

MicroRNA-409-3p suppresses cell proliferation and cell cycle progression by targeting cyclin D2 in papillary thyroid carcinoma

ZHIJUN ZHAO, FEI YANG, YAN LIU, KAI FU and SHANGHUA JING

Department of Head and Neck Surgery, The Fourth Hospital of Hebei Medical University,
Shijiazhuang, Hebei 050000, P.R. China

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Abstract. MicroRNAs (miRs) function as tumor suppressors or oncogenes in tumor development and progression. The present study reported that miR-409-3p was significantly downregulated in human papillary thyroid carcinoma (PTC) tissues and cell lines. Overexpression of miR-409-3p suppressed cell proliferation and induced cell cycle G₀/G₁ phase arrest in PTC cells. Further study revealed that the transcriptional regulator cyclin D2 was a target of miR-409-3p, as miR-409-3p bound directly to its 3'-untranslated region and the miR-409-3p mimic reduced the protein expression levels of cyclin D2. In addition, restoration of cyclin D2 expression reversed the inhibitive effect of miR-409-3p on PTC cells. To the best of our knowledge, these findings demonstrate for the first time that miR-409-3p functions as a tumor suppressor in PTC and could serve as an efficient agent for therapy of PTC.

Introduction

Thyroid cancer is the most common endocrine malignancy in the world, with a rapid increasing incidence (1); it has been classified into several subtypes, including papillary thyroid cancer (PTC), follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer (2). PTC is the most common type and is derived from follicular thyroid cells and accounts for ~80% of thyroid cancer cases (3). Currently, the majority of PTC patients that undergo surgical resection in combination with radioiodine and levothyroxine have a good prognosis; however, recurrence is observed in ~30% of patients (4). Therefore, understanding the molecular mechanism underlying the development and progression of PTC is necessary to elucidate more effective therapeutic strategies.

MicroRNAs (miRNAs/miRs) are a group of small endogenous non-coding RNAs with a length of 19-22 nucleotides, which are derived from double-stranded RNA (5). miRs are capable of regulating many biological processes, including cell differentiation, apoptosis and development through the complementary binding of the 3'-untranslated region (3'-UTR) of target genes, resulting in the degradation or silencing of the target mRNA (6,7). Evidence indicates that the alteration of miRNAs was frequently associated with cancer progression, the miRNAs acting as tumor suppressors or oncogenes depending on the cellular function of their targets (8,9). A number of miRNAs have been identified to contribute to PTC development and progression, including miR-199a-3p (10), miR-101 (11) and miR-34a (12). Previously, miR-409-3p has been reported to be a potential tumor suppressor in variety of cancer types, including colorectal (13), prostate (14) and breast (15) cancer. However, to the best of our knowledge, no evidence of miR-409-3p function in PTC has been documented.

In this study, the expression of miR-409-3p in human PTC tissues and cells was determined. *In vitro* experiments were performed to investigate the function role of miR-409-3p in PTC cells, as well as the underlying mechanisms. The findings of the present study suggest that miR-409-3p may be a potential target for PTC treatment and serve an important role in development and progression of PTC.

Materials and methods

Clinical tissue specimens and cell lines. Samples of 20 pairs of primary PTC tissues and adjacent non-tumor tissues (≥ 3 cm away from the primary site) were obtained from patients with thyroid cancer, who had undergone surgical resection at the Fourth Hospital of Hebei Medical University (Hebei, China). Specimens were collected, immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA was extracted. In the present study, each patient voluntarily signed written informed consent and the collection and use of patient samples was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

Human PTC B-CPAP, TPC-1 and GLAG-66 cell lines and the human thyroid epithelial Nthy-ori3-1 cell line were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific,

Correspondence to: Mr. Shanghua Jing, Department of Head and Neck Surgery, The Fourth Hospital of Hebei Medical University, 12 Health Road, Shijiazhuang, Hebei 050000, P.R. China
E-mail: jingshua1962@126.com

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Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Merck, KGaA, Darmstadt, Germany) in a humidified incubator of 5% CO₂ at 37°C.

