **$p<0.01$.

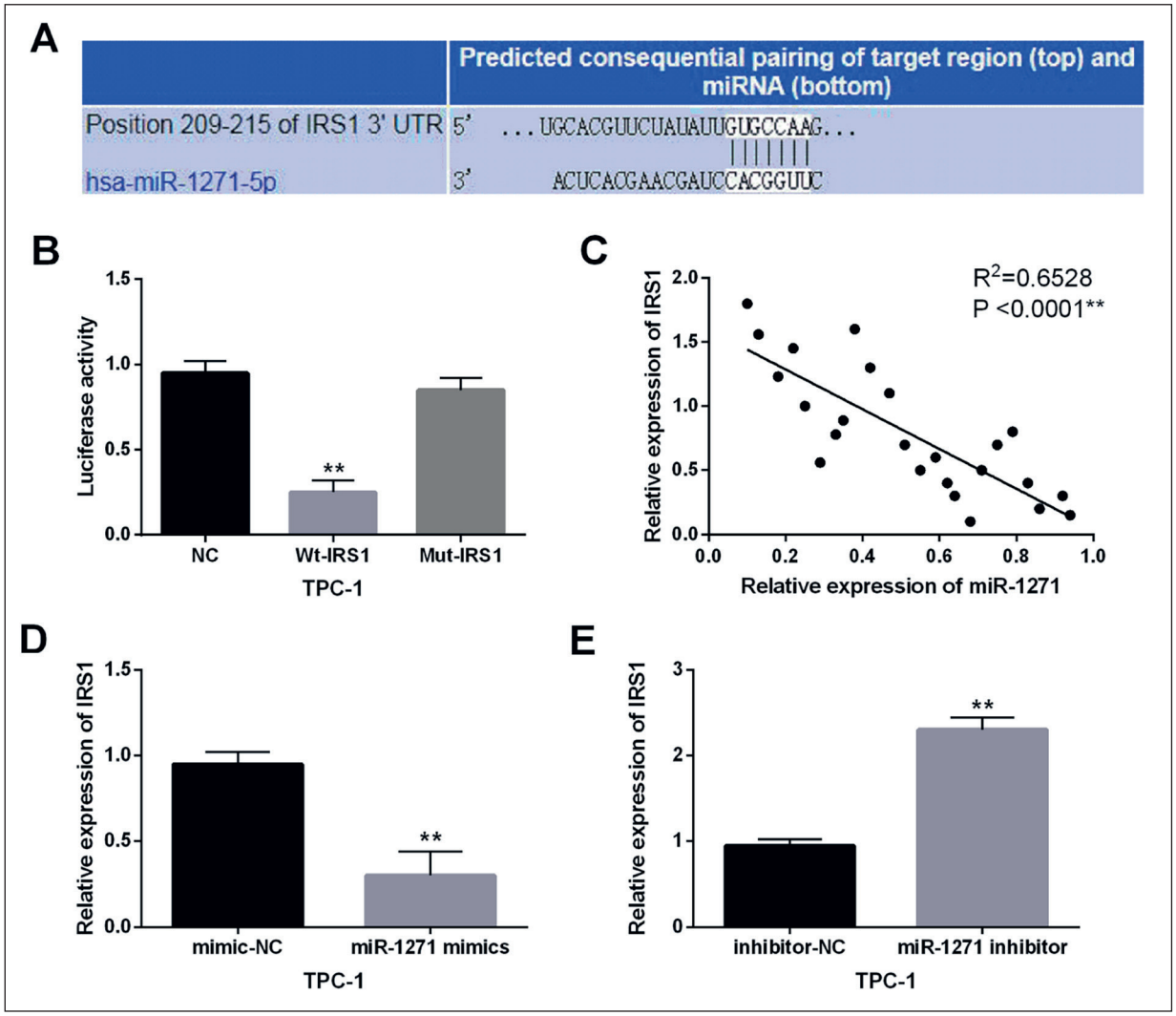

Figure 3. IRS1 was a direct target gene of miR-1271. **A,** IRS1 has binding sites with miR-1271. **B,** Luciferase reporter assay. **C,** MiR-1271 had negative correlation with IRS1 expression. **D, E,** The expression of IRS1 were observed in TPC-1 cells containing miR-1271 mimics or inhibitor $^{**}p<0.01$.

lation. Consistently, miR-1271 mimics reduced IRS1 expression (Figure 3D), and miR-1271 inhibitor promoted IRS1 expression (Figure 3E). IRS1 was a direct target gene of miR-1271, and its expression was negatively regulated by miR-1271 in PTC tissues.

