European Review for Medical and Pharmacological Sciences 2018; 22: 3804-3811

MiR-381-3p inhibits proliferation, migration and invasion by targeting LRP6 in papillary thyroid carcinoma

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Abstract. – OBJECTIVE: MiR-381-3p plays an essential role in the progression of a variety of cancers, but its expression and role in papillary thyroid carcinoma (PTC) progression have not been investigated. The aim of this study was to investigate the expression of miR-381-3p and its function in PTC.

PATIENTS AND METHODS: The expression levels of miR-381-3p and low-density lipoprotein receptor-related protein 6 (LRP6) mRNA in PTC tissues and cell lines were measured using RT-PCR. Cell proliferation, migration and invasion were assessed by cell viability assay and transwell assay. Luciferase assays and Western blotting were performed to demonstrate miR-381-3p target gene.

RESULTS: We found that miR-381-3p was significantly down-regulated in PTC tissues and cell lines. *In vitro* assay indicated that up-regulation of miR-381-3p significantly suppressed PTC cell proliferation, migration and invasion. Moreover, luciferase reporter gene assay demonstrated that miR-381-3p could target LRP6 by binding to the 3' UTR. Western blot and Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) showed that miR-381-3p overexpression suppressed the expression of LRP6 at both mRNA and proteins levels. In addition, functional experiment confirmed that LRP6 was involved in the suppressive effect of miR-381-3p-mediated PTC on cell proliferation, migration and invasion.

CONCLUSIONS: Our findings suggested, for the first time, that miR-381-3p was lowly expressed in PTC tissues, and its up-regulation inhibited tumorigenesis of PTC by targeting LRP6.

Key Words:
miR-381-3p, Papillary thyroid cancer, LRP6, Proliferation, Migration, Invasion.

Introduction

Thyroid carcinoma is the most common endocrine malignancy, accounting for 5-10% of cancers in women[1]. Papillary thyroid carcinoma (PTC), which represents 70-80% of thyroid carcinoma, is the most common endocrine malignancy in thyroid carcinoma[2]. Although the majority of PTC is effectively managed by surgical resection followed by radioiodine and levothyroxine, the prognosis of about 10% of PTC patients presenting recurrence or metastasis remains poor[3,4]. In order to find better therapeutic strategies to cure PTC, a better understanding of the underlying mechanisms of human PTC development and progression is necessary.

MicroRNAs (miRNAs) are a novel class of short, endogenous, non-coding RNA with 18-25 nucleotides in length[5]. It has been proved that miRNAs regulate genes expression by binding to complementary sites in their 3'-untranslated region (3'-UTR)[6]. Growing evidence[7] indicates that miRNAs influence multiple biological events, especially the development and progression of malignancies. With the advancement of DNA microarray-based miRNA expression profiles, more and more abnormally expressed miRNAs were identified in various tumors[8]. A large number of functional researches revealed that miRNAs functioned as tumor suppressors or oncogenes according to the roles of their target genes[9-11]. Among these miRNAs, miR-381-3p was reported to serve as oncogenic or tumor-suppressive miRNAs based on the types of tumors[12-14]. However, the expression and function of miR-381-3p in PTC have not been reported.

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The low-density lipoprotein receptor-related protein 6 (LRP6), localized on chromosome 12p13.2, is a member of the low-density lipoprotein receptor family[15]. It has been observed that LRP6 is highly expressed in many cancer tissues and serves as a positive regulator in the progression of tumors[16,17]. Growing evidence[18,19] has shown that LRP6 is essential for the activation of Wnt/β-catenin pathway, which is involved in the development and progression of various tumors. Previous research[20] revealed that LRP6 exerted a highly tumor-promoting function in PTC and might serve as an ideal therapeutic target. However, the association between miR-381-3p and LRP6 in PTC has not been investigated.

In the present study, we firstly determined the expression levels of miR-381-3p in PTC tissues and cell lines. The biological function of miR-381-3p was explored by *in vitro* assay. Moreover, whether LRP6 was a direct target of miR-381-3p was also determined. Our results suggested miR-381-3p as an important biomarker and therapeutic target for PTC patients.