Cell transfection. The synthesized oligos for miR-409-3p mimics (5'-GAAUGUUGCUCGUGAACCCCU-3') or negative control (NC: 5'-ACTACTGAGTGACAGTAGA-3') were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). For cyclin D2 overexpression, the coding sequence of human cyclin D2 was cloned into a pcDNA3.1 vector (performed by Thermo Fisher Scientific, Inc.). The empty pcDNA3.1 was used as a negative control. Next, TPC-1 and GLAG-66 cells were seeded into 24-well plates at a density of 4x10⁵ cells per well and allowed to attach prior to transfection to ensure 60-70% cell confluence and transfected with the aforementioned vectors using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The miRNA mimics were used at a final concentration of 50 nM. Cell samples were collected at 48 h after transfection for further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from tissues and PTC cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA templates were reverse transcribed into cDNA using a One Step PrimeScript cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. miR-409-3p and *CCND2* expression levels were quantified using a ABI 7500 FAST real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green PCR Master Mix (Takara Bio, Inc.). The thermocycling conditions of the PCR reaction were as follows: Initial denaturation for 1 min at 95°C, denaturation for 5 sec at 95°C, and annealing for 40 cycles at 60°C. The expression of U6 and GAPDH were used to normalize miR-409-3p and *CCND2* expression levels in each group, respectively, using the 2^{-ΔΔC_q} method (16). The primer sequences were as follows: miR-409-3p forward, GAA TGTGCTCGGTGAACCCCT and reverse, GAAUGUUGC UCGGUGAACCCCU; *CCND2* forward, TGCAACCGA CGATTCTTCTACTCAA and reverse, CAAGCAGTGATG TATCTGATAAACAAGG; U6 forward, CTCGCTTCGGCA GCACA and reverse, AACGCTTCACGAATTTGCGT; and GAPDH forward, AGAGGCAGGGATGATGTTCTG and reverse, GACTCATGACCACAGTCCATGC.

Cell proliferation assay. For cell proliferation assay, approximately 4x10³ TPC-1 and GLAG-66 cells following transfection were seeded in a 96-well plate. After removing the medium, Cell Counting Kit-8 solution (CKK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to cells and incubated at 37°C for an additional 2 h. The absorbance of the solution at a wavelength of 450 nm was measured with a MRX II absorbance reader (Dynex Technologies, Worthing, UK).

Colony formation assay. After 48 h transfection, TPC-1 and GLAG-66 cells at a density of 500 cells per well were seeded into 6-well plates and incubated for 7 days at 37°C. Subsequently, the cells were washed twice with PBS, fixed in 70% ethanol and stained with 1% crystal violet solution for 30 min at room temperature. Colonies containing >50 cells

were photographed and counted using a light microscope (x200 magnification).

Cell cycle analysis. Cell cycle distribution was determined using flow cytometry with propidium iodide (PI) staining (Sigma-Aldrich, Merck KGaA Darmstadt Germany). Briefly, cells were plated in 6-cm dishes at a density of 1.0x10⁵ cells per dish after transfection for 48 h. Next, cells were washed with PBS and fixed in 70% ethanol overnight at 4°C. The following day, the cells were stained with a PI/RNase Staining Solution (Beyotime Institute of Biotechnology) at 4°C for 25 min. Cellular DNA was analyzed on fluorescence-activated cells sorting (FACS) Calibur flow cytometer (Gallios, Beckman Coulter, Inc., Brea, CA, USA).

Dual-luciferase reporter assays. The potential target genes of miR-409-3p were predicted using TargetScan database (http://www.targetscan.org/vert_71/). The 3'-UTR of *CCND2* containing predicted miR-409-3p binding sites (5'-UGACAU UCCCAUCACAACAUUC-3') was amplified from human cDNA and cloned into the psiCHECK-2 dual-luciferase expression vector by Promega Corporation (Madison, WI, USA). The mutation of the predicted 3'-UTR was obtained using PCR site-directed mutagenesis by Promega Corporation (Madison, WI, USA). 293T cells were seeded into 48-well plates, then co-transfected with miR-409-3p mimics or NC and luciferase reporter plasmids (50 ng) containing the psiCHECK-cyclin D2-3'-UTR wild type or mutant (5'-UGACAUUCCCAUCAG TTGTAAC-3') using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) for 24 h at 37 °C. Firefly and *Renilla* luciferase activities were measured at 48 h after transfection using a dual-luciferase reporter assay system (Promega Corporation). As per the previous experiment an empty vector control was used to validate that there was no-specific effects from vector integration into the hosts genome during transfection. Results were presented as the ratio of *Renilla* luciferase activity to firefly luciferase activity.

