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MicroRNA-26a suppresses the malignant biological behaviors of papillary thyroid carcinoma by targeting ROCK1 and regulating PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: Papillary thyroid carcinoma (PTC) is an endocrine malignancy, the morbidity of which has kept rising in recent years. MicroRNA (miRNA/miR) is emerging as a key regulator in carcinogenesis, including PTC. The current study concentrates on the biological roles and mechanisms of miR-26a in the PTC progression.

PATIENTS AND METHODS: 51 pairs of PTC tissue samples and matched adjacent thyroid tissues were collected from PTC patients who received surgical excisions at The People's Hospital of Linqing between July 2015 and June 2018 with informed consent. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect expressions of miR-26a and rho-associated coiled-coil–containing protein kinase 1 (ROCK1) mRNA in PTC tissues and cells. Functional assays, including (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) MTT assays and transwell assays were performed to determine the roles of miR-26a in the PTC progression. Western blot was used to detect expression levels of the related proteins.

RESULTS: Findings demonstrated prominently down-regulated miR-26a in PTC tissues and cells. Down-regulated miR-26a indicated malignant clinicopathologic characteristics and shorter overall survival rate of PTC patients. MTT assay and transwell assay indicated that miR-26a up-regulation significantly repressed PTC cell viability, invasion, and metastasis. Western blot analysis revealed that miR-26a exerted its anti-PTC effects via phosphatidylinositol 3-kinase/protein kinase B pathway (PI3K/AKT) and epithelial-to-mesenchymal transition (EMT). ROCK1 was a target of miR-26a in PTC cells and ROCK1 was mediated by miR-26a, as a regulatory mechanism in PTC.

CONCLUSIONS: Taken together, these findings demonstrated the anti-tumor functions of miR-26a in PTC, providing novel strategies for PTC diagnosis and therapy.

Key Words:
PTC, MiR-26a, ROCK1, PI3K/AKT, EMT.

Introduction

Papillary thyroid carcinoma (PTC) is one common thyroid malignancy and its morbidity is rising worldwide, accounting for more than 80% of all thyroid tumors[1]. It is generally believed that PTC patients have a better prognosis and several patients could be cured with surgery or radiotherapy[2] However, increasing studies[3] have revealed that the prominent biological properties of PTC, including high incidence of lymph node metastases, strong invasion ability, and aberrant proliferation, occasionally result in recurrence and poor prognosis of PTC patients. Therefore, investigation of PTC pathogenesis is indispensable to develop new therapeutic strategies in the PTC treatment. Moreover, the PTC-specific biomarkers can be able to assess tumor invasiveness, thereby guiding surgical parameters or providing novel targets for treatment.

MiRs participate in regulating the expressions of target mRNAs via base pairing with its 3′-UTRs, leading to translational silencing or degradation[4]. Researchers[5,6] have focused on the correlation between tumor progression and miRs, which serve as tumor suppressors or oncogenes. Hafshar et al[7] have shown that invasive tumors present aberrant expression profile of certain miRs, which is associated with clinicopathologic characteristics. MiR-142-3p functioned as an anti-tumor miR in human breast cancer tumorigenicity via regulation of Bach-1 expressions[8]. Moreover, in triple-negative breast cancer, the

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epigenetic regulation of another miR, miR-200, was found to be the potential therapy strategy[9]. Up-regulated miR-552 in human colorectal cancer was a potential biomarker of poor prognosis[10]. However, the specific functions of miR26a in PTC have yet to be analyzed.

As a multifunctional member of the serine/threonine kinase family, rho-associated coiled-coil–containing protein kinase 1 (ROCK1) is implicated in a wide range of ubiquitous biological processes, such as metastasis, cell movement, cell detachment, differentiation, evasion from apoptosis, survival, and establishment of unlimited proliferation potential[11]. ROCK1 over-expression has been identified in a variety of tumors, being associated with poor prognosis and strong invasion capacity. For example, in glioma, ROCK1 was confirmed to promote cell invasion, being regulated by miR-145[12]. Similarly, oncoprotein ROCK1 was found to participate in breast cancer cell metastasis and growth[13]. Here, we were interested in whether ROCK1 was mediated by miR-26a, as a regulatory mechanism in PTC.

Patients and Methods

Tissue Samples

51 pairs of PTC tissue samples and matched adjacent thyroid tissues were collected from PTC patients who received surgical excisions at The People's Hospital of Linqing between July 2015 and June 2018 with informed consent. All tissues were snap-frozen in liquid nitrogen and preserved at –80°C. The research was approved by the Ethics Committee of The People's Hospital of Linqing.