Knockdown of IRS1 Inhibited PTC Cell Migration, Invasion and Proliferation

Subsequently, alternation of IRS1 expression was examined in PTC tissues and cell lines. The results showed that IRS1 was upregulated in PTC tissues compared to normal tissues (Figure 4A). Furthermore, the expression of IRS1 was also increased in TPC-1, K1 and BCPAP cell lines compared to Nthyori3-1 cells (Figure 4B). Next, IRS1 siRNA was transfected into TPC-1 cells to explore its role in PTC. The expression of IRS1 was remarkably decreased by IRS1 siRNA (Figure 4C). We found that knockdown of IRS1 inhibited cell proliferation in PTC (Figure 4D). Moreover, migration of TPC-1 cells was suppressed by knockdown of IRS1 (Figure 4E). Similarly, knockdown of IRS1 inhibited invasion of TPC-1 cells (Figure 4F). Briefly, knockdown of IRS1 inhibited cell migration, invasion and proliferation in PTC.

MiR-1271 Inhibited the Progression of PTC by Targeting IRS1

To confirm the interaction between miR-1271 and IRS1, miR-1271 mimics and IRS1

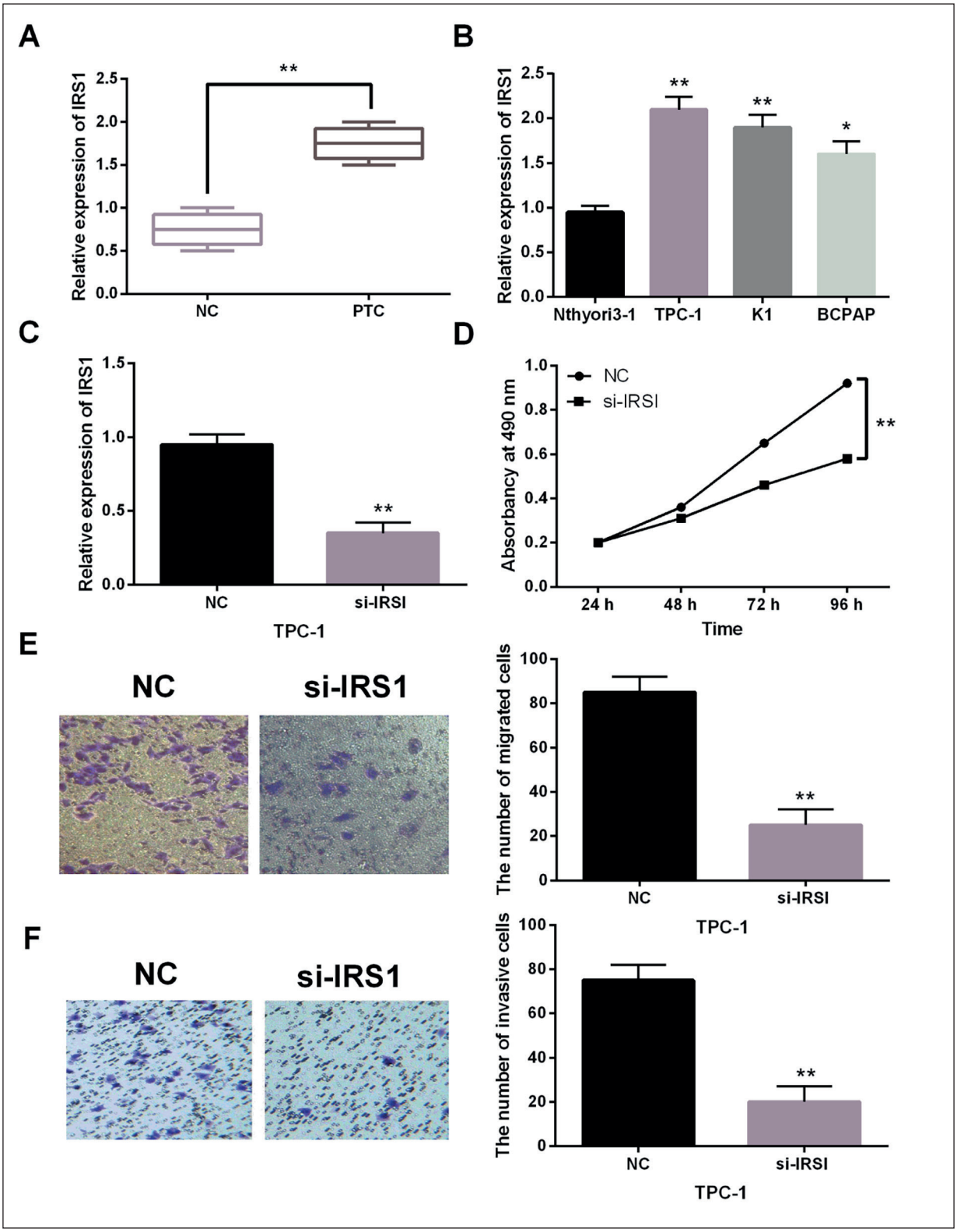