Western blot analysis. At 48 h after transfection, cells were harvested, lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration in samples was quantified with the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Equivalent quantities (30 μg per lane) of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked for 1 h with 5% non-fat milk at room temperature and then incubated overnight at 4°C with primary antibodies, including anti-cyclin D2 (1:1,000, ab81359, Abcam, Cambridge, UK) and anti-GAPDH (1:10,000, 10494-1-AP, ProteinTech Group, Inc., Chicago, IL, USA). Next, the membranes were washed three times in TBST containing 0.1% Tween-20 and incubated with the corresponding horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; sc-2054, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. The blots were visualized using an enhanced chemiluminescence system (BeyoECLPlus; Beyotime Institute of Biotechnology). Band density was measured by Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

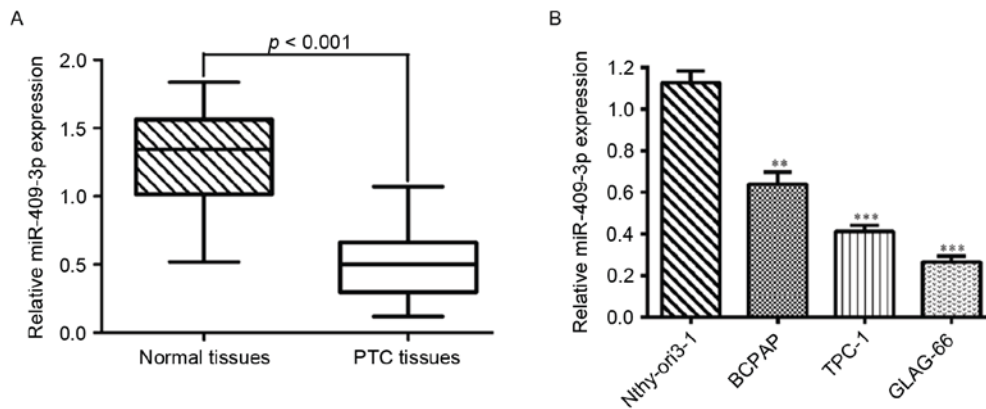


Figure 1. Downregulation of miR-409-3p expression in PTC tissues and cell lines compared with the corresponding controls. Reverse transcription-quantitative polymerase chain reaction was used to analyze the relative expression levels of miR-409-3p in (A) 20 surgically resected specimens of PTC tissues and matched non-tumor tissues, as well as in (B) three PTC cell lines (B-CPAP, TPC-1 and GLAG-66) and a normal human thyroid epithelial cell line (Nthy-ori3-1). Data are shown as the mean and standard deviation of expression levels relative to U6 expression levels. ** $P < 0.01$, *** $P < 0.001$ vs. Nthy-ori3-1. PTC, papillary thyroid carcinoma; miR-409-3p, microRNA-409-3p.

Statistical analysis. All experiments were repeated in triplicate and the results were expressed as the mean \pm standard deviation. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). A two-tailed Student's t-test was applied for comparisons between the two groups. Multiple comparisons were performed using a one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-409-3p is downregulated in PTC tissues and cell lines. The expression levels of miR-409-3p were first determined by RT-qPCR in 20 pairs of human PTC tissues and adjacent normal tissues. As shown in Fig. 1A, the expression level of miR-409-3p was significantly lower in tumor tissues compared with matched non-tumor tissues ($P < 0.001$). Also, the expression levels of miR-409-3p were measured in three PTC cell lines (B-CPAP, TPC-1 and GLAG-66) and a normal human thyroid epithelial cell line (Nthy-ori3-1). miR-409-3p was significantly downregulated in all PTC cell lines compared with Nthy-ori3-1 (Fig. 1B; $P < 0.001$). Thus, it was concluded that a decrease in miR-409-3p expression may serve a role in PTC development.