Cell Cultures

Human thyroid epithelial cell Nthy-ori3-1 and PTC cells (BCPAP, TPC-1, K1, and HTH83) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37°C.

Transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized to transfect miR-26a mimic, inhibitor, and the corresponding negative controls (NC; GenePharma Co., Ltd., Shanghai, China) into PTC cells. 48 h post-transfection, the transfected cells were harvested for further assays.

QRT-PCR

The total RNAs were extracted from cultured cells or tissue specimens by TRIzol (Invitrogen, Carlsbad, CA, USA). For detection of miR26a expressions, reverse transcriptions were performed to generate cDNA by a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For ROCK1, PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China) was used for cDNA synthesis. Next, qPCR was carried out with TaqMan MicroRNA PCR Kit (Applied Biosystems, Foster City, CA, USA) or SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China) on ABI Prism 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA) for miR26a or ROCK1 respectively. Finally, the expressions were analyzed using the $2^{-\Delta\Delta Ct}$ method. ROCK1 mRNA expression and miR26a expression were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6. The primers used were list in Table I.

Cell Viability Assay

The transfected cells were seeded into 96-well plate and incubated for 24, 48, and 72h. Then, (3-(4,5-dimethylthiazol-2-yl)-2,5-di- phenyl tetrazolium bromide (MTT) solution was added and incubated at 37°C for 4 h followed by the addition of dimethyl sulfoxide (DMSO) into each well. The absorbance at 490 nm was measured using a microplate reader (BioTek, Winooski, VT, USA).

Transwell Assay

Cell invasion and migration assay were performed with transwell chamber (8-μm pore size; Corning, Corning, NY, USA). For the invasion assay, cells were plated into the top chambers of Matrigel-coated insert in free-serum medium, while a medium with 10% fetal bovine serum (FBS) was added into the bottom chambers. Having been incubated for 48 h, the invasive cells were fixed and stained. Finally, the cells were counted under an inverted microscope (X71; Olympus, Tokyo, Japan). The transwell migration assay was conducted as invasion assay, without the Matrigel coating.

Western Blot

The whole-cell lysate was obtained by lysing cells with radioimmunoprecipitation (RIPA) lysis

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-26a forward	5'-CGCGTTCAAGTAATCCAGGA-3'
miR-26a reverse	5'-AGTGCAGGGTCCGAGGTATT-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
ROCK1 forward	5'-CGGTATCTCTACATGGTGATGGAG-3'
ROCK1 reverse	5'-ATCCAATGCAAGAACTACTTCTGC-3'
GAPDH forward	5'-CCAGGTGGTCTCCTCTGACTT-3'
GAPDH reverse	5'-GTTGCTGTAGCCAAATTCGTTGT-3'

U6: small nuclear RNA, snRNA; ROCK1: rho-associated coiled-coil-containing protein kinase 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

buffer (Beyotime, Shanghai, China). The concentrations of the extracted proteins were determined by bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Then, the proteins were subjected to separation by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). 2 h post the blocking with 5% skimmed milk in Tris-Buffered Saline and Tween-20 (TBST), the membranes were probed overnight at 4°C with primary antibodies. The antibodies were as follows: AKT (1:1000, Abcam, Cambridge, MA, USA), p-AKT (1:1000, Abcam), PI3K (1:1000, Abcam), p-PI3K (1:2000, Abcam), E-cadherin (1:2000, Abcam), N-cadherin(1:1000, Abcam), Vimentin (1:1000, Abcam), and GAPDH (1:1000, Abcam), which served as an internal control. Afterward, the membrane was incubated with appropriate secondary antibody (1:4,000, ab7090; Abcam) and proteins were visualized with enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA).

Luciferase Reporter Assay

The human ROCK1 3′-UTRs containing mutant (MUT) or wild-type (WT) miR-26a binding sequences were cloned into the pGL3 vectors (Promega, Madison, WI, USA). Then, the vectors along with miR-26a mimics were transfected into PTC cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) was used to examine the luciferase activity 48h post-transfections.

Statistical Analysis

All experiments were conducted at least thrice.SPSS software version 17.0 (SPSS Inc., Chicago, IL) was used to perform statistical analysis. Comparisons of two or more groups were determined by the Student's *t*-test or One-way ANOVA analysis together with Tukey's post-hoc test. The Kaplan-Meier method along with log-rank test was applied to analyze the overall survival (OS) of PTC patients. $p<0.05$ indicated a statistically significant difference.