Patients and Methods

Patients and Tissue Samples

Fresh frozen tissues from PTC patients were obtained from Affiliated Hospital of Taishan Medical University. No patients had received any adjuvant treatment before the surgery. These samples were immediately frozen and stored in liquid nitrogen. The study was approved by the Research Ethics Committee of Affiliated Hospital of Taishan Medical University (Taishan, China), and written informed consents were obtained from these patients.

Cell Culture and Transfection

Human PTC cell lines (TPC-1, BCPAP, K1) and the human thyroid epithelial cell line Nthy-ori3-1 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All above cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (Invitrogen, Carlsbad, CA, USA) with 0.023 IU/ml insulin and 10 % fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) in 5% CO_2 cell culture incubator.

The miR-381-3p mimic (miR-381-3p) and mimic negative control (NC) were purchased from GenePharma (Huangpu, Shanghai, China). The coding sequences LRP6 were amplified by PCR and inserted into pcDNA3.1 vector to generate LRP6 overexpression vectors. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection.

RNA Extraction and RT-PCR

Total RNA from the cultured cells and tissues samples were extracted by TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The RNA was then purified using a RNeasy Mini Kit (Qiagen, Haidian, Beijing, China) following the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). The primer of miR-381-3p and LRP6 were shown in Table I. GAPDH was used as internal control. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Assay

The cell proliferation assay was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Dojindo, Gaithersburg, MD, USA) according to the manufacturer's protocol. Briefly, 1000 cells from different groups were seeded in 96-well culture plates and were cultured for indicated days. At the indicated time point, 5 μL MTT solution (5 mg/ml) were added into each well and incubated 2 h at room tempera-

Table I. Sequence of the primers used in this study.

Genes	Primer sequences (5′-3′)
MiR-381-3p	(Sense) TAATCTGACTATACAAGGGCAAGCT (Antisense) TATGGTTGTTCTGCTCTCTGTCTC
LRP6	(Sense) GCTCAGAGTCCCAGTTCCAG (Antisense) TCCCTTCATACGTGGACACA
GAPDH	(Sense) ATTCCATGGCACCGTCAAGGCTGA (Antisense) TTCTCCATGGTGGTGAAGACGCCA

ture. Absorbance was assessed at 490 nm by a spectrophotometer (BioTek, Chaoyang, Beijing, China).

Cell Migration and Invasion Assay

Cell migration and invasion were detected using transwell chambers (Corning Incorporated, Corning, NY, USA) with or without 50 μL Matrigel (8.0 μm, Millipore, Billerica, MA, USA). 40,000 indicated PTC cells suspended in serum-free medium were seeded in the upper chambers of a 24-well transwell insert (Millipore, Billerica, MA, USA). The chambers were then incubated for 24 h in culture medium with 10% fetal bovine serum (FBS) as chemoattractant in the bottom chambers before examination. After incubating for 24 h at 37°C under 5% CO_2, non-invading cells were removed from the upper chamber by cotton-tipped swabs. Cells that had migrated or invaded through the membrane were stained with methanol. Finally, cells were counted under a microscope and the relative number was calculated.

Dual Luciferase Assay

The interaction between miR-381-3p and LRP6 was determined by dual luciferase assay. Briefly, PTC cells (6×10^4) were seeded in triplicate in 24-well plates 24 h before transfection. Plasmids containing LRP6-3'UTR and LRP6-3'UTR mutations were constructed via technical support from Dajin Co. (Pudong, Shanghai, China). Then, cells were co-transfected with miR-381-3p mimics and either pcDNA3.1-LRP6 or control miR. About 48 h after transfection, the luciferase activity was determined using the Dual-Luciferase system (Promega, Madison, WI, USA). The activity of Renilla luciferase was normalized to Firefly luciferase.

Western Blot Analysis

Proteins were extracted by radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The total protein concentration was quantified with a bicinchoninic acid (BCA) assay kit. Lysates were separated by 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked at room temperature for 2 h with blocking solution incubated overnight at 4°C. Membranes were blocked in 5% milk-TBST, then incubated with primary antibodies followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The relative protein expression was analyzed using Image-Pro plus software 6.0. β-actin was used as the internal reference.