Overexpression of miR-409-3p induces cell growth inhibition and G_0/G_1 phase arrest in PTC cells. The results demonstrated that TPC-1 and GLAG-66 cells presented lower expression of miR-409-3p. Therefore, synthetic miR-409-3p mimics and NC mimics were separately transfected into TPC-1 and GLAG-66 cells to investigate the biological function of miR-409-3p in PTC. RT-qPCR assays revealed that miR-409-3p expression was significantly upregulated following transfection with miR-409-3p mimics, compared with NC control cells in TPC and GLAG-66 cells ($P < 0.001$; Fig. 2A). The CCK-8 assay demonstrated that transfection with miR-409-3p mimics significantly inhibited cell proliferation in TPC-1 and GLAG-66 cells ($P < 0.05$; Fig. 2B). The colony formation assay indicated that the ability of colony formation in TPC-1

and GLAG-66 cells transiently transfected with miR-409-3p mimics was impaired compared with that in NC-transfected cells ($P < 0.05$; Fig. 2C). Flow cytometric analysis revealed that overexpression of miR-409-3p led to G_0/G_1 phase arrest by elevating the percentage of cells at G_0/G_1 phase in TPC-1 and GLAG-66 cells, but decreasing the cell numbers at S and G_2/M phase in TPC cells and cells at G_2/M phase in GLAG-66 cells ($P < 0.05$; Fig. 2D).

miR-409-3p downregulates cyclin D2 expression by targeting its 3'UTR. On the basis of the aforementioned results, a possible role for miR-409-3p in repressing G_0/G_1 -associated genes was hypothesized. As shown in Fig. 3A, *CCND2* exhibited miR-409-3p-binding sequences in its 3'-UTR, which was identified as a putative target of miR-409-3p by miRNA target gene prediction. To confirm the direct targeting of *CCND2* by miR-409-3p, a dual-luciferase reporter assay was performed by integrating sequences of the *CCND2* 3'UTR containing the binding sites for miR-409-3p or corresponding mutated sequences into 293T cells. Luciferase assays revealed that miR-409-3p mimics significantly decreased the relative activity of the luciferase reporter containing the wild-type 3'-UTR of *CCND2* mRNA ($P < 0.05$), but did not affect the luciferase activity of the mutant *CCND2* 3'-UTR (Fig. 3B). Next, the effect of miR-409-3p on the expression of *CCND2* was measured at the mRNA and protein levels in PTC cells. As shown in Fig. 3C, transfection with miR-409-3p mimics downregulated the mRNA and protein expression levels of *CCND2* and cyclin D2, respectively, compared with cells transfected with NC controls ($P < 0.05$). These results indicated that *CCND2* may be a direct target of miR-409-3p in PTC.

Enhanced expression of *CCND2* partially rescues miR-409-3p-induced cell growth inhibition and G_0/G_1 phase arrest in PTC cells. To determine whether *CCND2* mediated the inhibitory effect of miR-409-3p on cell proliferation and cell cycle progression, rescue experiments were performed by transfecting pcDNA/*CCND2* into PTC cells with higher miR-409-3p expression levels and cell proliferation and cell cycle were then examined. The results revealed that