Results

MiR-26a Down-regulation Was Associated with Aggressive Clinicopathological Features of PTC Patients

We first detected miR-26a expressions in PTC tissues and matched non-carcinoma tissues. QRT-PCR analysis indicated that PTC tissues presented significant lower miR-26a expressions than the non-tumor tissues (Figure 1A). Furthermore, to clarify the clinicopathological value of miR-26a in PTC patients, the enrolled patients were assigned to high and low miR-26a subgroups according to the mean miR-26a level. In brief, patients in low miR-26a subgroup exhibited aggressive clinicopathological features (Table II). Consistently, data of Kaplan-Meier analysis also demonstrated that the OS of patients in low miR-26a subgroup was shorter than that in high miR-26a subgroup (Figure 1B).

MiR-26a Up-regulation Suppressed PTC Cell Viability

We measured miR-26a expressions in PTC cells. As expected, the qRT-PCR analysis showed that miR-26a was dramatically underexpressed in PTC cells when compared to the controls (Figure 2A). To explore the potential bio-functions of miR-26a in PTC, we ectopically over-expressed

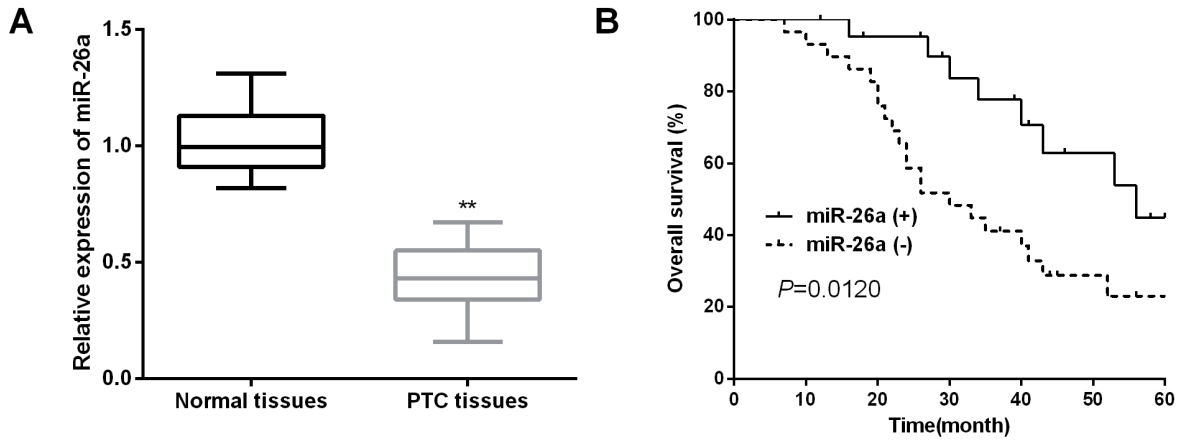

Figure 1. MiR-26a was under-expressed in PTC tissues. **A,** QRT-PCR analysis of the relative miR-26a expressions in PTC tissues. **B,** Overall survival of PTC patients with different miR-26a expressions were examined by Kaplan-Meier analysis. $^{**}p<0.01$.

or inhibited miR-26a levels in TPC-1 cell lines. Efficient miR-26a over-expression or inhibition in TPC-1 cells was confirmed by qRT-PCR (Figures2B and 2C). Subsequently, we carried out the MTT assay to examine the viability ability of miR-26a-overexpressed or -inhibited TPC-1 cells. Results revealed that miR-26a over-expression obviously repressed TPC-1 cell viability while miR-26a silence had the opposite functions in TPC-1 cell viability (Figure 2D).

MiR-26a Repressed PTC Cell Invasion and Migration

The transwell assay was applied to determine the role of miR-26a in PTC cell invasion and migration. As shown in Figures 3A and 3B, the invasion and migration abilities in TPC-1 cells with transfections of miR-26a mimics were evidently restricted. In the meantime, miR-26a inhibition in TPC-1 cells significantly facilitated the invasion and migration capacities (Figures 3C and 3D).

Table II. Correlation of miR-26a expression with the clinicopathological characteristics of the PTC patients.

