

# MiR-181a promotes growth of thyroid cancer cells by targeting tumor suppressor RB1

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**Abstract. – OBJECTIVE:** MicroRNAs (miRs) are critical regulators in cancer development and progression. The current study aimed to investigate the expression and potential function of miR-181a in thyroid cancer.

**PATIENTS AND METHODS:** A total of 15 paired thyroid cancer tissues and adjacent normal tissues were subjected to Real-time Polymerase Chain Reaction (PCR) to evaluate miR-181a expression.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, enzyme linked immunosorbent assay (ELISA) or flow cytometry was employed to assess the growth activity, apoptosis and cell cycle, respectively, upon modulation of the miR-181a expression in TPC-1 cells. Western blot was used to assess protein expression. The interaction between miR-181a and RB1 was tested by luciferase activity assay.

**RESULTS:** The expression of miR-181a was significantly upregulated in thyroid cancer tissues compared with the adjacent tissues. Inhibition of miR-181a attenuated cell growth, which could be abrogated by miR-181a co-transfection. MiR-181a overexpression reduced apoptosis and promoted cell cycle progression; inhibition of miR-181a exerted opposite effects on both cell cycle and apoptosis. MiR-181a directly suppressed RB1 expression. RB1 expression in tumor tissues was downregulated and negatively correlated with miR-181a expression.

**CONCLUSIONS:** miR-181a plays an oncogenic role in thyroid cancer; by targeting RB1, it promotes cell cycle progression and inhibits apoptosis.

*Key Words:*

Thyroid cancer, miR-181a, Cell cycle, Apoptosis, RB1.

## Introduction

Thyroid cancer represents one of the most common malignancies in the endocrine system. Its in-

cidence is increasing yearly worldwide<sup>1-3</sup>. Due to the recent efforts that have been achieved in its early detection and systematic treatment beyond surgical resection (radiotherapy and levothyroxine administration)<sup>4</sup>, the survival of patients with thyroid cancer has been greatly improved. Nonetheless, some of the thyroid cancer cases are still highly aggressive and metastatic, which often lead to treatment failure or recurrence. Understanding the molecular basis that underlies the development of thyroid cancer will certainly help us to identify new therapies for this malignant disease. Emerging evidence has highlighted that microRNAs (miRs) are important regulators in cancer development<sup>5,6</sup>. These small molecules are often composed of 20-25 nt nucleotides, and they rationally take effect via binding to the 3' untranslated region (UTR) of given mRNAs<sup>7</sup>. The binding affinity of miRs and mRNAs depends on their complete or incomplete base pair matches. In thyroid cancer, it was well documented that a group of miRs is aberrantly expressed<sup>8</sup>; a gene profile study showed that miR-146b, miR-221, and miR-222 are overexpressed in papillary thyroid cancer (PTC), and that they confer a high-risk of extrathyroidal extension, lymph node metastasis, distant metastasis, and recurrence<sup>9-13</sup>. In addition to these miRs, numerous recent studies have now expanded our understanding on multiple other miRs in the molecular basis of thyroid cancer. For example, miR-497, miR-195, miR-144, and miR-338-3p, inhibit cell proliferation and invasion in PTC<sup>14-17</sup>.

While some studies identified miR-181a as an oncogene in cancers in prostate, ovary, and stomach<sup>18-21</sup>, the role of miR-181a in some other cancer types is still undetermined. Previous studies have shown that miR-181a is upregulat-

ed in thyroid cancer tissues<sup>9</sup>. However, whether it plays an essential role for thyroid cancer development is unknown. In the present study, we analyzed the expression profile of miR-181a in the paired tumor tissue and adjacent normal tissue from patients with thyroid cancer. We show that miR-181a is constantly upregulated in thyroid cancer. Functional tests reveal that miR-181a serves as an oncogenic factor that is involved in the critical biological events such as cell cycle progression and apoptosis. Mechanistically, the tumor suppressor RB1 is identified to be the direct target of miR-181a in thyroid cancer cells. Thus, our study reveals a novel miR-181a-related regulatory mechanism essential for thyroid cancer development and progression.

## Patients and Methods

### *Tumor Specimens*

The tumor specimens were collected in Jiangxi Cancer Hospital from August 2016 to February 2017. A total of 15 pairs of specimens were collected. Patients did not receive chemotherapy or radiotherapy before surgical treatment. The adjacent normal tissues were all obtained from the surgical resection and were at least 3 cm away from the tumor. The tumor tissues and the negative resection margins of normal tissues were histologically examined. The tissues were snap frozen in liquid nitrogen. Informed consent was obtained from each individual. This study was approved by the Ethics Review Board of Jiangxi Cancer Hospital.

### *Cell Source and Culture Condition*

We obtained thyroid cancer cell lines 8505C, SW1736, and TPC-1 and the normal human thyroid follicular cell line Nthy-ori3-1 from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS), and the medium was changed every other day. Cells were cultured at constant 37°C in a humidified incubator, and the concentration of CO<sub>2</sub> was 5%. For the transfection of miR-181a mimic or inhibitor, lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as per manufacturer's protocol. Transfection concentration of this oligonucleotide was 100 nM.