Figure 4. Knockdown of IRS1 inhibited PTC cell migration, invasion and proliferation. **A,** The expressions of IRS1 in PTC tissues. **B,** The expression of IRS1 in TPC-1, K1, BCPAP and Nthyori3-1 cell lines. **C,** The expression of IRS1 was examined in TPC-1 cells with IRS1 siRNA. **D,** Cell proliferation was measured in TPC-1 cells with IRS1 siRNA. **E, F,** Cell migration and invasion analysis in TPC-1 cells with IRS1 siRNA (magnification: 40×). *p<0.05, **p<0.01.

overexpression plasmid were co-transfected into TPC-1 cells. We found that the decreased expression of IRS1 induced by miR-1271 mimics was restored by upregulation of IRS1 (Figure 5A). More importantly, the inhibitory effect of miR-1271 on cell proliferation was hindered by IRS1 vector in PTC (Figure 5B). The same results of cell migration and invasion were also identified in TPC-1 cells (Figure 5C, 5D). Combining these results, we considered that miR-1271 inhibited the progression of PTC by targeting IRS1.

MiR-1271 Blocked EMT and AKT Pathway in PTC

Finally, the effect of miR-1271 on EMT and AKT pathway was investigated in PTC. Primarily, overexpression of miR-1271 was found

