

# Circular RNA circ-ABCB10 promotes the proliferation and invasion of thyroid cancer by targeting KLF6

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**Abstract.** – **OBJECTIVE:** Recent researches have proved that circular RNAs (circRNAs) play important roles in many diseases. Thyroid cancer is one of the most common malignant tumors worldwide. Therefore, the aim of this study was to investigate the role of circ-ABCB10 in thyroid cancer.

**PATIENTS AND METHODS:** Circ-ABCB10 expression in 40 paired thyroid cancer tissues and adjacent normal tissues was monitored by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Subsequently, circ-ABCB10 was silenced or overexpressed in thyroid cancer cells. Function assays were conducted to explore the role of circ-ABCB10 in the proliferation and invasion of thyroid cancer *in vitro*. Furthermore, RT-qPCR and Western blot assay were performed to elucidate the potential underlying mechanism.

**RESULTS:** Circ-ABCB10 expression was significantly higher in thyroid cancer tissues than that in adjacent tissues. The growth ability of thyroid cancer cells was significantly inhibited after circ-ABCB10 silence. However, the growth ability of thyroid cancer cells was remarkably promoted after circ-ABCB10 was overexpressed *in vitro*. Similarly, the invasion of thyroid cancer cells was significantly inhibited or promoted after circ-ABCB10 silence or overexpression, respectively. Besides, the expression of KLF6 was markedly up-regulated by the silence of circ-ABCB10. However, KLF6 expression was down-regulated by overexpression of circ-ABCB10.

**CONCLUSIONS:** Circ-ABCB10 was first identified as a novel oncogene in thyroid cancer. Furthermore, it significantly enhanced the proliferation and invasion of thyroid cancer by targeting KLF6. Our findings suggested that circ-ABCB10 could be used as a potential therapeutic target in thyroid cancer.

**Key Words:**

Circular RNA, Circ-ABCB10, Thyroid cancer, KLF6.

## Introduction

Thyroid cancer is one of the most common malignant cancers, which originates from follicular or parafollicular thyroid cells. It is estimated that almost approximately 90% of all cases are papillary thyroid carcinoma (PTC) annually<sup>1</sup>. Due to the application of ultrasonography, the number of newly diagnosed TC patients has been increasing worldwide over the past few decades<sup>2</sup>. However, despite numerous efforts have been made in surgery and radioactive iodine, there are still no significant improvements in the survival rate of thyroid cancer<sup>3</sup>. Current studies have found that the recurrence rate is approximately 20-30% or even higher in thyroid cancer. Therefore, searching for new biomarkers for early diagnosis and targeted therapy of aggressive TC is essential for improving the poor prognosis of thyroid cancer patients.

As a novel class of noncoding RNAs, circular RNAs (circRNAs) are formed by a covalently closed loop. They have emerged as a new hot topic in the noncoding RNAs network<sup>4</sup>. In the past decades, the roles of circRNAs have been widely explored, remaining still poorly understood<sup>5</sup>. Some studies have revealed that cellular circRNAs play important roles in cancers *via* targeting cancer-related regulators. Acting as a sponge of miR-153-3p, circ\_0084043 accelerates the proliferation and migration of malignant melanoma *via* up-regulating the expression Snail<sup>6</sup>. Overexpression of circ PVT1 promotes cell proliferation in gastric cancer by serving as a sponge for the miR-125 family<sup>7</sup>. Hsa\_circ\_0005986 functions as a tumor suppressor gene in hepatocellular carcinoma by serving as a miR-129-5p sponge. Meanwhile, it may be a novel biomarker for hepatocellular carcinoma<sup>8</sup>. By sponging miR-370,

the knockdown of hsa\_circ\_0061140 inhibits cell growth and metastasis in ovarian cancer<sup>9</sup>. Overexpression of circ\_0067934 serves as an oncogene and facilitates the progression of cervical cancer by modulating the miR-545/EIF3C axis<sup>10</sup>. A recent work has suggested that circ-ABCB10 is dysregulated in multiple types of cancers, including thyroid cancer. However, the exact role of circ-ABCB10 in thyroid cancer and the potential molecular mechanism have not been elucidated. This drives us to explore the function of circ-ABCB10 in thyroid cancer.