Statistical Analysis

All statistical analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). Independent and paired *t*-test was used to compare the data. The multi-group comparison was performed using one-way analysis of variance. The paired comparison was performed by the Student-Newman-Keuls (SNK) approach. $p < 0.05$ was considered statistically significant.

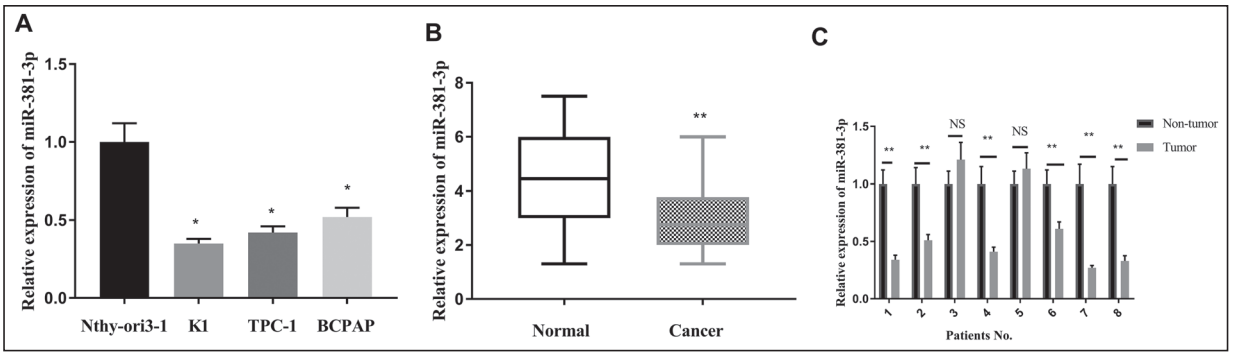

Figure 1. MiR-381-3p is significantly downregulated in PTC tissues and cell lines. ***(A)*** qRT-PCR analysis of miR-381-3p expression in human PTC cell lines (TPC-1, BCPAP and K1) and normal thyroid epithelial cell line (Nthy-ori3-1). ***(B)*** The qPCR analysis of miR-381-3p for 24 normal thyroid tissues and 53 PTC tissues. ***(C)*** The qPCR analysis of miR-381-3p for 8 paired PTC tissues. Each experiment was performed three times in triplicate. *$p < 0.05$ and **$p < 0.01$.

Results

miR-381-3p was Decreased in PTC Tissues and Cell Lines

To investigate the role of miR-381-3p in PTC, we first examined miR-381-3p expression in three PTC cell lines (TPC-1, BCPAP and K1) and Nthy-ori3-1 using RT-PCR. As shown in Figure 1A, the results indicated that miR-381-3p was significantly decreased in PTC cells compared with Nthy-ori3-1 cells. Next, we explored whether miR-381-3p was downregulated in clinical PTC tissues. As shown in Figure 2B, we observed that miR-381-3p expression levels were decreased in PTC tissue, compared with normal thyroid tissues ($p < 0.01$). Then, in order to further demonstrate the inhibition of miR-381-3p in PTC patients, we detected the levels of miR-381-3p in 8 PTC tissues and corresponding nontumor tissues. As shown in Figure 1C, down-regulation of miR-381-3p was observed in 6 of 8 PTC samples compared with corresponding nontumor tissues. Taken together, our results suggested that miR-381-3p expression was frequently down-regulated in PTC patients.

miR-381-3p Inhibited Proliferation, Migration and Invasion in PTC Cells

To study the role of miR-381-3p in the growth of PTC, K1 and TPC-1 cells were transfected with miR-381-3p mimic or miR-NC. As shown in Figure 2A, the increased levels of miR-381-3p can be detected in the K1 and TPC-1 cells after transfection. The MTT assay was used to measure the PTC proliferation ability. We observed that overexpression of miR-381-3p significantly suppressed the proliferation of both K1 and TPC-1 cells (Figure 2B and 2C). Then, we investigated the effect of miR-381-3p on their migration and invasion ability. As shown in Figure 3D and 3E, we found that miR-381-3p overexpression resulted in reduced migration rate and invasion rate of K1 and TPC-1 cells compared with the control. Taken together, these data suggested that miR-381-3p was involved in regulating the proliferation, migration and invasion of PTC cells.