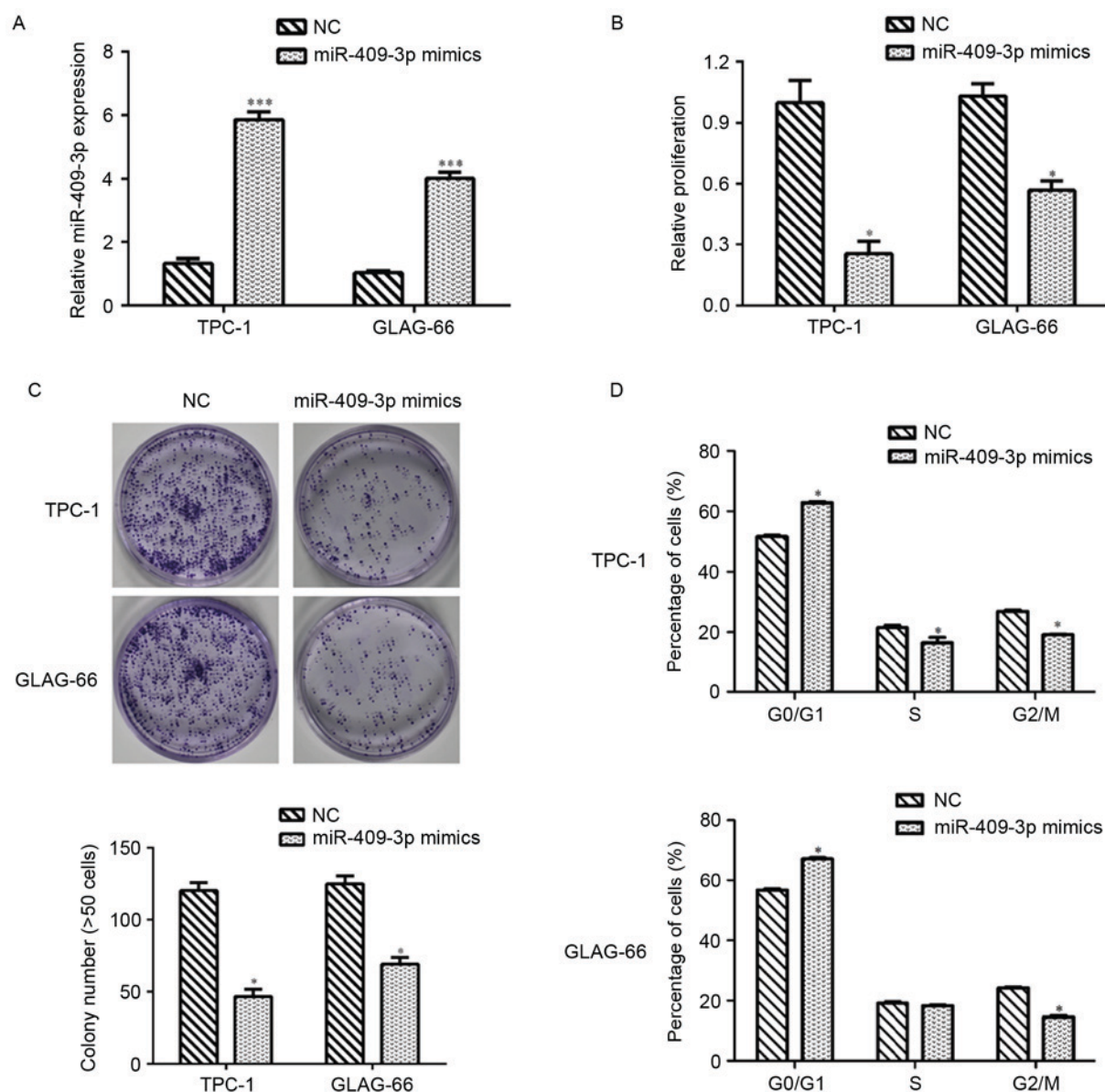


Figure 2. Overexpression of miR-409-3p inhibits PTC cell proliferation by cell cycle arrest at G₀/G₁ phase. (A) Reverse transcription-quantitative polymerase chain reaction detection of miR-409-3p expression in TPC-1 and GLAG-66 cells stably transfected with miR-409-3p mimics or NC. U6 small nuclear RNA was used as an internal control. (B) Effect of miR-409-3p overexpression on PTC cell viability using a Cell Counting Kit-8 assay. (C) Representative micrographs of cell colony formation assay (top) and quantification (bottom) from three independent experiments. (D) Flow cytometry cell cycle analysis revealed the effect of miR-409-3p mimic transfection on the percentage of cells in G₀/G₁, S and G₂/M phase in TPC-1 and GLAG-66 cells. Values represent the mean from three separate experiments and error bars represent the standard deviation. *P<0.05 and ***P<0.001 vs. NC. PTC, papillary thyroid carcinoma; miR-409-3p, microRNA-409-3p; NC, negative control.

overexpression of *CCND2* partially rescued the impaired cell proliferation induced by miR-409-3p mimics in TPC-1 and GLAG-66 cells (P<0.05; Fig. 4A). It was also found that restoration of *CCND2* reversed the cell cycle G₀/G₁ phase arrest imposed by miR-409-3p mimics in TPC-1 (P<0.05; Fig. 4B) and GLAG-66 cells (P<0.05; Fig. 4C). These data indicated that *CCND2* may mediate the suppressive functions of miR-409-3p in PTC cells.