In our report, circ-ABCB10 was highly expressed in thyroid cancer tissues. Meanwhile, it promoted the proliferation and invasion of thyroid cancer cells. Furthermore, we investigated the related protein and mechanism.

## Patients and Methods

### Tissue Specimens

Totally, 40 paired thyroid cancer tissues and adjacent tissues were collected from patients who received surgeries at the Weihai Central Hospital. Before surgery, no patients received treatment of chemotherapy or radiotherapy. All the collected fresh tissues were stored immediately at  $-80^{\circ}\text{C}$  for use. This research was approved by the Ethics Committee of Weihai Central Hospital. Signed written informed consents were obtained from all participants before the study.

### Cell Culture

Human thyroid cancer cell lines (K1, TPC-1, and SW579) and normal human thyroid cell line (Nthy-ori 3-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) consisting of 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1% penicillin, and streptomycin. (Sigma-Aldrich, St. Louis, MO, USA).

### Cell Transfection

The complementary deoxyribose nucleic acid (cDNA) oligonucleotides specifically targeting circ-ABCB10 (shRNA) or control cDNA oligonucleotides was synthesized by GenePharma (Shanghai, China). Subsequently, it was inserted into the shRNA expression vector pGPH1/Neo. Cell transfection in TPC-1 cells was performed according to the instructions of Lipofectamine

3000. Lentivirus specifically targeting circ-ABCB10 (lentivirus; Biossetia Inc., San Diego, CA, USA) or empty vector was also synthesized and transfected into SW579 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 h later, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to monitor the transfection efficiency.

### RNA Extraction and RT-qPCR

Total RNA in tissues and cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary deoxyribose nucleic acid (cDNA) was synthesized according to the instructions of the PrimeScript<sup>TM</sup> RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 30 sec at  $95^{\circ}\text{C}$ , 5 sec for 40 cycles at  $95^{\circ}\text{C}$ , and 35 sec at  $60^{\circ}\text{C}$ . The relative expression level of the target gene was calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method. Primers used in this study were as follows: circ-ABCB10 primers forward 5'-CTAAGGAGTCACAGGAAGACATC-3', reverse 5'-GTAGAATCTCTCAGACTCAAGGTTG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward 5'-GGGAGCCAAAAGGGTCAT-3' and reverse 5'-GAGTCCTTCCACGATACCAA-3'.

### Cell Proliferation Assay

Cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was used to detect the growth ability of thyroid cancer cells. Transfected cells in 96-well plates were assessed at 24, 48, and 72 h, respectively. Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA) was utilized to measure the absorbance at 450 nm.

### Colony Formation Assay

Thyroid cancer cells were first seeded into 6-well plates and cultured for 10 days. Subsequently, colonies were treated with 10% formaldehyde for 30 min and stained with 0.5% crystal violet for 5 min. Image-Pro Plus 6.0 (Media Cybernetics, Silver Springs, MD, USA) was used for data analysis.

### Transwell Assay

$2 \times 10^5$  transfected cells in 100  $\mu\text{L}$  serum-free DMEM were transformed into the upper chamber of 8- $\mu\text{m}$  culture inserts (Corning, Corning, NY, USA) coated with 50  $\mu\text{g}$  Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, 20 %

FBS-DMEM was added to the lower chamber of culture inserts. 24 h later, these inserts were treated with methanol for 30 min and stained with hematoxylin for 20 min. The number of invaded cells was counted under an inverted microscope. Three fields were randomly selected for each sample.

### Western Blot Analysis

Cells were washed with pre-cooled phosphate-buffered saline (PBS) and lysed with cell lysis Radioimmunoprecipitation Assay solution (RIPA; Beyotime, Shanghai, China). Protein concentration was determined using the bicinchoninic acid method (BCA; Thermo Fisher Scientific, Rockford, IL, USA). Subsequently, proteins were separated and transferred on to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in tris buffered saline-tween (TBST; 25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk for 2 h, the membranes were incubated with primary antibodies of KLF6 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, MA, USA) at 4°C overnight. After washing ( $3 \times 10$  min) with Tris-Buffered Saline and Tween (TBST), the membranes were incubated with corresponding secondary antibody at room temperature for 1 h. Immunoreactive bands were analyzed by Image J software (NIH, Bethesda, MD, USA).