LRP6 was a Direct Target of miR-381-3p in PTC Cells

Emerging data imply that miR-381-3p serves as a tumor suppressor or oncogene by binding the 3' UTR tumor-related genes. In order to explore the molecular mechanism of miR-381-3p function in PTC cells, our attention focused on the target gene of miR-381-3p. We predicted the target of miR-381-3p by publicly available algorithms (Target Scan) and found that LRP6 mRNA harbored a putative miR-381-3p binding site in its 3'-UTR (Figure 3A). Subsequently, luciferase reporter assay was used to determine whether LRP6 was directly regulated by miR-381-3p. As shown in Figure 3B, the results indicated that the luciferase activity decreased in K1 and TPC-1 cells with miR-381-3p mimics and pGL3-LRP6 vectors. However, there was no significant difference on luciferase activity when K1 and TPC-1 cells were transfected with pGL3- LRP6-MUT vector. To further identify the targeting of LRP6 by miR-381-3p, RT-PCR and Western blot were performed to determine the LRP6 mRNA and proteins levels after the up-regulation of miR-381-3p. As shown in Figure 3C and 3D, we found that mRNA and protein expression levels of LRP6 were significantly suppressed in miR-381-3p transfectants as compared with control groups. On the other hand, we detected the expression levels of LRP6 mRNA in clinical tissues. The results indicated that LRP6 mRNA was significantly higher in PTC tissues compared to normal thyroid tissues, indicating that LRP6 served as a tumor promoter in PTC. Taken together, these data indicated that LRP6 was a direct target of miR-381-3p in PTC.

miR-381-3p Attenuated the Promotion of LRP6 Mediated Cell Development in Vitro

To explore the effect of miR-381-3p and LRP6 in PTC cells behavior, Western blot was used to determine the expression levels of miR-381-3p and LRP6 in K1 cells transfected with pcDNA3.1-LRP6 and/or miR-381-3p. As shown in Figure 4A, we found that the expression of LRP6 was increased in pcDNA3.1-LRP6 transfected cells compared to control cells, but forced expression of miR-381-3p suppressed the levels of LRP6. Then, MTT and transwell assays were used to study the association between miR-381-3p and LRP6 in cells proliferation and metastasis. The results indicated that up-regulation of LRP6 promoted PTC proliferation, invasion and migration, while miR-381-3p could largely reverse the promotive effect of LRP6 on PTC cell proliferation, migration and invasion (Figure 4B, 4C and 4D). Overall, our results suggested that miR-381-3p served as a tumor suppressor by downregulating LRP6.