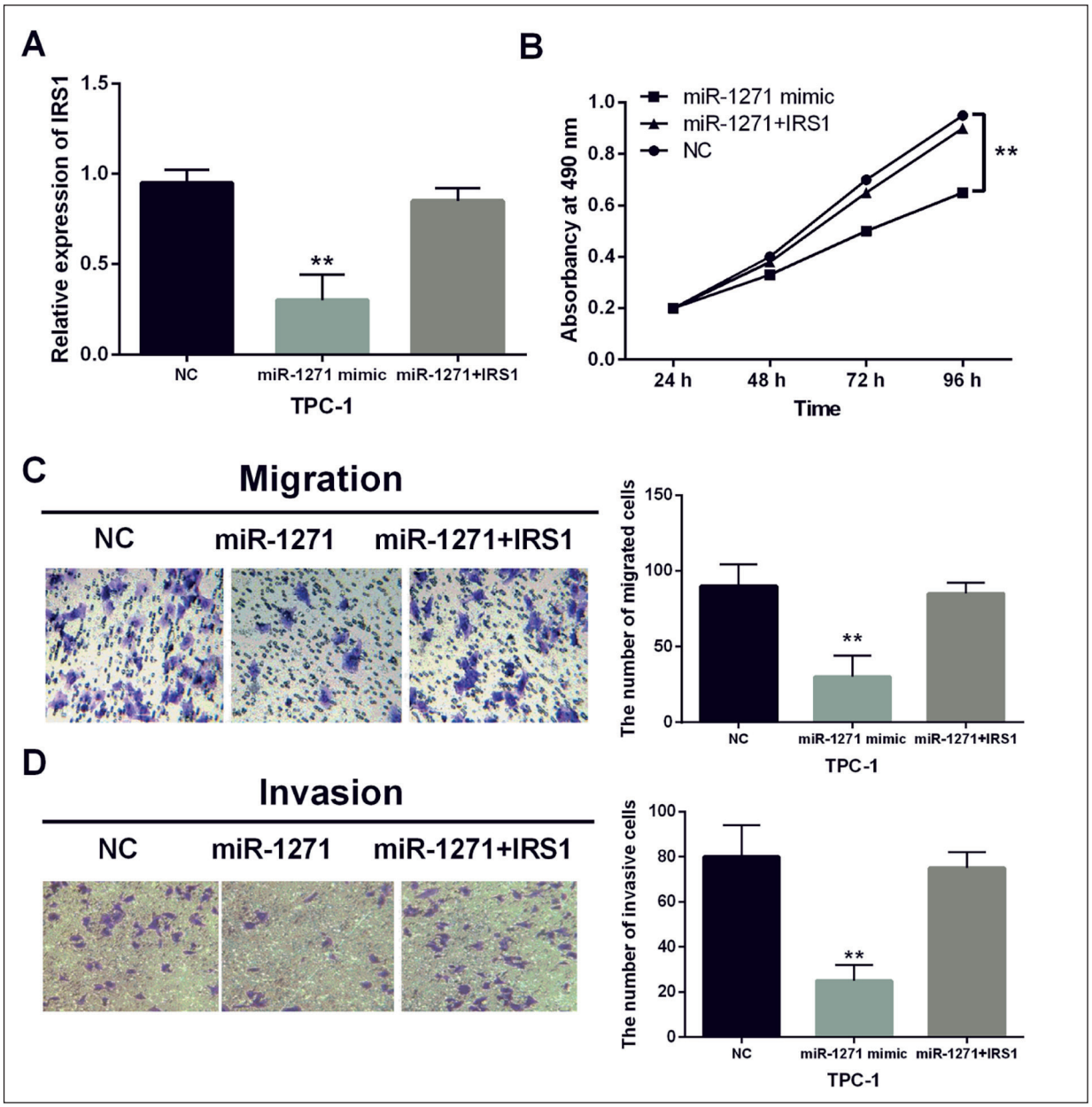

Figure 5. MiR-1271 inhibited the progression of PTC by targeting IRS1. **A,** The expression of IRS1 was measured in TPC-1 cells with IRS1 vector and miR-1271 mimics. **B,** Cell proliferation was measured in TPC-1 cells with IRS1 vector and miR-1271 mimics. **C, D,** Cell migration and invasion in TPC-1 cells with IRS1 vector and miR-1271 mimics (magnification: 40×) $^{**}p<0.01$.

to reduce N-cadherin and Vimentin expressions and enhance E-cadherin expression in TPC-1 cells (Figure 6). In contrast, downregulation of miR-1271 had an opposite effect on the expression of the three markers (Figure 6). As for the AKT pathway, upregulation of miR-1271 repressed the expression of p-AKT (Figure 6), while downregulation of miR-1271 promoted p-AKT expression (Figure 6). However, the expression of AKT was not affected by miR-1271 mimics and inhibitor. These results indicated that miR-1271 blocked EMT and AKT pathway to regulate the progression of PTC.

Discussion

In recent years, some miRNAs have been reported to be involved in tumorigenesis, progression and prognosis of PTC[17,18]. In the current study, miR-1271 was identified as a novel miRNA in PTC. We demonstrated that miR-1271 was a tumor inhibitor in PTC by inhibiting cell migration, invasion and proliferation. In addition, IRS1 was found to take part in the regulatory mechanism of miR-1271 in PTC. Furthermore, the inhibitory effect of miR-1271 on EMT and AKT pathway was identified. Our study revealed that miR-1271 might act as a valuable therapeutic biomarker for PTC.