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for all statistical analysis. Indepen-

dent-sample *t*-test was selected when appropriate.  $p < 0.05$  was considered statistically significant.

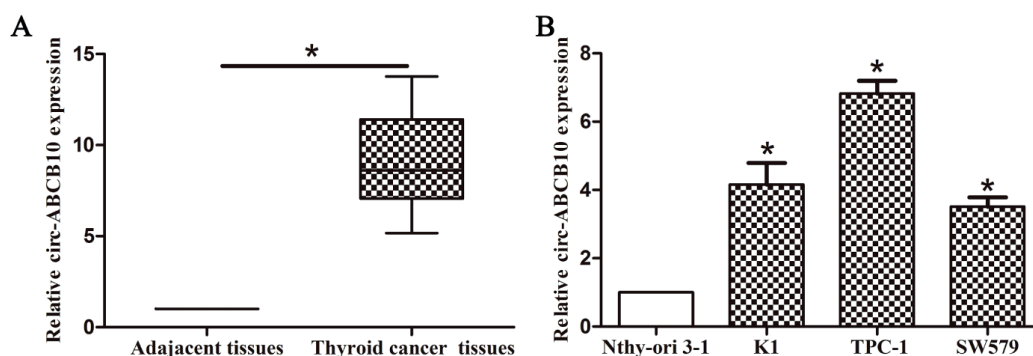
## Results

### Circ-ABCB10 Expression in Thyroid Cancer Tissues and Cells

RT-qPCR was used to detect circ-ABCB10 expression in 40 paired thyroid cancer tissues and adjacent tissues, as well as three thyroid cancer cell lines. As shown in Figures 1A and B, circ-ABCB10 was significantly up-regulated in thyroid cancer tissues when compared with adjacent tissues. Meanwhile, it was also significantly higher in thyroid cancer cells than that of Nthy-ori 3-1 cells. The results suggested that up-regulated circ-ABCB10 might be associated with the progression of thyroid cancer.

### Silence of Circ-ABCB10 Suppressed Proliferation and Invasion of Thyroid Cancer Cells

TPC-1 cell line was chosen for the silence of circ-ABCB10. Circ-ABCB10 expression in transfected cells was detected by RT-qPCR (Figure 2A). Cell proliferation assay showed that the silence of circ-ABCB10 significantly repressed the growth ability of TPC-1 cells (Figure 2B). Colony formation assay was conducted to further confirm the results of cell proliferation assay. As shown in Figure 2C, the number of colonies was remarkably reduced after circ-ABCB10 silence in TPC-1 cells. To further explore the effect of circ-ABCB10 on cell invasion, the transwell assay was conducted.