Clinicopathological features	Cases (n=51)	miR-26a# expression		*p*-value
		High (n=18)	Low (n=33)	
Age (years)				0.5133
>60	28	10	18	
≤60	23	8	15	
Gender				0.4352
Male	24	7	17	
Female	27	11	16	
Tumor size (cm)				0.3126
≥ 5.0	26	6	20	
<5.0	25	12	13	
Lymph node metastasis				0.0119*
Yes	18	14	4	
No	33	4	29	
TNM stage				0.0049*
I +II	20	13	7	
III+IV	31	5	26	
Distant metastasis				0.0063*
Yes	32	4	28	
No	19	14	5	

PTC: papillary thyroid carcinoma; TNM: tumor-node-metastasis; #The mean expression level of miR-26a was used as the cutoff; *Statistically significant.

Findings suggested that miR-26a served as an anti-tumor miR in PTC.

Identification of ROCK1 As a Direct Target of MiR-26a in PTC Cells

TargetScan was used to search the potential targets of miR-26a and data revealed that ROCK1 was a candidate target. The putative binding sites of miR-26a in ROCK1 3'UTRs were shown in-Figure. 4A. Then, the luciferase reporter assay was used to confirm the association between ROCK1 and miR-26a. Luciferase activities of the ROCK1 3'UTR-WT-transfected cells were markedly decreased by miR-26a mimics and luciferase activity of ROCK1 3'UTR-MUT-transfected cells remained unaffected (Figure 4B). Furthermore, qRT-PCR was conducted to investigate whether miR-26a negatively modulated ROCK1. As expected, results revealed that miR-26a over-expression evidently decreased the ROCK1 expressions while miR-26a inhibition increased ROCK1 expressions (Figures 4C and 4D). All data revealed that miR-26a targeted ROCK1 by directly binding to its 3'-UTR.

Effects of MiR-26a in PTC cell PI3K/AKT Pathway and Epithelial-To-Mesenchymal Transition

As ROCK1 was an efficient target of miR-26a, the prognostic significance of ROCK1 was further explored. ROCK1 expressions in PTC tissues and cell lines were first examined. As demonstrated by qRT-PCR analysis, ROCK1 level was markedly increased both in PTC tissues and cells (Figures 5A and 5B). Moreover, the prognostic value of ROCK1 in PTC patients was determined