### *Real-time PCR*

The total RNA from tissue samples or cell samples was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), using a standard protocol provided by the manufacturer. The RNA was then purified with a Tiangen RNA Clean Kit (Tiangen, Beijing, China). Reverse transcription using the GoScript Reverse Transcription System (Promega, Madison, WI, USA) was performed following purification. Stem-loop primer sets for miR-181a reverse transcription were purchased from RiboBio (Guangzhou, China). The cDNA first strand was then amplified using GoTaq qPCR SYBR GREEN Master Mix (Promega, Madison, WI, USA) with the ABI 7900 system (Applied Biosystems, Foster City, CA, USA). U6 was used as a normalization control for miR-181a quantification. GAPDH was used for RB1 normalization. To compare the relative fold changes of genes, the 2<sup>-ΔΔCt</sup> method was used.

### *Apoptosis Assay*

Apoptosis was quantified by the cell death ELISA kit from Roche Diagnostics (Basel, Switzerland) according to the provided protocol, and three independent replicates were performed.

### *Cell Cycle Analysis*

Cell cycle was analyzed using the cell cycle detection kit (Beyotime, Shanghai, China). Briefly, fixed cells were stained with propidium iodide and subjected to FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

### *Luciferase Activity Assay*

The cDNA fragment of RB1 3'UTR containing the putative miR-181a binding site was sub-cloned into pmirGLO (Promega, Madison, WI, USA) to construct a reporter containing the RB1 3'UTR. The construct was then co-transfected with miR-181a mimic or miR-181a inhibitor into TPC-1 cells for 48 h; luciferase activity was measured using Dual luciferase activity assay system as per manufacturer's protocol.

### *Western blot*

To assess protein content, Western blot was performed. Cells grown in 6-well plate were treated as described and 72 h after transfection, cells were washed and collected using 2X SDS sample buffer (Tiangen). Next, the protein samples were boiled for 3 min. The protein samples were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

gel (Beyotime) at constant 120 V for 1 h by using the Tanon electrophoresis system (Shanghai, China). The proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat milk and incubated with primary antibodies anti BCL2, BAX, RB1 and  $\beta$ -actin at 4°C overnight. The primary antibodies were all purchased from Cell Signaling Technology (CST, Danvers, MA, USA) and used at dilution of 1:1000. Horseradish peroxidase (HRP) linked goat anti rabbit secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Finally, membranes were detected by electro chemiluminescence (ECL) plus kit (Beyotime, Shanghai, China); the images were quantified by ImageJ software, and  $\beta$ -actin was used as normalization control.

### **MTT assay**

MTT assay was used to assess cell growth activity. The cells were seeded in 24-well plate at the concentration of  $4 \times 10^4$ /ml. MTT was added to the culture medium 4 h before final test. The MTT product was dissolved with dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and the absorbance value at the 490 nm wavelength was measured using a Biotek Synergy2 spectrophotometer (Winooski, VT, USA).

### **Statistical Analysis**

The data were presented as means $\pm$ SEM. Student's *t*-test was used to compare the values between two groups. One way analysis of variance (ANOVA) was used for comparison between three groups; the pairwise comparison was performed using Tukey post-hoc test. Correlation analysis between RB1 and miR-181a expression was performed using Spearman correlation test.  $p < 0.05$  was considered to be statistically significant.

## **Results**

### ***MiR-181a is Overexpressed in Thyroid Cancer Tissues and Cells***

To assess the potential function of miR-181a in thyroid cancer development and progression, we have collected 15 paired samples of thyroid cancer tissues and adjacent normal tissues. The endogenous level of miR-181a was quantified by Real-time PCR. We found that miR-181a was expressed at a significantly higher level in thyroid tumor tissues compared with the adjacent tissues (Figure 1A). Moreover, if we compare the chang-