Discussion

In recent years, many miRNA signatures have been well characterized for PTC (17); however, but further investigation on the roles of dysregulated miRs in PTC development

and progression is necessary for seeking more effective therapeutic strategies. In the present study, it was observed that miR-409-3p was frequently downregulated in PTC samples specimens and cell lines, consistent with its reduced expression in lung (18) and bladder cancer (19). Functional analysis revealed that overexpression of miR-409-3p caused a reduction in proliferation rate, which was accompanied by G₀/G₁ phase arrest of the cell cycle, indicating its suppressive role in PTC cells.

Evidence of miR-409-3p as a tumor growth inhibitor has been well documented in several types of cancer. For example, miR-409-3p inhibited growth and induced apoptosis in gastric cancer (20). In breast cancer, miR-409-3p has been demonstrated to suppress cell growth by targeting protein

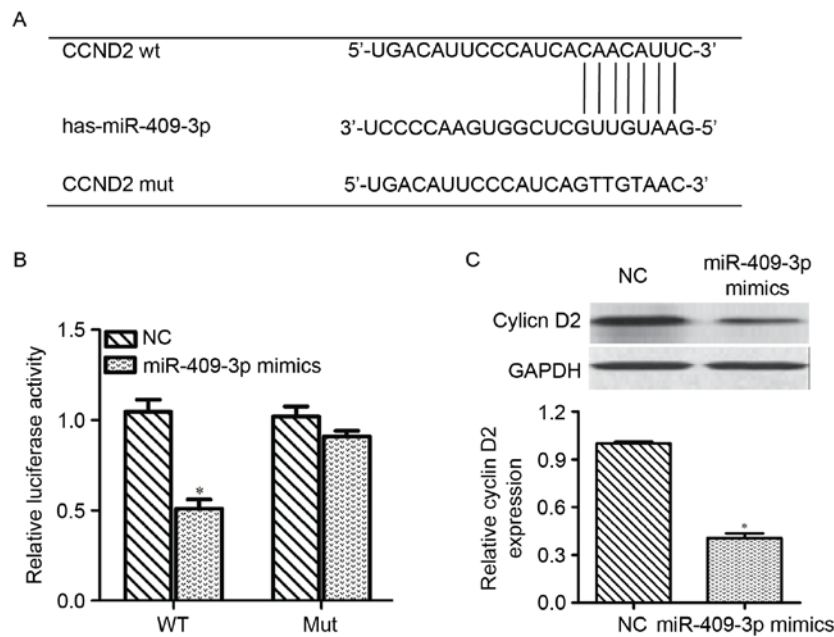


Figure 3. miR-409-3p directly binds to the 3'-UTR of *CCND2*. (A) Predicting binding sites and the corresponding mutated sequences within the *CCND2* 3'UTR for miR-409-3p were presented. (B) 293T cells were co-transfected with either miR-409-3p mimics or NC and luciferase reporter psiCHECK vector comprising Wt or Mut 3'UTR of *CCND2*. The relative firefly luciferase activity normalized with *Renilla* luciferase was measured 48 h after transfection. The data shown represents the mean \pm standard deviation of three independent experiments. (C) The relative *CCND2* mRNA and protein levels were detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively, in PTC cells stably transfected with miR-409-3p mimics or NC. GAPDH was used as an internal control. * $P < 0.05$ vs. NC. 3'UTR, 3' untranslated region; NC, negative control; *CCND2*, cyclin D2; miR-409-3p, microRNA-409-3p; wt, wild type; mut, mutant; PTC, papillary thyroid carcinoma.