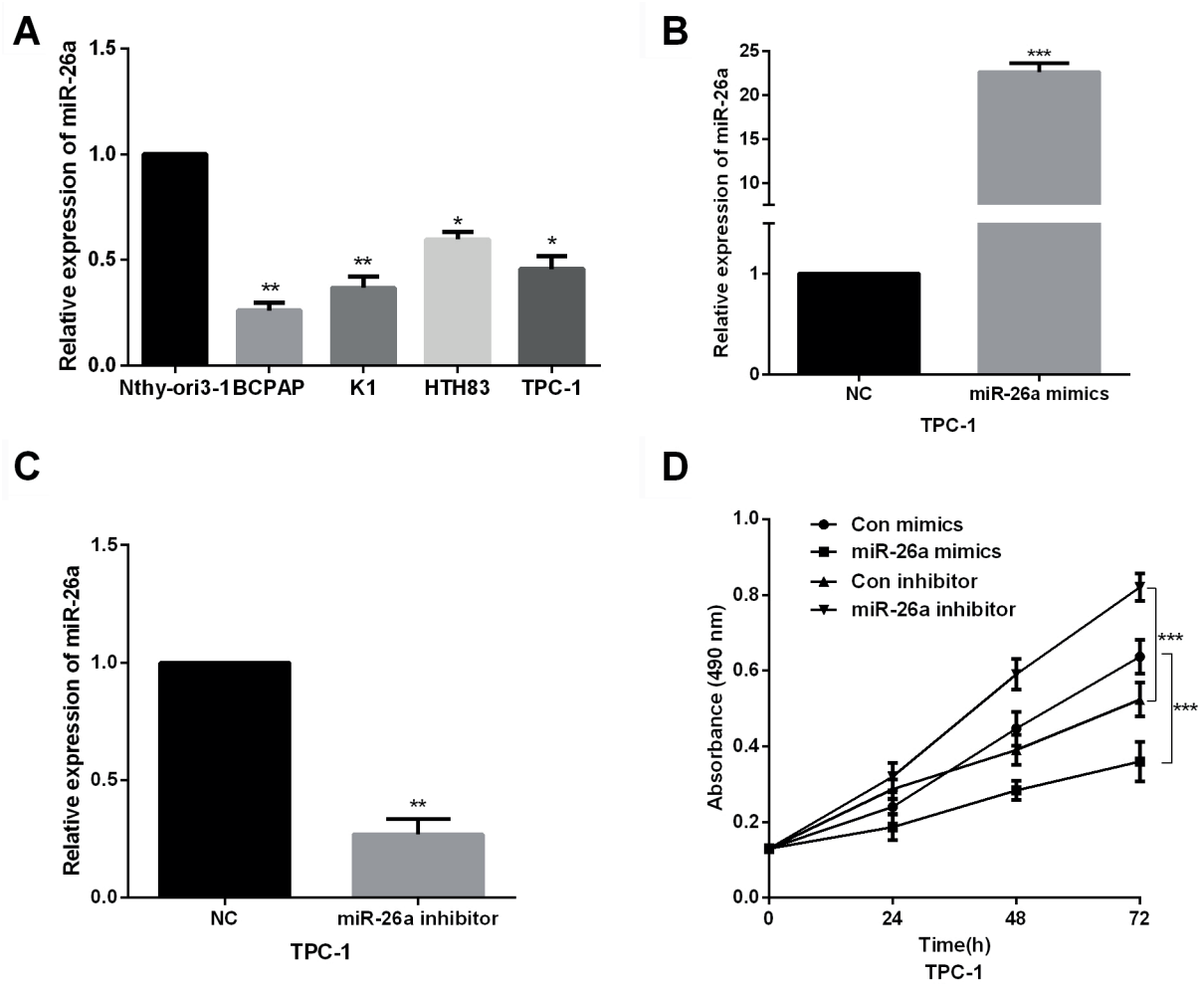

Figure 2. MiR-26a was down-regulated in PTC cells and miR-26a up-regulation inhibited PTC cell proliferation. **A,** MiR-26a level in PTC cells was measured using qRT-PCR. **B-C,** MiR-26a up-regulation or inhibition in PTC cells was certified with qRT-PCR. **D,** Influence of miR-26a on PTC cell viability was analyzed by MTT assay. *p<0.05,** p<0.01,*** p<0.001.