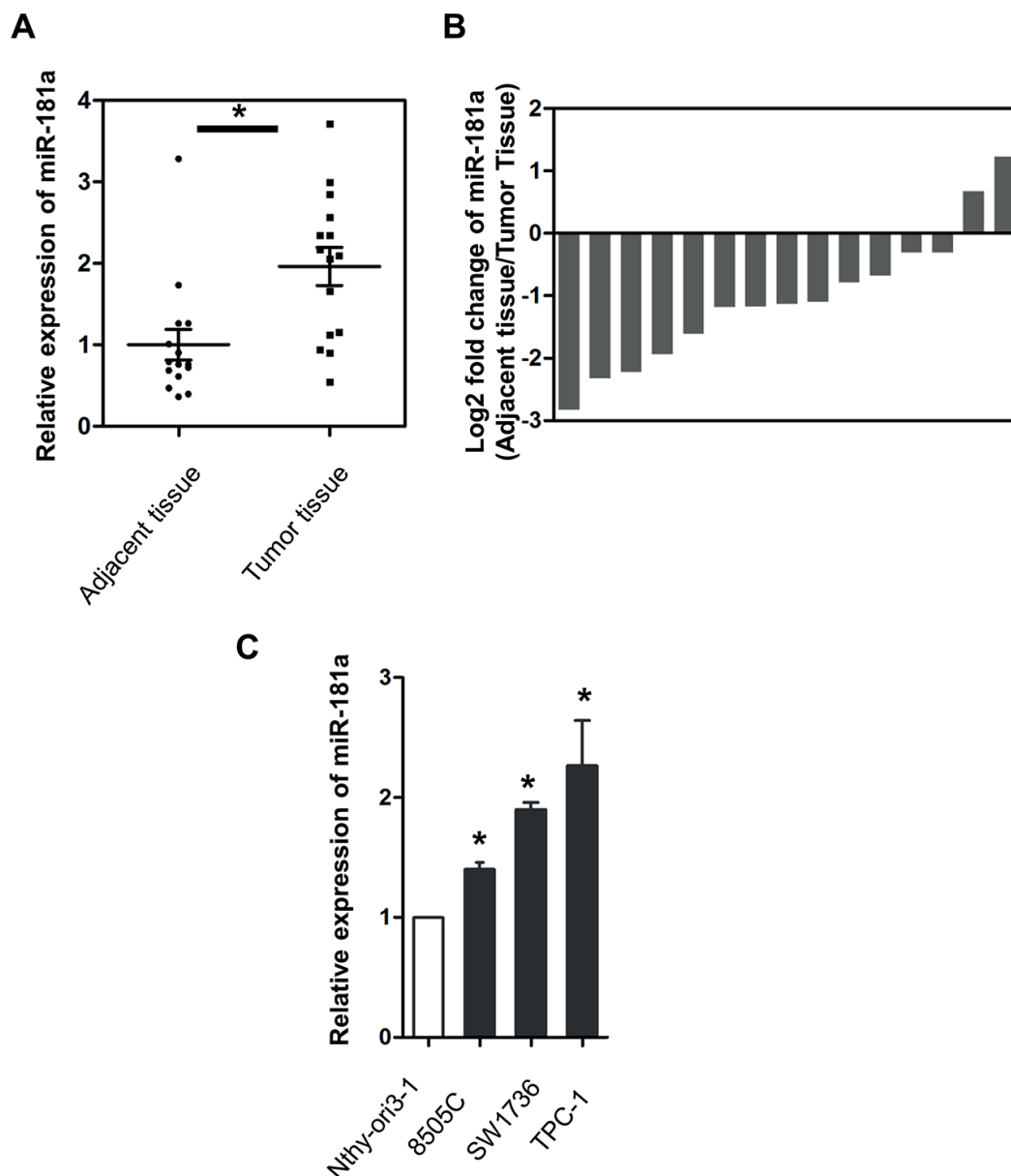
es of miR-181a content in each pair, we could find that miR-181a expression was upregulated in 13 out of 15 patients (Figure 1B). Next, we examined the content of miR-181a in various thyroid cancer cells (8505C, SW1736 and TPC-1) and the normal follicle epithelial cell (Nthy-ori3-1). The expression of miR-181a was significantly overexpressed in all the cancer cells tested (Figure 1C), and its expression was highest in TPC-1 cell line. Thus, TPC-1 was later used for further study. These data show that miR-181a is upregulated in thyroid cancer tissues, and suggest its possible role in thyroid cancer.

### ***MiR-181a Positively Regulates Thyroid Cancer Cell Growth***

To further explore the function of miR-181a in thyroid cancer, we transfected miR-181a mimic and miR-181a inhibitor into TPC-1 cells to overexpress and inhibit miR-181a, respectively. Real-time PCR confirmed the overexpression and inhibition by mimic and inhibitor (Figure 2A). By using MTT assay to construct a 4-day growth curve, we monitored a significant decline of growth activity in miR-181a inhibitor transfected cells from 72 h post-transfection. The growth inhibition effect of miR-181a inhibitor was almost completely abrogated by miR-181a (Figure 2B). This finding suggests that miR-181a positively regulates thyroid cancer cell growth.