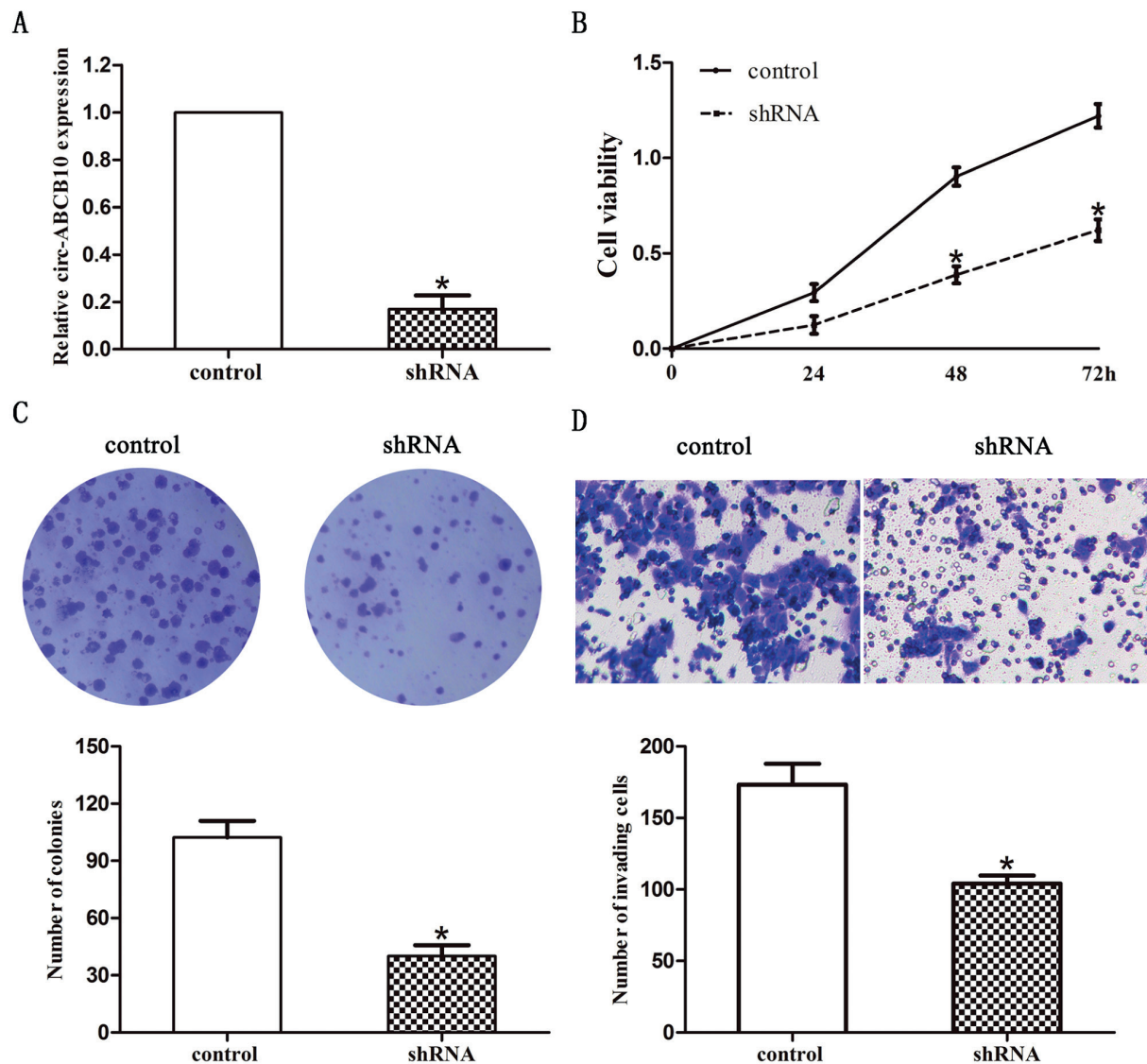
**Figure 1.** Expression levels of circ-ABCB10 in thyroid cancer tissues and cell lines. **A**, Circ-ABCB10 expression was significantly up-regulated in thyroid cancer tissues compared with adjacent tissues. **B**, Expression levels of circ-ABCB10 were detected in human thyroid cancer cell lines and Nthy-ori 3-1 cell by RT-qPCR. Data were presented as mean  $\pm$  standard error of the mean. \* $p < 0.05$ .

As shown in Figure 2D, the number of invaded cells decreased significantly after circ-ABCB10 was silenced in TPC-1 cells.