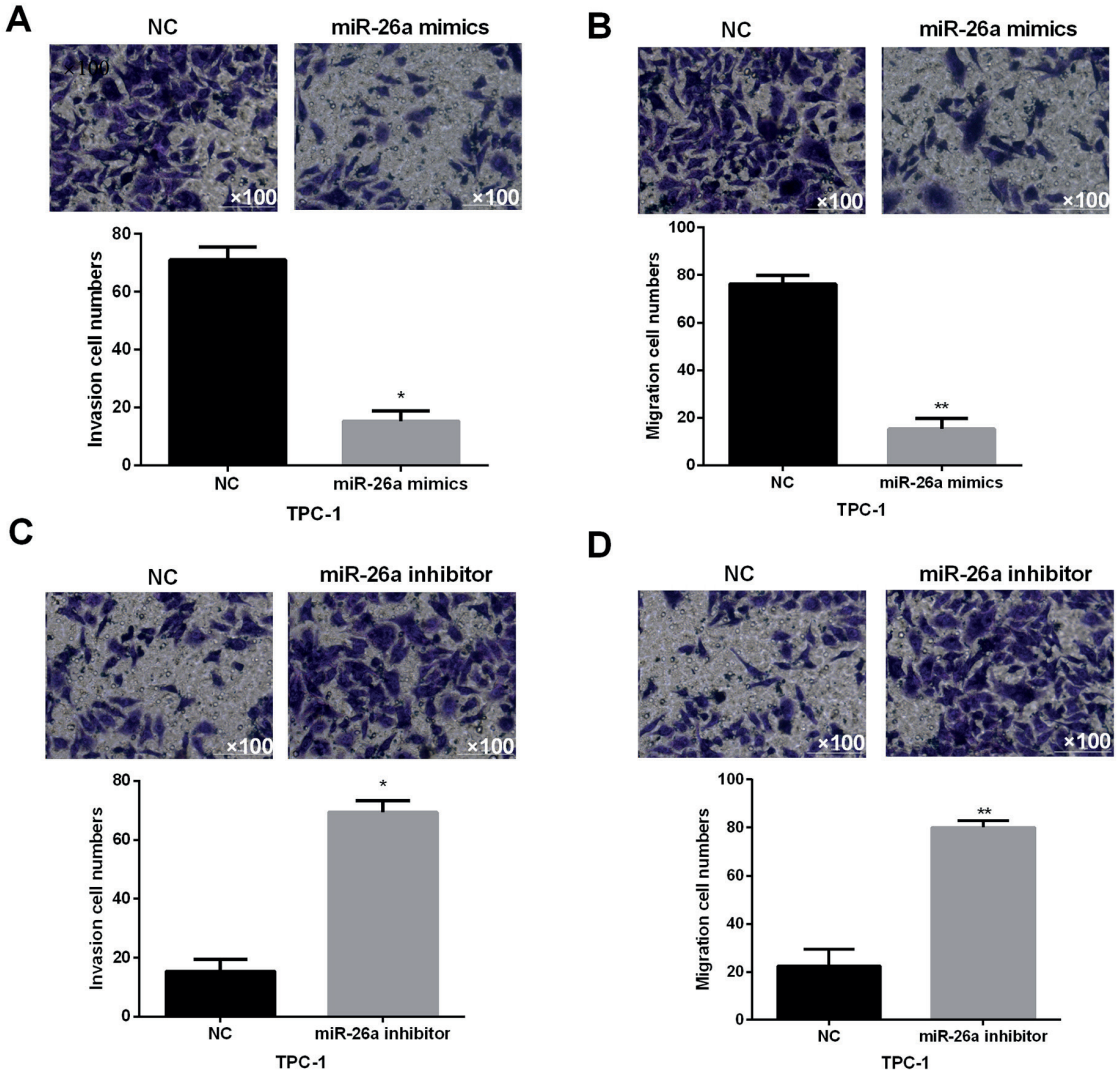

Figure 3. MiR-26a restoration suppressed PTC cell invasion and migration. **A-B,** Transwell assay indicated that miR-26a mimics inhibited PTC cell invasion and migration. **C-D,** Invasion and migration abilities were promoted by miR-26a inhibition in PTC cells as demonstrated by transwell assays. *p<0.05,** p<0.01.

by Kaplan-Meier analysis. It was shown that patients with high ROCK1 levels presented prominent shorter OS than patients with low ROCK1 levels (Figure 5C). Western blot was applied to explore the functions of miR-26a in PI3K/AKT and EMT. In brief, decreased expressions of related proteins in the PI3K/AKT pathway, such as p-PI3K and p-AKT, were identified in cells transfected with miR-26a mimics (Figure 5D). In the meantime, among EMT-related proteins, E-cadherin was up-regulated whereas Vimentin and N-cadherin were underexpressed in miR-26a mimics-transfected cells (Figure 5E). Additionally, in miR-26a-inhibited cells, results were reversed.

Discussion

The potential of unrestrained growth is one important biological feature of cancers. Abnormal expressions of tumor suppressor genes and oncogene activation are considered as major factors in tumorigenesis[14,15]. A growing body of evidence[16] has indicated that aberrant miR expressions promoted the development and metastases of PTC by inhibiting their targets, suggesting that miR may function as a molecular biomarker and therapeutic target for predicting and prognosing PTC. MiR-431 down-regulation was reported to promote PTC cell invasion and be related to lymph node metastasis[17]. MiR-101 targeted MCL-

1 and c-met in PTC to promote TRAIL-induced mitochondrial apoptosis[18]. MiR-150 inhibited malignant behaviors of PTC and blocked cell proliferation by down-regulating MUC4[19].

The PI3K/AKT pathway has important effects in tumor propagation. Over-activation of this pathway was found[20] to promote tumor migration and proliferation. Zheng et al[21] showed that over-activation of PI3K/AKT was involved in PTC genesis and development. EMT is an essential biological process during tumor metastasis. The most significant features of EMT were the changes in gene expressions, including the acquisition of mesenchymal markers (such as N-cadherin and vimentin) and loss of epithelial markers (such as E-cadherin), resulting in increased tumor cell migration and invasion ability[22]. Zhou et al[23] revealed that EMT was implicated in PTC invasion. In consistency with these findings, we also found that the expressions of p-PI3K and p-AKT were decreased by miR-26a up-regulation. Over-expression of miR-26a also led to up-regulated E-cadherin and down-regulated vimentin, as well as N-cadherin.