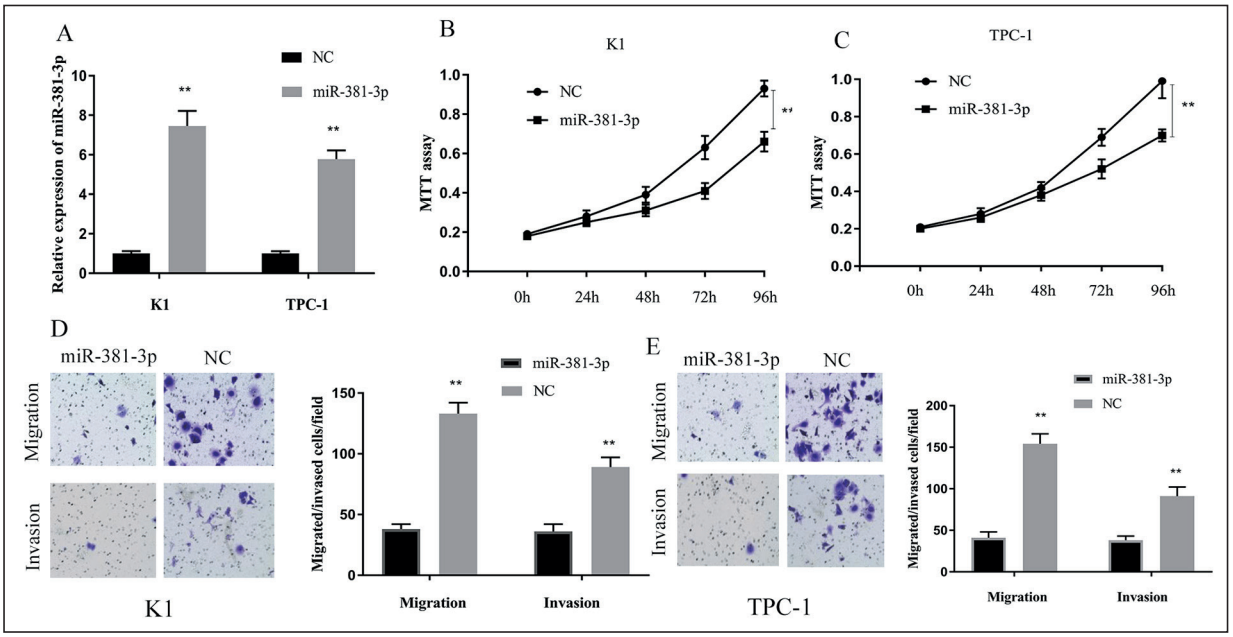

Figure 2. Overexpression of miR-381-3p suppressed PTC cell proliferation, migration and invasion. ***(A)*** The expression level of miR-381-3p was measured by real-time PCR after K1 and TPC-1 cells were transfected with miR-381-3p mimic or their relevant controls. (***B, C)*** MTT assays were conducted to determine the proliferation of K1 and TPC-1 cells. ***(D, E)*** Inhibitory effect of miR-381-3p toward the migration and invasion of K1 and TPC-1 cells. *$p < 0.05$ and **$p < 0.01$.