**Overexpression of Circ-ABCB10 Promoted Cell Proliferation and Invasion of Thyroid Cancer Cells**

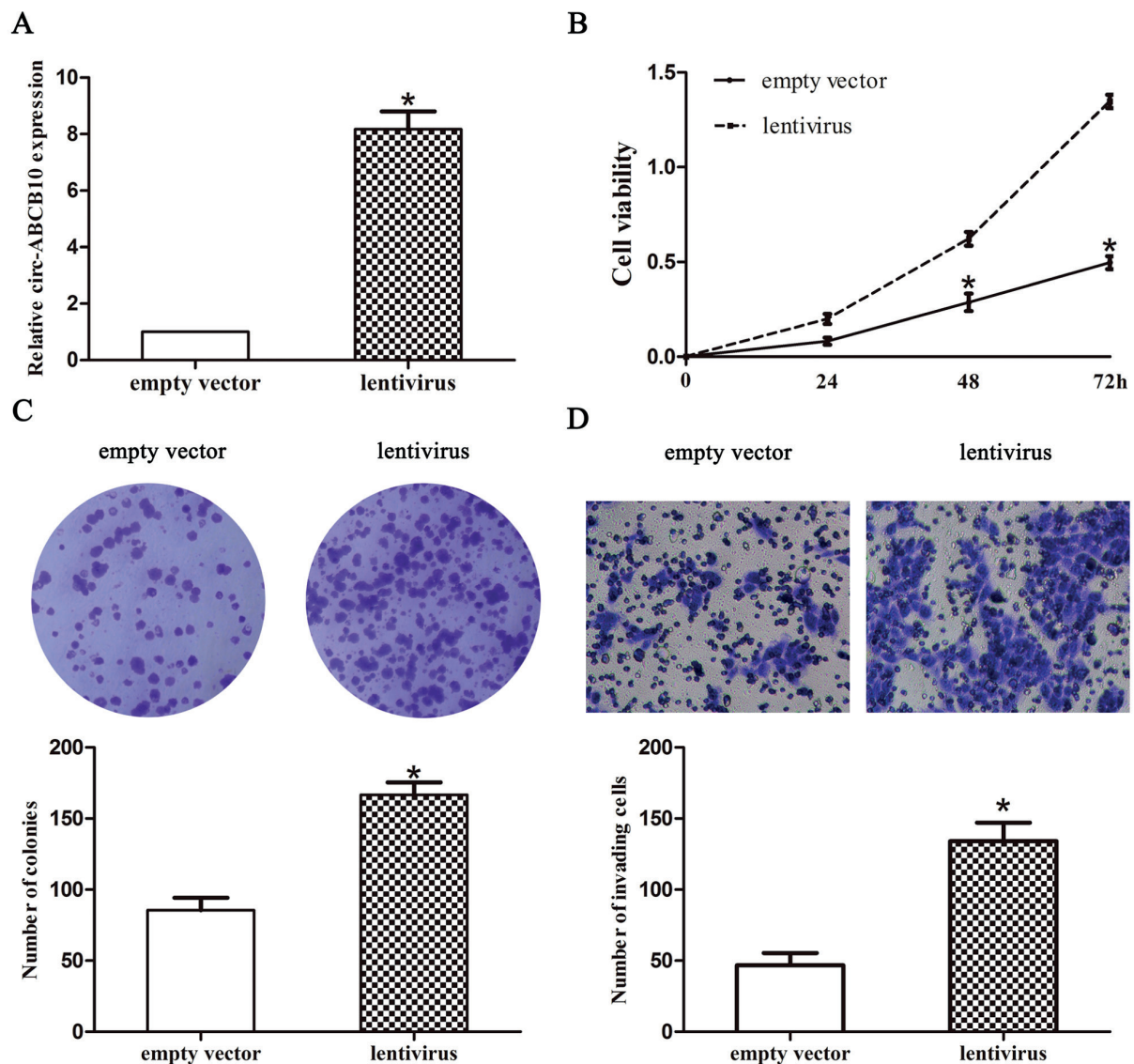
SW579 cell line was chosen for overexpression of circ-ABCB10 *in vitro*. Circ-ABCB10 expression in transfected cells was also detected by

RT-qPCR (Figure 3A). Cell proliferation assay showed that overexpression of circ-ABCB10 significantly promoted the growth ability of SW579 cells (Figure 3B). Colony formation assay demonstrated that the number of colonies increased remarkably after circ-ABCB10 overexpression in SW579 cells (Figure 3C). Transwell assay indicated that the number of invaded cells increased markedly after circ-ABCB10 was overexpressed in SW579 cells (Figure 3D).



**Figure 2.** Silence of circ-ABCB10 inhibited thyroid cancer cell proliferation and invasion. **A**, Circ-ABCB10 expression in TPC-1 cells of shRNA group and control group was detected by RT-qPCR. **B**, CCK-8 assay showed that silence of circ-ABCB10 significantly repressed viability of TPC-1 cells. **C**, Colony formation assay showed that the number of colonies was significantly reduced via silence of circ-ABCB10 in TPC-1 cells (magnification: 10×). **D**, Transwell assay showed that silence of circ-ABCB10 significantly repressed the invasion of TPC-1 cells (magnification: 40×). The results represented the average of three independent experiments (mean ± standard error of the mean). \* $p < 0.05$ .



