European Review for Medical and Pharmacological Sciences 2020; 24: 1271-1277

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

X.-T. HAN[1], J.-Q. JIANG[2], M.-Z. LI[1], Q.-M. CONG[3]

[1]Department of Mammary and Thyroid Surgery, Weihai Central Hospital, [illegible] Ch
[2]Department of Pathology, Weihai Central Hospital, Weihai, China
[3]Department of Oncology, Weihai Central Hospital, Weihai, China

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 monito[illegible] Real Time-quantitative Polymerase Ch[illegible] action (RT-qPCR). Subsequently, circ-A[illegible]10 was silenced or overexpressed in thyroid[illegible] cer cells. Function assays were conducted to[illegible] plore the role of circ-ABCB10 in the proliferati[illegible] and invasion of thyroid cancer [illegible]. Further[illegible] more, RT-qPCR and Wester[illegible] ay were performed to elucidate the [illegible]ential [illegible]erlying mechanism.

RESULTS: Circ-ABCB[illegible] press[illegible] nificantly higher in th[illegible]sues than[illegible] that in adjacent tis[illegible]. The[illegible]h ability of thyroid cancer ce[illegible]as signi[illegible] inhibited after circ-ABCB1[illegible]nce. Howeve[illegible] growth ability of thyro[illegible] cells was [illegible]arkably promoted aft[illegible]rc-A[illegible] was overexpressed in vitro. Similarly, the inv[illegible] of thyroid cancer cells was [illegible]ificantly inhib[illegible] promoted after circ[illegible]B10 silence or ov[illegible]expression, respecti[illegible]. Besi[illegible] the expression of KLF6 was mar[illegible]y up-r[illegible]ated by the silence of circ-ABCB[illegible] we[illegible]LF6 e[illegible]ssion was down-regulated[illegible]xpress[illegible] of circ-ABCB10.

CONCL[illegible]NS: [illegible]ABCB10 was first identi[illegible] a no[illegible]ne in thyroid cancer. Furthe[illegible]e, it sig[illegible]ntly enhanced the proliferation[illegible] invasion[illegible] of thyroid cancer by targeting KL[illegible] [illegible]ings suggested that circ-ABCB10 c[illegible] [illegible]as a potential therapeutic target [illegible]yroid cancer.

[illegible]rds:
[illegible] RNA, Circ-ABCB10, Thyroid cancer, KLF6.

Intro[illegible]ion

[illegible]yroid cancer is one of the most common ma[illegible]ant cancers, [illegible]ich originates from follicular [illegible]arafollicular [illegible]roid cells. It is estimated that [illegible]st approxim[illegible]y 90% of all cases are papill[illegible]roid ca[illegible]oma (PTC) annually[1]. Due to the a[illegible] ultrasonography, the number of newly diagnosed TC patients has been increasing [illegible]orldwide over the past few decades[2]. However, [illegible]merous efforts have been made in sur[illegible]ry a[illegible] radioactive iodine, there are still no significant improvements in the survival rate of thyroid cancer[3]. 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[4]. In the past decades, the roles of circRNAs have been widely explored, remaining still poorly understood[5]. 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[6]. Overexpression of circ PVT1 promotes cell proliferation in gastric cancer by serving as a sponge for the miR-125 family[7]. 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[8]. By sponging miR-370,

Corresponding Author: Qiumei Cong, BM; e-mail: cqmlss@sina.com

the knockdown of hsa_circ_0061140 inhibits cell growth and metastasis in ovarian cancer[9]. Overexpression of circ_0067934 serves as an oncogene and facilitates the progression of cervical cancer by modulating the miR-545/EIF3C axis[10]. 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 Ho[illegible]. Before surgery, no patients received trea[illegible] chemotherapy or radiotherapy. All the co[illegible]ed fresh tissues were stored immediately at –[illegible] for use. This research was approved by the Et[illegible] Committee of Weihai Central Hospital. Signe[illegible] written informed consents wer[illegible] from all participants before the study[illegible]

Cell Culture

Human thyroid ca[illegible]r c[illegible]X1, TPC-1, and SW579) and no[illegible] huma[illegible]id cell line (Nthy-ori 3-1) we[illegible]tained from [illegible]merican Type Culture C[illegible] (ATCC; Ma[illegible]as, VA, USA). All cel[illegible]re c[illegible]d in Dulbecco's Modified Eagle's Medium (D[illegible] Gibco, Rockville, MD, USA) consisting of 10% [illegible] bovine serum (FBS; G[illegible]o, Rockville, MD, USA), 1% penicillin, a[illegible]treptor[illegible]n. (Sigma-Aldrich, St. Louis, MO, USA).