### ***MiR-181a Promotes cell Cycle Progression and Inhibits Apoptosis***

To investigate how the growth promoting effect by miR-181a was achieved, we performed further analyses on cell cycle and apoptosis, both of which could severely affect cancer cell growth activity. We found that overexpression of miR-181a by mimic transfection decreased the number of cells in G1 phase, suggesting that G1 to S phase transition was accelerated (Figure 3A). Overexpression of miR-181a resulted in a mild but significant decrease in apoptosis (Figure 3B). BCL2 and BAX are essential in regulating apoptosis; BCL2 exerts a negative effect on apoptosis, while BAX exerts a positive effect. Their ratio is a well-established marker to evaluate the extent of apoptosis. As shown in Figure 3C, after miR-181a transfection, BCL2 expression was increased, and BAX expression was decreased. Quantification of the Western blot results revealed an increase in BCL2/BAX ratio, suggesting a decreased apoptotic potential (Figure 3D). In contrast, transfection of miR-181a inhibitor resulted in cell cycle arrest at G1 phase



**Figure 1.** miR-181a is upregulated in thyroid cancer tissues and cells. **(A)** The relative expression of miR-181a in thyroid tumor tissues and adjacent normal tissue.  $*p < 0.05$ ,  $n = 15$ . **(B)** The Log2 transformed miR-181a expression in adjacent tissue and tumor tissue in each patient. **(C)** Comparison of the relative expression of miR-181a in normal follicle epithelial cell line Nthy-ori3-1 and thyroid cancer cell lines 8505C, SW1736 and TPC-1.  $*p < 0.05$  vs. Nthy-ori3-1,  $n = 3$ .

(Figure 4A). An increase in apoptosis was also observed (Figure 4B). Concurrently, BCL2/BAX ratio was significantly downregulated (Figure 4C and 4D). These data indicate that miR-181a positively regulates cell growth by promoting cell cycle progression and inhibiting apoptosis.

***RB1 is a Functional Target of miR-181a***

To mechanistically explore the possible mechanism of the above reported effects of miR-181a in

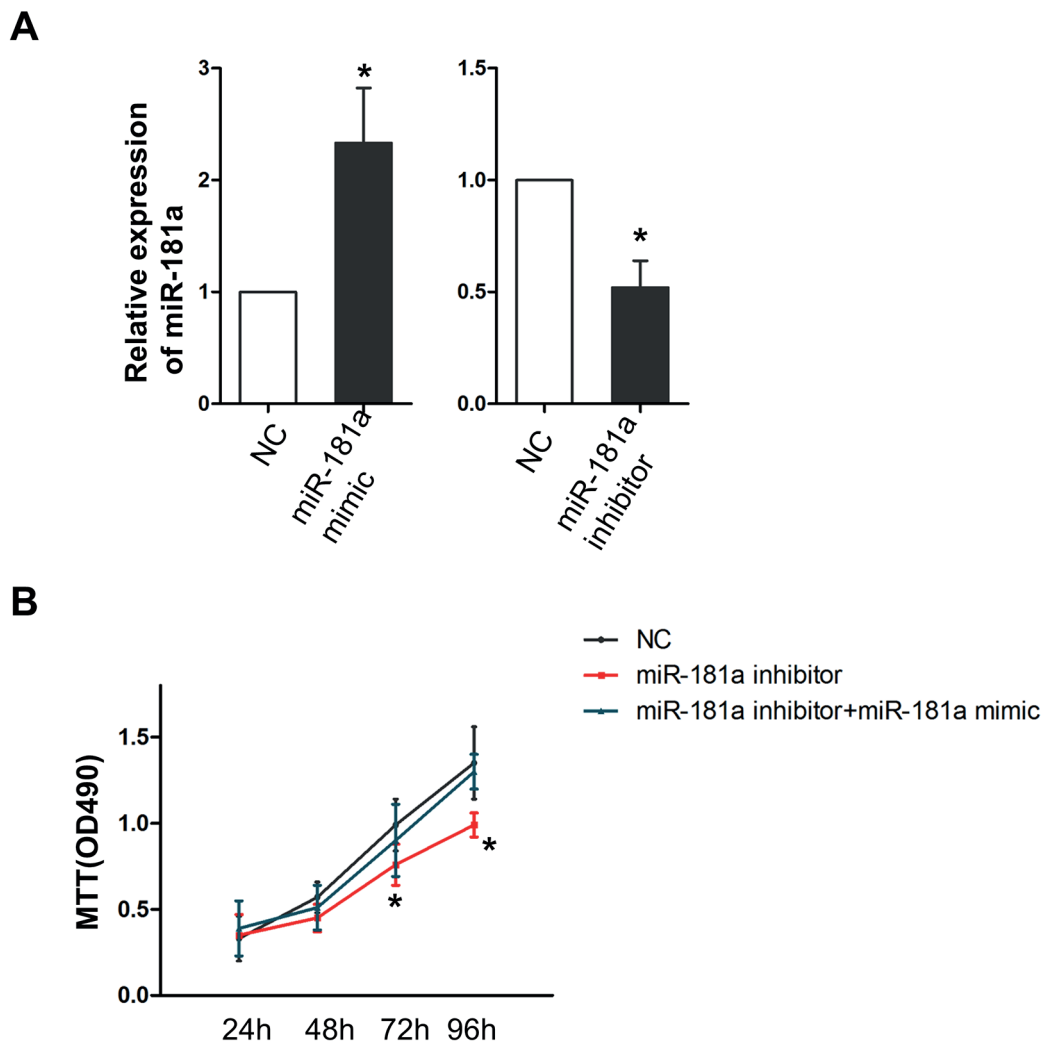
thyroid cancer cells, we searched the miRanda database ([www.microrna.org](http://www.microrna.org)). We found that RB1, an essential regulator for G1-S phase cell cycle transition, contains a putative binding site for miR-181a in its 3'UTR (Figure 5A). We again transfected miR-181 mimic and inhibitor into TPC-1 cells, analyzing the protein of RB1, which revealed that its expression was decreased upon mimic transfection, but increased upon inhibitor transfection (Figure 5B-C). Then, we cloned the 3'UTR fragment con-