Increasing evidence[24-26] has revealed that miR-26a expression is under-expressed in various cancers, such ashepatocellular carcinoma, osteosarcoma, and esophageal squamous cell carcinoma, suggesting its tumor suppressive roles. In the current study, miR-26a was under-expressed in PTC, and miR-26a expression was found to correlate with the clinicopathologic features of PTC patients. Furthermore, functional assays also revealed that miR-26a up-regulation markedly repressed PTC cell viability, invasion, and migration via the regulation of EMT and PI3K/AKT pathway. Moles[27] has demonstrated that miR exerts crucial roles in tumors by binding to their targets. Hence, the investigation of the correlation between miRs and their targets is pivotal to understand the mechanisms of miRs in tumorigenesis[28]. Recently, several target genes of miR-26a have been identified, such as PAK2[29] and SMAD1[30]. We further predicted another target of miR-26a, ROCK1, based

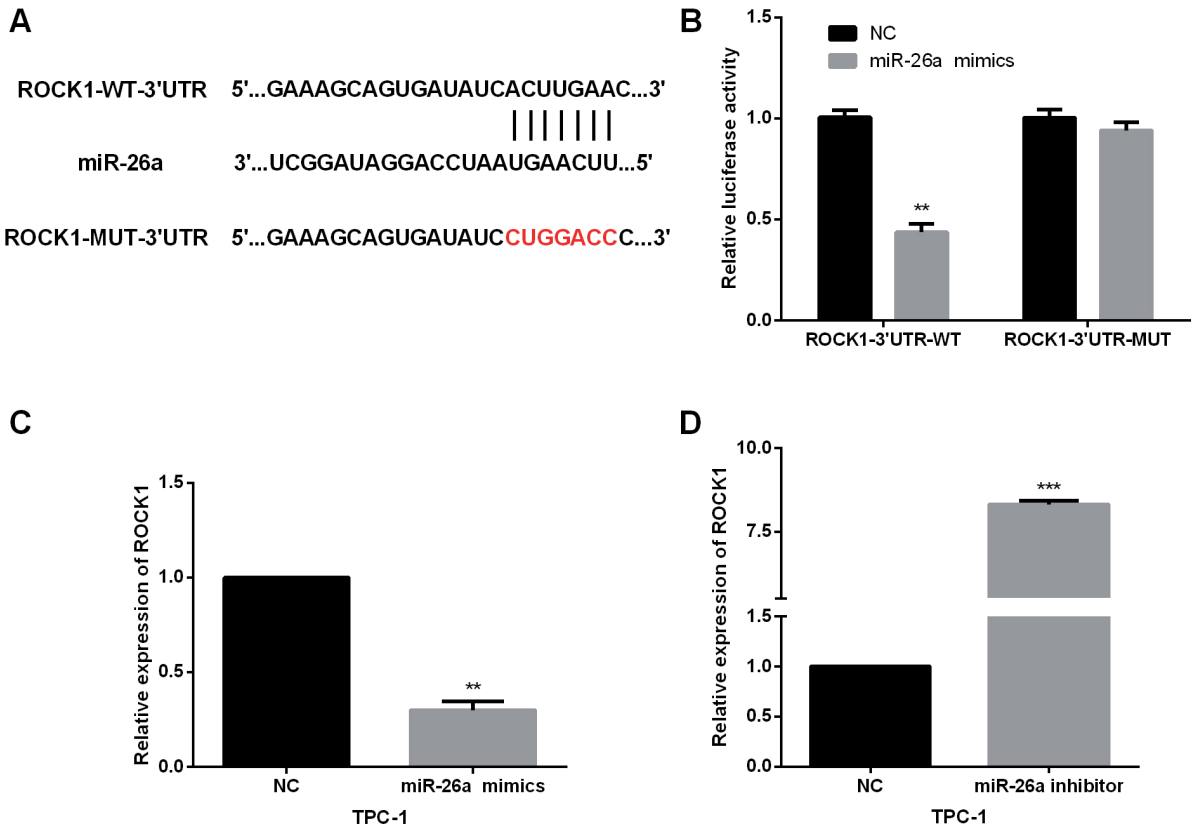

Figure 4. MiR-26a regulated expression of ROCK1 via base-pairing with its 3'UTRs. **A,** Putative binding sequences between ROCK1 and miR-26a. **B,** Luciferase activities in PTC cells transfected with luciferase reporters containing ROCK1-3'UTR-WT or -MUT and miR-26a mimics. **C-D,** Effects of miR-26a on ROCK1 expressions were determined by qRT-PCR. $^{**}p<0.01$,$^{***}p<0.001$.