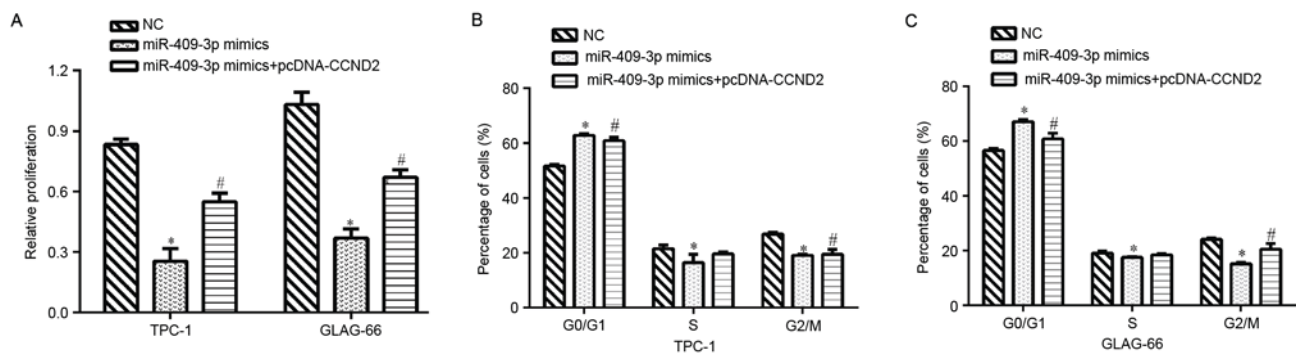


Figure 4. Overexpression of *CCND2* partially rescues the inhibitory effects of miR-409-3p on cell proliferation and cell cycle progression. TPC-1 and GLAG-66 cells were transfected with mimic NC or miR-409-3p mimics together with a plasmid expressing *CCND2* or an empty vector. (A) CCK-8 assays were performed to determine the proliferation rate of TPC-1 and GLAG-66 cells after 48 h transfection. Cell cycle analysis was performed 48 h after transfection in (B) TPC-1 and (C) GLAG-66 cells. Values represent the mean from three separate experiments; error bars represent the standard deviation. * $P < 0.05$ vs. NC and # $P < 0.05$ vs. miR-409-3p mimics. *CCND2*, gene encoding cyclin D2; miR-409-3p, microRNA-409-3p; NC, negative control.

kinase B (15). Consistent with the role of miR-409-3p in PTC cell proliferation, the present study identified *CCND2* as a target gene of miR-409-3p. Further analysis demonstrated that the enforced expression of miR-409-3p significantly decreased cyclin D2 protein expression levels, indicating that miR-409-3p may be able to negatively regulate the expression of *CCND2*. The identification of *CCND2* may account for one of the underlying mechanisms by which miR-409-3p is involved in PTC cell proliferation.

D-type cyclins are reported to be closely associated with cell cycle progression (21), of which *CCND2* is a key player in the cell cycle from the G₀/G₁ phase to S phase (22). The *CCND2* oncogene has been well noted in human thyroid cancer, which was downregulated by miR-1 (23). Similarly, upregulation

of *CCND2* has been documented in breast cancer (24) and prostate cancer (25). To confirm the role of *CCND2* in PTC further, the present study also demonstrated that when miR-409-3p-expressing cells resumed *CCND2* expression, the proliferation deficiencies and G₀/G₁ phase arrest were partly reversed. Therefore, it was concluded that miR-409-3p may inhibit cell proliferation and cell cycle progression in PTC by downregulating, at least partially, the protein expression of cyclin D2.

In conclusion, the results of the present study indicated that miR-409-3p is frequently downregulated in PTC and may negatively regulate PTC cell proliferation and cell cycle progression by downregulating its target gene *CCND2*. The data may help to further elucidate the molecular mechanisms

underlying PTC progression and provide a strategy for targeting the miR-409-3p/*CCND2* interaction as a novel prevention and treatment of PTC.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

ZZ, FY and YL performed the experiments. KF participated in the interpretation of data. SJ is major contributor and participated in the design of study. All authors revised the manuscript, read and approved the final manuscript.

Ethics approval and consent to participate

Each patient voluntarily signed written informed consent and the collection and the current study was approved by the Fourth Hospital of Hebei Medical University.

Patient consent for publication

The written informed consent for publication was signed by all patients in advance and they gave their consent for the publication of any associated data and images.

Competing interests

The authors declare that they have no competing interests.

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