taining the putative binding site and constructed luciferase activity assay. As shown in Figure 5D, miR-181a mimic suppressed the luciferase activity, whereas miR-181a inhibitor increased the luciferase activity. Of note, we found that the mRNA expression of RB1 in the tumor tissues was significantly downregulated (Figure 5E). Correlation analysis confirmed a negative association between the expression of RB1 and miR-181a (Figure 5F). This finding supports that miR-181a promotes thyroid cancer cell growth by directly targeting RB1.

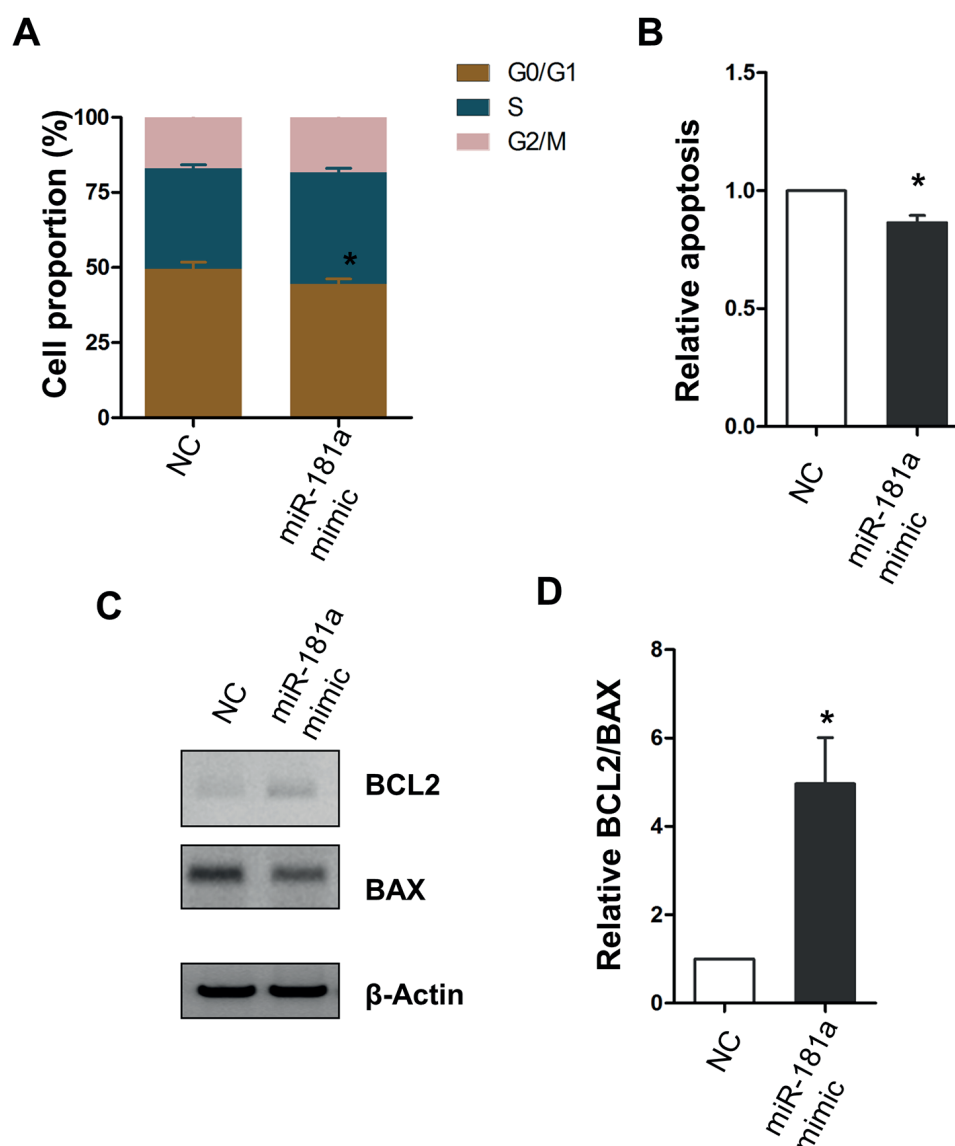
### Discussion

Although previous studies have identified that several miRs, including miR-181a, are aberrantly

expressed in thyroid cancer<sup>9</sup>, it is still largely unknown whether they are functionally linked to the pathogenesis of this malignant disease. In the present work, we have sought to address whether miR-181a is involved in the cell biology of thyroid cancer development and progression. We demonstrate that miR-181a is an oncogenic factor in thyroid cancer. Several lines of evidence support our conclusion. Firstly, miR-181a is constantly upregulated in the tumor tissue compared with the adjacent normal tissue. Secondly, inhibition of miR-181a attenuated, whereas overexpression of miR-181a promoted the growth activity of cancer cells. Thirdly, cell cycle progression and apoptosis are likely to be significantly affected after modulation of miR-181a expression. Lastly, the well-characterized tumor suppressor, RB1, is a direct target of miR-181a