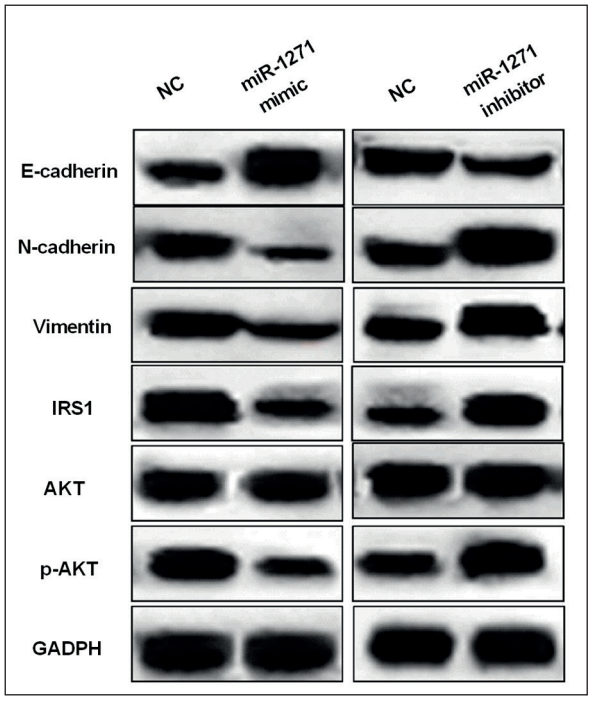

Figure 6. MiR-1271 blocked EMT and the AKT pathway in PTC. Western blot analysis of E-cadherin, N-cadherin, Vimentin, AKT and p-AKT in TPC-1 cells contained miR-1271 mimics or inhibitor.

Many previous researches have reported the dysregulation of miR-1271 in different human cancers. Liu et al[19] demonstrated that the expression of miR-1271 was decreased in pancreatic cancer, and upregulation of miR-1271 inhibited cell migration, invasion and EMT in pancreatic cancer *via* targeting ZEB1 and TWIST1. Here, the downregulation of miR-1271 was also detected in PTC. Furthermore, PTC patients with low expression of miR-1271 had a poor prognosis. Besides that, abnormal miR-1271 expression was closely related to lymph nodes metastasis and TNM stage of PTC patients. Similarly, miA-451 was identified as a prognostic marker for PTC with lymph node metastasis[20]. Functionally, miR-1271 negatively regulated AKT/MTOR signaling and promoted apoptosis *via* targeting PDK1 in pancreatic cancer[22]. It was also demonstrated that miR-1271 functioned as a metastasis and EMT inhibitor in human hepatocellular carcinoma by regulating the PTP4A1/c-Src axis. These studies support the inhibitory effect of miR-1271 in PTC. In addition, miR-1271 was found to inhibit the progression of PTC by targeting IRS1.

In this paper, the upregulation of IRS1 was found in PTC and knockdown of IRS1 inhibited cell migration, invasion and proliferation in PTC. Same as our results, an increase in IRS1 expression was detected in human hepatocellular carcinoma[23]. Moreover, overexpression of IRS1 promoted proliferation and invasion of hepatocellular carcinoma cells[24]. Furthermore, miR-1271 regulated cisplatin resistance in human gastric cancer by targeting IRS1[25], which was consistent with our results. Besides that, IRS1 has been reported to activate the AKT pathway, which is critical for the oncogenesis of human cancers[26]. Phosphorylated AKT has been reported to inhibit apoptosis and promote cellular proliferation[27]. Our work also showed that miR-1271 inhibited the phosphorylation of AKT, thereby regulating the proliferation of PTC cells. Luan et al[28] proposed that miR-126 acted as a tumor suppressor in glioma cells by targeting IRS1 and inactivating the PI3K/AKT pathway. In this report, we found that overexpression of miR-1271 suppressed IRS1 expression and inactivated the AKT pathway in PTC. These findings suggested that miR-1271 inhibited the progression of PTC by targeting IRS1 and blocking the AKT signaling pathway.

Conclusions

MiR-1271 functioned as a tumor inhibitor in the pathogenesis of PTC. Upregulation of miR-1271 suppressed migration, invasion and proliferation of PTC cells by targeting IRS1 and blocking EMT and AKT pathway. These findings suggest that miR-1271 may be a valuable therapeutic biomarker for PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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