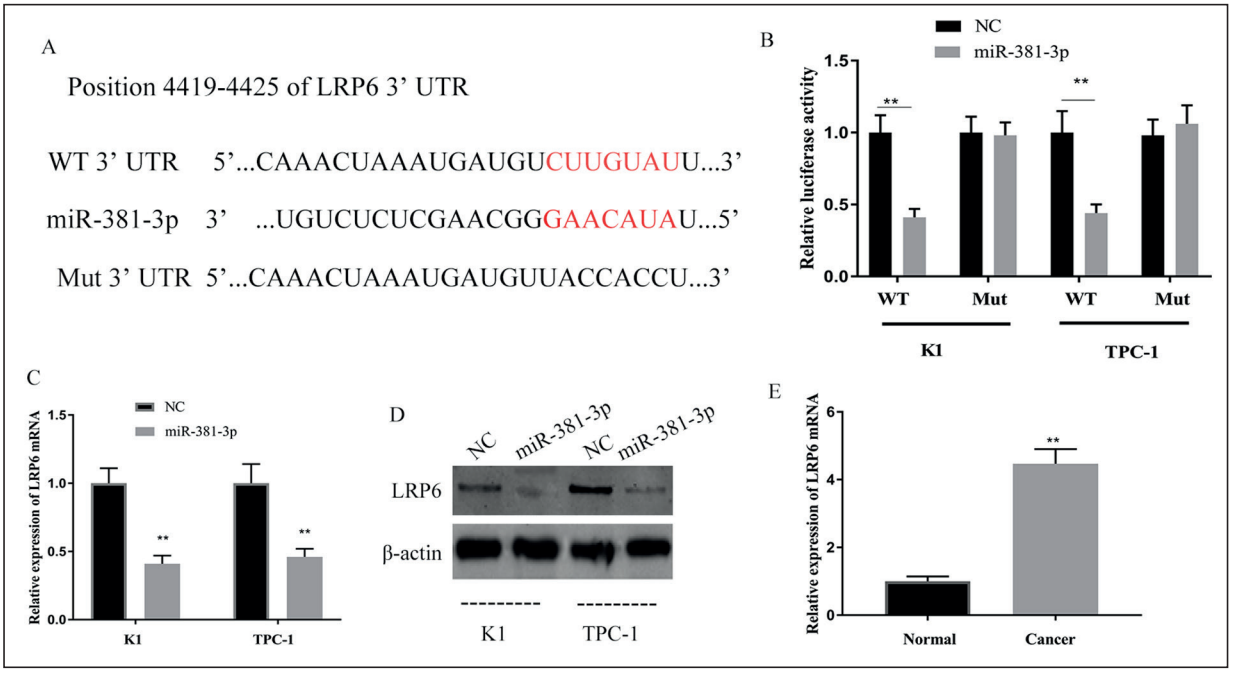

Figure 3. LRP6 was identified as a functional target of miR-381-3p in PTC. ***(A)*** Schematic representation of LRP6 3'UTR was showing the putative miR-381-3p target site. ***(B)*** Luciferase activity was measured in K1 and TPC-1 cells after co-transfected with miR-381-3p or miR-NC and Wt or Mut LRP6 3'UTR report plasmid. ***(C, D)*** miR-381-3p down-regulated LRP6 mRNA and proteins in K1 and TPC-1 cells. Cells were transfected with miR-381-3p or its control for 48 h, and then collected for RT-PCR and Western blot. ***(E)*** Relative expression levels of LRP6 mRNA in PTC tissues and adjacent normal tissues. *$p < 0.05$ and **$p < 0.01$.