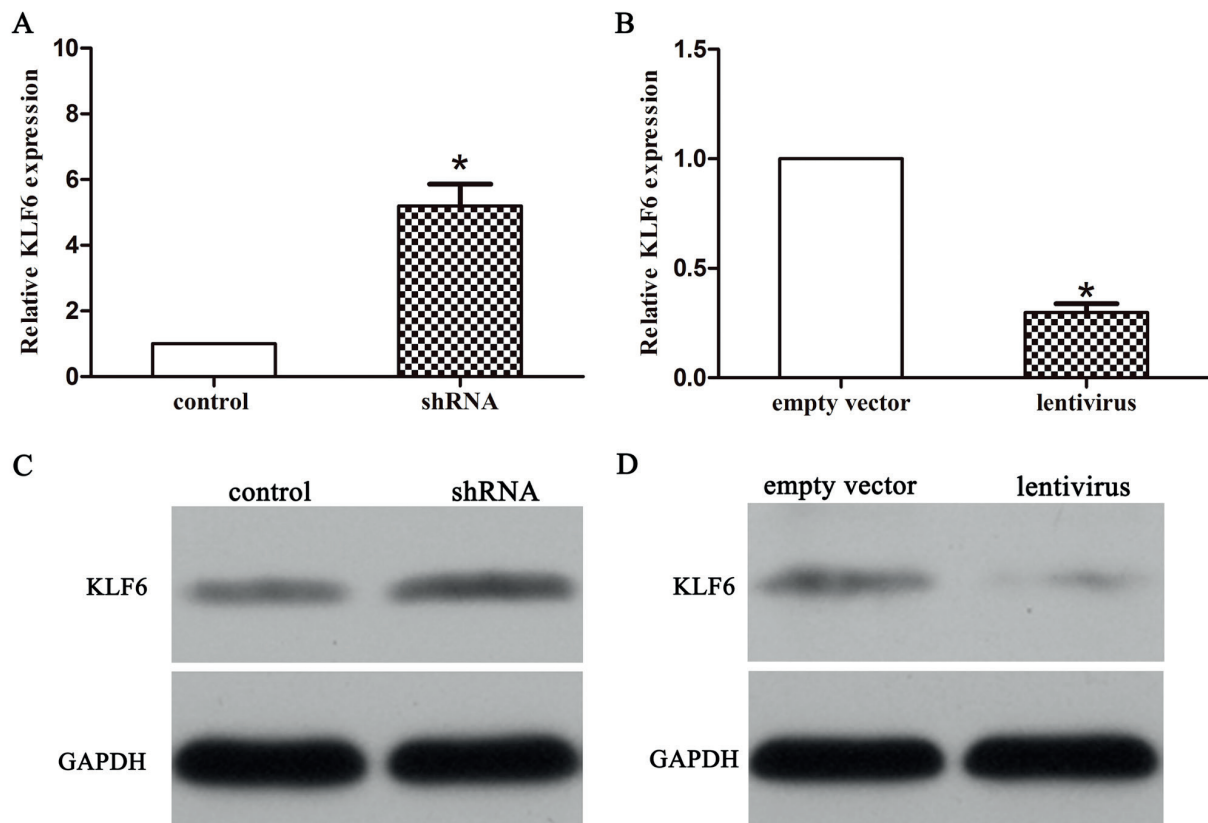


**Figure 3.** Overexpression of circ-ABCB10 enhanced thyroid cancer cell proliferation and invasion. **A**, Circ-ABCB10 expression in SW579 cells of lentivirus group and empty vector group was detected by RT-qPCR. **B**, CCK-8 assay showed that overexpression of circ-ABCB10 significantly promoted the viability of SW579 cells. **C**, Colony formation assay showed that the number of colonies increased significantly *via* overexpression of circ-ABCB10 in SW579 cells (magnification: 10 $\times$ ). **D**, Transwell assay showed that overexpression of circ-ABCB10 significantly promoted the invasion of SW579 cells (magnification: 40 $\times$ ). The results represented the average of three independent experiments (mean  $\pm$  standard error of the mean). \* $p < 0.05$ .

### The Interaction Between Circ-ABCB10 and KLF6 in Thyroid Cancer

Circular RNA Interactome (<https://circinteractome.nia.nih.gov/>) was used to predict the target proteins of circ-ABCB10. Finally, KLF6 was screened out as a tumor suppressor gene in thyroid cancer and was selected for our further experiments. RT-qPCR results showed that the expression level of KLF6 in TPC-1 cells of circ-ABCB10

shRNA group was remarkably higher than that of control group. However, the expression level of KLF6 in SW579 cells of circ-ABCB10 lentivirus group was significantly lower than empty vector group (Figures 4A and B). Western blot assay found out that protein expression of KLF6 was significantly up-regulated or down-regulated after circ-ABCB10 was silenced or overexpressed, respectively (Figures 4C and D).