**Figure 2.** miR-181a promotes thyroid cancer cell growth. (A) The relative expression of miR-181a after miR-181a mimic and inhibitor transfection in TPC-1 cells. \* $p < 0.05$  vs. NC,  $n = 3$ . (B) The MTT (OD490) value in TPC-1 cells transfected with NC, miR-181a inhibitor and miR-181a inhibitor+miR-181a mimic. \* $p < 0.05$  vs. NC,  $n = 3$ . NC: negative control.



**Figure 3.** Overexpression of miR-181a promotes cell cycle progression and inhibits apoptosis. **(A)** Cell cycle analysis of TPC-1 cells transfected with miR-181a mimic. \* $p < 0.05$  vs. NC for G0/G1 phase,  $n = 3$ . **(B)** Relative apoptosis of TPC-1 cells transfected with miR-181a mimic. \* $p < 0.05$  vs. NC,  $n = 3$ . **(C)** The effect of miR-181a mimic on BCL2 and BAX expression in TPC-1 cells. **(D)** The effect of miR-181a mimic on relative BCL2/BAX ratio. \* $p < 0.05$  vs. NC,  $n = 3$ .

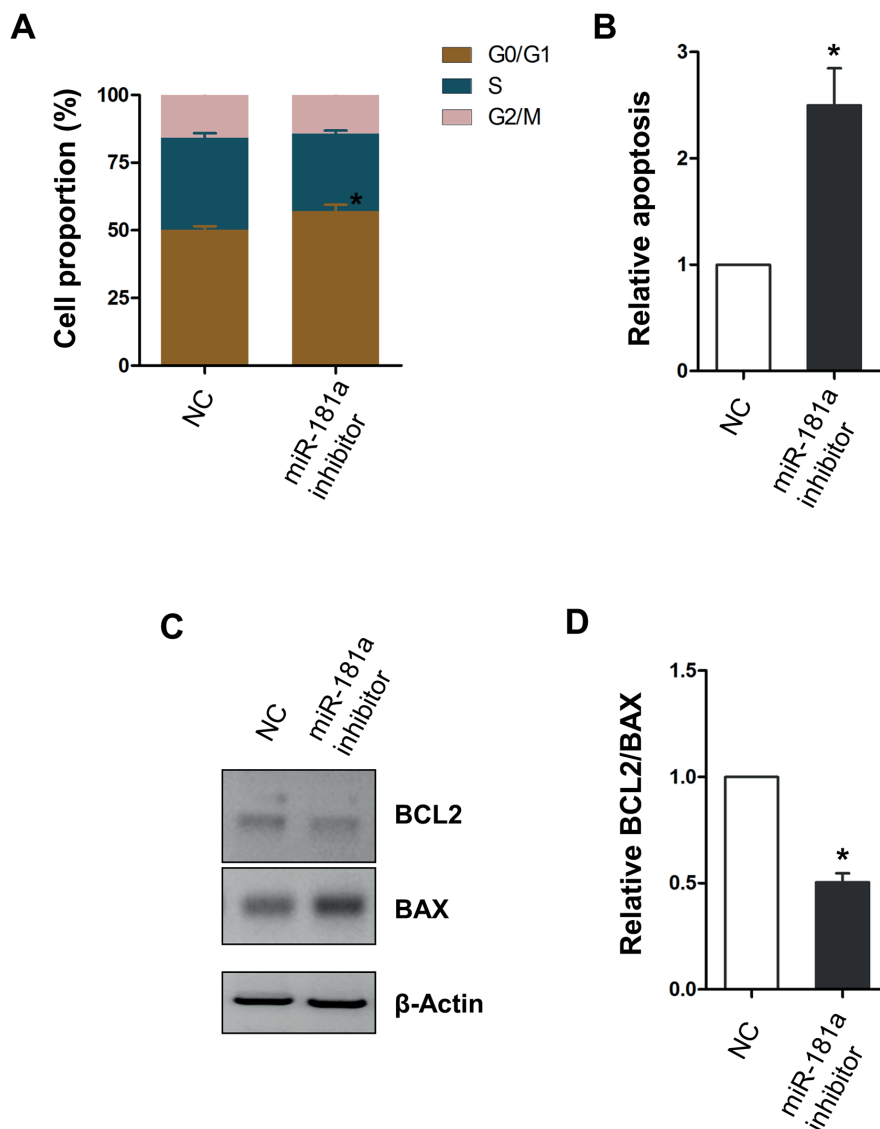
in thyroid cancer. To the best of our knowledge, our study is the first to demonstrate the function of miR-181a in thyroid cancer, and it provides novel insights into the regulatory mechanism of thyroid cancer development.