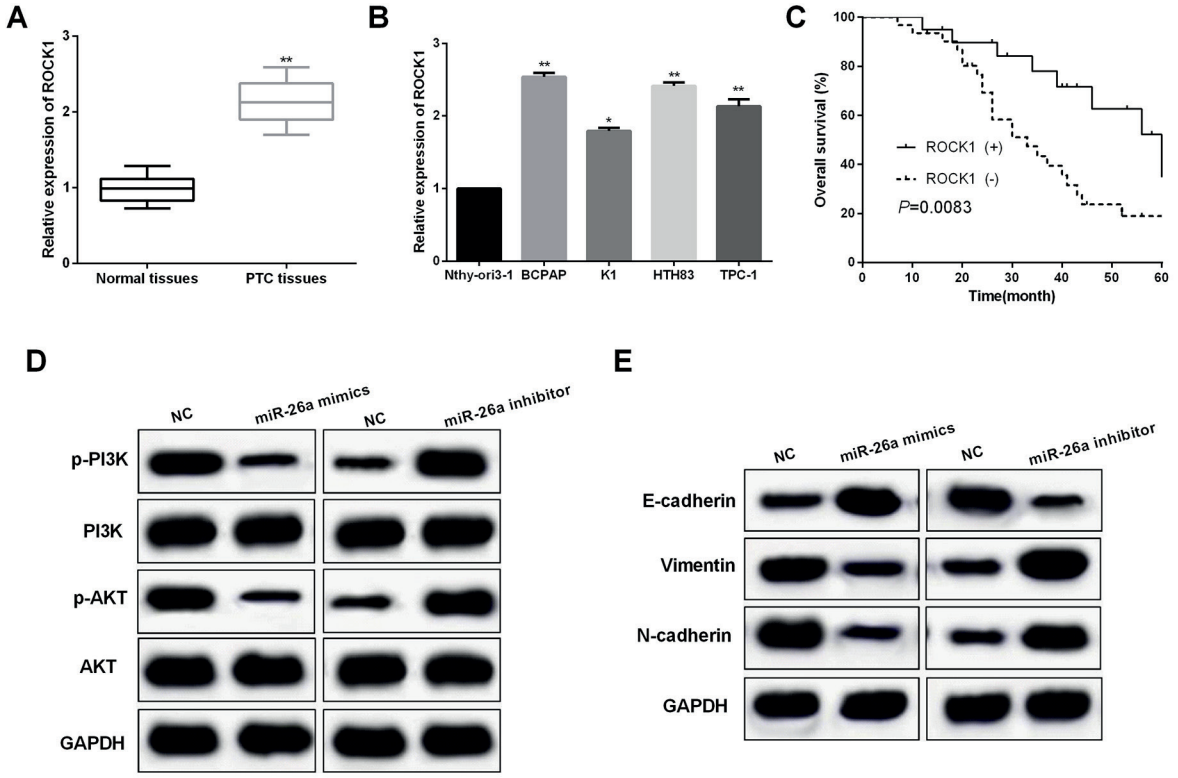

Figure 5. Up-regulated ROCK1 in PTC correlated with shorter overall survival of PTC patients and miR-26a regulated PI3K/AKT and EMT in PTC cells. **A-B,** Relative ROCK1 expressions in PTC tissues and cell lines were measured by qRT-PCR. ***C,*** Kaplan-Meier analysis of ROCK1 expressions in overall survival rates of PTC patients. **D-E,** MiR-26a regulated PTC cell PI3K/AKT and EMT. **p*<0.05,** *p*<0.01.

on the bioinformatics analysis. Data in our study showed that ROCK1 was involved in miR-26a-mediated functions in PTC cells.

Conclusions

MiR-26a was frequently under-expressed in PTC tissues and cells. Moreover, the down-regulated miR-26a correlated with aggressive clinicopathologic features of PTC patients. MiR-26a restoration could inhibit PTC cell viability, migration, and invasion via regulating EMT and PI3K/AKT pathway. Additionally, ROCK1 was verified as one functional target for miR-26a in PTC cells, being involved in the anti-PTC functions of miR-26a. These findings revealed that miR-26a was a tumor repressor in PTC, providing insights into the potential therapeutic values of miR-26a in PTC therapies.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' Contributions

YW wrote the manuscript. SL performed qRT-PCR, MTT, transwell, and luciferase reporter assays, and Western blot. YJ contributed to observation indexes analysis. The final version was read and adopted by all the authors. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of The People's Hospital of Linqing. Patients who participated in this research signed the informed consent and had complete clinical data. Signed written informed consents were obtained from the patients and/or guardians.

Conflicts of interest

The authors declare no conflicts of interest.

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