**Figure 4.** Interaction between circ-ABCB10 and KLF6. **A**, RT-qPCR results showed that KLF6 expression increased significantly in shRNA group compared with control group in TPC-1 cells. **B**, RT-qPCR results showed that KLF6 expression decreased remarkably in lentivirus group compared with empty vector group in SW579 cells. **C**, Western blot results showed that the protein level of KLF6 was up-regulated in shRNA group compared with control group in TPC-1 cells. **D**, Western blot results showed that the protein level of KLF6 was down-regulated in lentivirus group compared with empty vector group in SW579 cells. The results represented the average of three independent experiments (mean  $\pm$  standard error of the mean). \* $p < 0.05$ .

## Discussion

Thyroid cancer is one of the most common malignancies in the world. Currently, the recurrence rate is remarkably high in patients with thyroid cancer. Effective targeted therapies for thyroid cancer depends on a better understanding of the molecular mechanism of the proliferation and metastasis of thyroid cancer. Recently, circRNAs have been reported dysregulated in thyroid cancer and involved in tumorigenesis. Bi et al<sup>11</sup> observed that circRNA\_102171 functions as an oncogene in PTC and enhances the development of thyroid cancer *via* activating the  $\beta$ -catenin pathway. Circ\_0025033 is up-regulated in PTC and promotes cell invasion by sponging miR-1231 and miR-1304<sup>12</sup>. Our previous study has demonstrated that circ-ABCB10 is up-regulated in thyroid cancer. The aim of this work was to uncover the role of circ-ABCB10 in the proliferation and invasion of thyroid cancer.

Circ-ABCB10 is 724 length in gene symbol ABCB10, which is located at chr1:229665945-229678118. Liang et al<sup>13</sup> have discovered that circ-ABCB10 facilitates cell proliferation and tumorigenesis by sponging miR-1271 in breast cancer. In the present study, the role of circ-ABCB10 was firstly researched in thyroid cancer. Our results showed that the proliferation of thyroid cancer cells was significantly inhibited or promoted after circ-ABCB10 was silenced or over-expressed, respectively. Therefore, circ-ABCB10 participated in regulating the proliferation of thyroid cancer.

Metastasis is the major cause of mortality in thyroid cancer. Evidence has demonstrated that circRNAs participate in the progression of thyroid cancer. In our research, we also explored whether circ-ABCB10 affected the aggressiveness of thyroid cancer *in vitro*. Results showed that the invasion of thyroid cancer cells was significantly inhibited after circ-ABCB10 was silenced. How-

ever, the invasion ability of thyroid cancer cells was markedly promoted after circ-ABCB10 over-expression *in vitro*. These results indicated that circ-ABCB10 participated in regulating the invasion of thyroid cancer.

Previous studies have revealed that circRNAs participate in the development of multiple cancers *via* targeting special regulators. The bio-informative analysis was performed to predict the related proteins of circ-ABCB10. Among them, Kruppel-like factor (KLF) has been reported to play an important role in malignant tumors, including thyroid cancer. KLF is a cluster of crucial regulators of gene expression. Meanwhile, KLF6 is a ubiquitously expressed zinc finger transcription factor, which participates in the modulation of cell proliferation, differentiation, and signal transduction<sup>14-16</sup>. In this work, we found out that KLF6 expression was significantly up-regulated by the silence of circ-ABCB10. On the other hand, KLF6 expression was down-regulated after overexpression of circ-ABCB10. Above results indicated that circ-ABCB10 participated in the development of thyroid cancer by targeting KLF6.

## Conclusions

We detected that circ-ABCB10 expression was remarkably up-regulated in thyroid cancer patients. Meanwhile, it facilitated the proliferation and invasion of thyroid cancer cells *in vitro* by targeting KLF6. All our findings suggested that circ-ABCB10/KLF6 axis could be a candidate therapeutic target for thyroid cancer patients.

## Conflict of Interests

The Authors declare that they have no conflict of interests.

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