Before the current work, several other studies have identified a batch of miRs to be critical in thyroid cancer. By comparing the miRs expression profile in 30 paired PTC samples and normal tissue samples, He et al<sup>9</sup> firstly identified that 23 miRs are differentially expressed; they proposed that miR-146b, miR-221, and miR-222

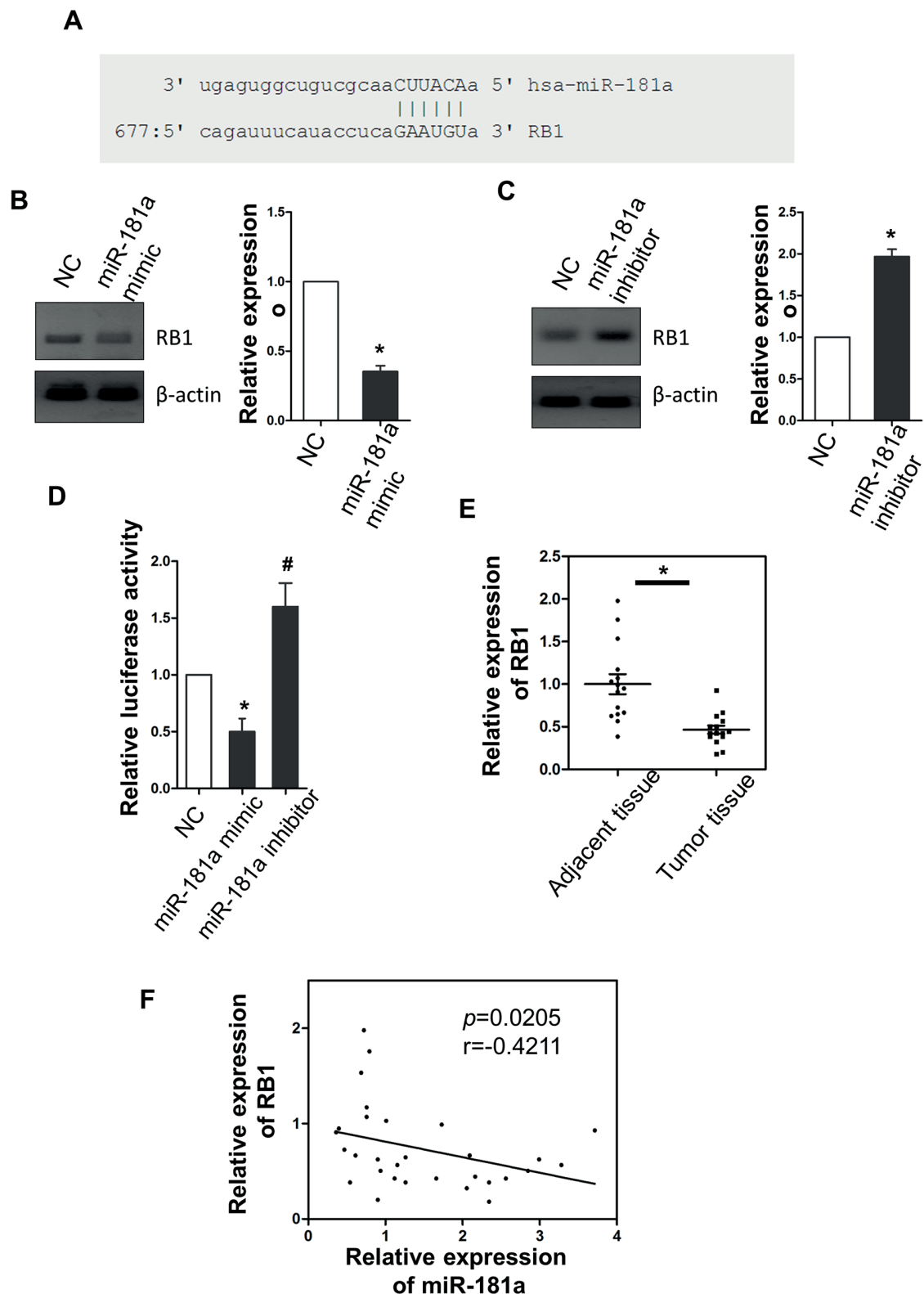
are three most significantly upregulated miRs. The following studies<sup>11,13,22-24</sup> have investigated their function and highlighted that these miRs might be important in the diagnosis and treatment of thyroid cancer. Recently, numerous miRs have been identified to regulate the cell biological behaviors in thyroid cancer. For instances, Sui et al<sup>16</sup> demonstrated that miR-338-3p serves as a tumor suppressive gene by regulating the pro-survival gene AKT3, and that miR-338-3p tends to be negatively associated with lymph node metastasis and mainly influences