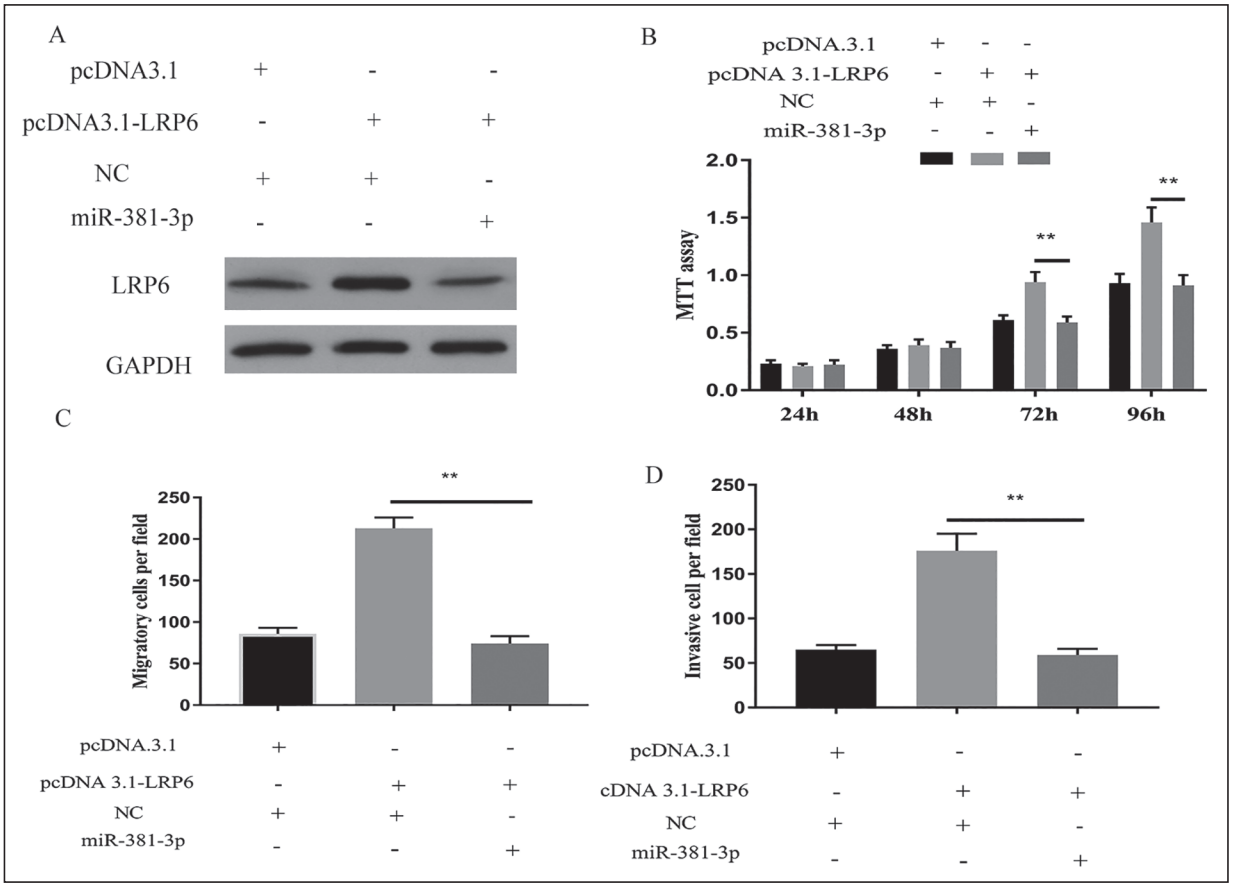

Figure 4. LRP6 involved in miR-381-3p mediated suppression of PTC cells proliferation, migration and invasion. ***(A)*** Western blot assay of K1 transfected with pcDNA3.1 + NC miRNA, pcDNA3.1-LRP6 + NC miRNA or pcDNA3.1-LRP6 + miR-381-3p. MTT assay ***(B)***, migration assay ***(C)*** and invasion assay ***(D)*** were performed on mentioned cells. *$p < 0.05$ and **$p < 0.01$.

Discussion

Identify specific miRNAs and their targets involved in tumorigenesis is important for prevention and treatment of PTC. In this study, we found that the miR-381-3p expression is greatly downregulated in PTC tissues and cell lines. A series of experiments confirmed overexpression of miR-381-3p suppressed proliferation, migration and invasion in PTC cells. Luciferase assays confirmed that miR-381-3p exerted its tumor-suppressive role by directly binds the 3'-UTR of LRP6 mRNA. Therefore, we speculated that miR-381-3p was involved in LRP6-mediated metastasis in PTC cells.

Recently, miRNAs become hotspots for their multiple biological functions. The expression and function of miR-381-3p in various tumors were reported. For instance, Cao et al[21] found that up-regulation of miR-381 suggested a favorable prognosis of gastric cancer and suppressed cell proliferation and invasion via targeting TME-M16A. Zhang et al[22] reported that miR-381-3p was lowly expressed in hepatocellular carcinoma and its forced expression inhibited cell growth and invasion by targeting the liver receptor homolog-1. Yang et al[23] also suggested miR-381-3p as a tumor suppressor by directly targeting FGFR2 inhibiting the proliferation of oral squamous cell carcinoma cells. On the contrary, miR-381-3p was reported to be up-regulated and act as a tumor promoter in osteosarcoma and glioma[24,25]. Those findings indicated that the functional role of miR-381-3p in cancer was still controversial. To our best knowledge, the expression and function of miR-381-3p in PTC have not yet been reported. A series of experiments in our study showed miR-381-3p as a tumor suppressor in PTC.

As an essential Wnt co-receptor to activate Wnt/β-catenin signaling, LRP6 has been recognized as a key tumor promoter in the regulation of tumorgenesis[26]. For instance, LRP6 was high-

ly expressed in hepatocellular carcinoma, and its up-regulation enhanced cell invasion[27]. LRP6 silencing suppressed breast cancer cells proliferation[28]. Several studies have shown that LRP6 was regulated by several miRNAs. For example, Zhang et al[29] reported that miR-202 suppressed hepatocellular carcinoma growth by targeting LRP6. Wang et al[30] observed that forced miR-183 expression inhibited retinoblastoma cell growth and metastasis by targeting LRP6. Importantly, Wen et al[31] found that upregulation of miR-126 inhibited cell proliferation, migration and invasion in PTC by targeting LRP6. In order to explore the potential mechanism by which miR-381-3p suppressed PTC cell proliferation and metastasis, we used TargetScan to search possible target genes of miR-381-3p and found that LRP6 may be a target gene of miR-381-3p. Then, we performed luciferase assays and Western blot and confirmed the results of TargetScan. Furthermore, *in vitro* assay showed that miR-381-3p overexpression rescued the promoting effects, which induced by LRP6. Overall, our findings suggest a potential mechanism for the tumor suppressor role of miR-381-3p mediated by the downregulation of LRP6.

Conclusions

We found that miR-381-3p was downexpressed in PTC cells and tissues, while the opposite trend was observed in the protein level of LRP6. Furthermore, miR-381-3p inhibited the proliferation and metastasis of PTC cells by targeting LRP6. In the future, miR-381-3p/LRP6 signaling has great potential to serve as a therapeutic target for PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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