the cell migration process. MiR-1-CCND2 signaling axis is involved in the growth regulation by long non-coding RNA HOTAIR<sup>25</sup>. Several other miRs, such as miR-195, miR-144 and miR-125b, are also involved in multiple aspects of thyroid cancer by targeting various genes<sup>15,17,26</sup>. A previous work on miRs expression profile in thyroid cancer has revealed that miR-181a, albeit not very significant, has a 2.6 fold of increase in PTC<sup>9</sup>. In the study cohort of the current investigation, a similar increase in miR-181a expression has been detected. Reseaches in other systems and organs showed that miR-181a has multiple

functions. Howerer contradictory results are often seen in the literature. In gastric cancer, overexpression of miR-181a contributed to an increased proliferative potential<sup>18,27</sup>. In cervical cancer, Luo et al<sup>28</sup> observed that miR-181a negatively regulates cell growth and drug resistance, whereas two reports supported an oncogenic role of miR-181a<sup>29,30</sup>. The reason for this discrepancy is unknown, but it might reflect the heterogeneity of different cancer types or subtypes. Cancer cells often adopt a phenotype that is resistant to programmed cell death and increased cell cycle progression, which ultimately results in over-



**Figure 4.** Inhibition of miR-181a inhibits cell cycle transition and promotes apoptosis. **(A)** Cell cycle analysis of TPC-1 cells transfected with miR-181a inhibitor. \* $p < 0.05$  vs. NC for G0/G1 phase,  $n = 3$ . **(B)** Relative apoptosis of TPC-1 cells transfected with miR-181a inhibitor. \* $p < 0.05$  vs. NC,  $n = 3$ . **(C)** The effect of miR-181a inhibitor on BCL2 and BAX expression in TPC-1 cells. **(D)** The effect of miR-181a inhibitor on relative BCL2/BAX ratio. \* $p < 0.05$  vs. NC,  $n = 3$ .



**Figure 5.** RB1 is a target of miR-181a. **(A)** Putative binding site of miR-181a and RB1 3'UTR. **(B)** The effect of miR-181a mimic on RB1 expression in TPC-1 cells. \* $p < 0.05$  vs. NC,  $n = 3$ . **(C)** The effect of miR-181a inhibitor on RB1 expression in TPC-1 cells. \* $p < 0.05$  vs. NC,  $n = 3$ . **(D)** The relative luciferase activity after miR-181a mimic and miR-181a inhibitor transfection in TPC-1 cells. \* $p < 0.05$  vs. NC, # $p < 0.05$  vs. NC,  $n = 3$ . **(E)** The relative mRNA expression of RB1 in tumor tissues and adjacent normal tissue. \* $p < 0.05$ ,  $n = 15$ . **(F)** Correlation analysis of the expression level of RB1 and miR-181a in all clinical samples.



growth of cells. Our present study showed that miR-181a regulates the growth activity in thyroid cancer at least partially by modulating apoptosis and cell cycle progression. The upregulation of miR-181a in thyroid cancer, thus, might underline one of its pathogenic causes. Given that the function of miR-181a in thyroid cancer has previously been completely unknown, our study might be important to expand our understanding towards miRs related molecular pathogenesis of this disease. We identified that tumor suppressor RB1 is the target of miR-181a in thyroid cancer cells; this finding is consistent with the observed effects on cell cycle and apoptosis, since RB1 is a well-defined essential negative regulator of cell cycle. When phosphorylated, RB1 loses its binding activity with E2F, thus promoting G1 to S phase cell cycle progression. This change in cell cycle progression may consequently lead to a higher risk of tumorigenesis<sup>31</sup>. RB1 mutations have been linked to various cancers, and previous reports also demonstrated the involvement of RB1 in thyroid tumors<sup>31-33</sup>. Our study highlights the regulation of RB1 at the expression level, thus, miR-181a-RB1 signaling axis might be an important mechanism for thyroid cancer development. However, it is still to be determined whether this regulation works in a context dependent manner. While cell cycle arrest can lead to increased apoptosis, it is worth noting that cellular apoptosis caused by miR-181a inhibition in our study might be caused by other independent factors. A previous study suggests that miR-181a can target multiple anti-apoptosis genes in leukemia<sup>34</sup>; we speculate that some of these genes may also be regulated by miR-181a in thyroid cancer cells. Nevertheless, our study only reveals a tip of iceberg for the regulatory roles of miR-181a. Further studies are still required to fully explore the multiple functions of miR-181a in thyroid cancer.

### Conclusions

We found that miR-181a was constantly upregulated in thyroid cancer, and this upregulation probably contributes to decreased apoptosis and increased cell cycle progression. Mechanistically, miR-181a mediates these oncogenic effects by directly suppressing the expression of the tumor suppressor RB1. Our study suggests that miR-181a might be effective in controlling the growth of thyroid tumors in clinical treatment.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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