Cell Tra[illegible]tion

[illegible] comp[illegible] deoxyribose nucleic acid (cD[illegible] oligon[illegible]tides specifically targeting circ[illegible]CB10 (shRNA) or control cDNA oligonu[illegible]s synthesized by GenePharma [illegible]nghai, China). Subsequently, it was inserted [illegible] the shRNA expression vector pGPH1/Neo. [illegible]nsfection in TPC-1 cells was performed acco[illegible]g to the instructions of Lipofectamine 3000. Lentivirus specifically targeting circ-ABCB10 (lentivirus; Biosettia Inc., San [illegible] USA) or empty vector was also sy[illegible]ized [illegible] transfected into SW579 cells usin[illegible]ofectamine 3000 (Invitrogen, Carlsbad, CA[illegible]). 48 h later, Real Time-quantitative Polymera[illegible]in Reaction (RT-qPCR) was used t[illegible]onitor [illegible]sfection efficiency.

RNA Extraction a[illegible]RT-qP

Total RNA in tiss[illegible]d c[illegible]was ext[illegible]d by TRIzol reagent (I[illegible]itro[illegible]lsbad, USA). The RNA con[illegible]ation wa[illegible]rmi[illegible] using an ultraviolet [illegible]rophotometer[illegible]ni, Tokyo, Japan). Th[illegible]mentary deox[illegible]bose nucleic acid (cD[illegible] was [illegible]sized according to the instructions of the Prim[illegible]t™ RT MasterMix kit (Im[illegible], Carlsbad, C[illegible]SA). QRT-PCR reac[illegible] conditions were as follows: 30 sec at 95°C, [illegible]c for 40 cycl[illegible] at 95°C, and 35 sec at 60°C. [illegible] relative exp[illegible]ion level of the target gene [illegible] calculated b[illegible]e $2^{-\Delta\Delta Ct}$ method. Primers used i[illegible] study w[illegible]s follows: circ-ABCB10 primers [illegible]TAAGGAGTCACAGGAAGACATC-3′, reverse 5′-GTAGAATCTCTCAGACTCAAGGTTG-3′; glyceraldehyde 3-phosphate [illegible]nase (GAPDH) primers forward [illegible]AGCCAAAAGGGTCAT-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 µL serum-free DMEM were transformed into the upper chamber of 8-µm culture inserts (Corning, Corning, NY, USA) coated with 50 µg 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 × 10 min) with Tris-Bu d Saline and Tween (TBST), the membra incubated with corresponding secondar ti-body at room temperature for 1 h. Immu active bands were analyzed by Image J softw (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product a Service lutions (SPSS) 20.0 (IBM Co leased SPSS Statistics for Wi lows , NY, USA) was used for all tical a Indepen-dent-sample *t*-test was selected when appropriate. $p<0.05$ was considered statistically sig

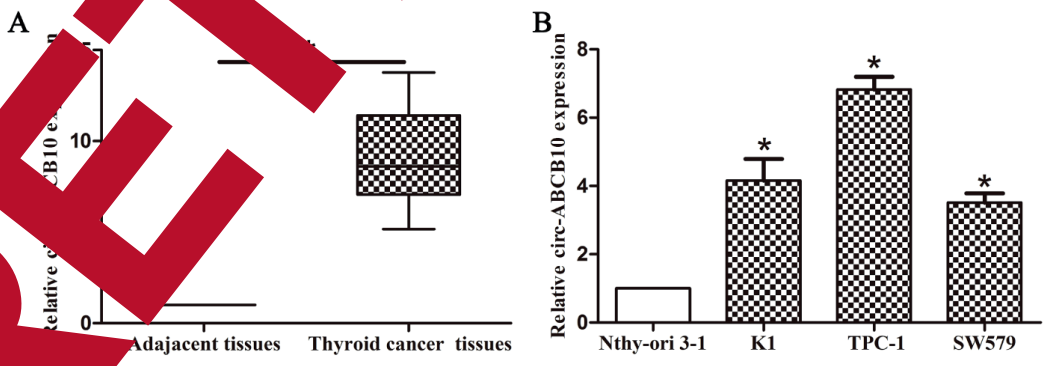
Results

Circ-ABCB10 Expressio n Thy Cancer Tissues and C

RT-qPCR was used tect ci ABCB1 pression in 40 pair roid c er tissues and adjacent tissues, a as e thy can-cer cell lines. A ho gures and B, circ-ABCB10 signifi up ulated in thyroid can sues when c with adja-cent tissu hile, it was a significantly higher i roid cells than that of Nthy-ori 3-1 cells. The re ggested that up-regulate BCB10 migh associated with the p ssion of thyroid cancer.

ence of Circ BCB10 Suppressed liferation a Invasion of Thyroid C r Cells

T e was chosen for the silence of circ-ABCB10. Circ-ABCB10 expression in nsfected cells was detected by RT-qPCR 2A). Cell proliferation assay showed at 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.



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

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-A nificantly promoted the growth abi SW cells (Figure 3B). Colony formati say demonstrated that the number of colo creased remarkably after circ-ABCB10 ov ssion in SW579 cells (Figure 3C). nswell indicated that the number of aded cells remarkedly after circ-A 10 wa verexpr in SW579 cells (Fig D).

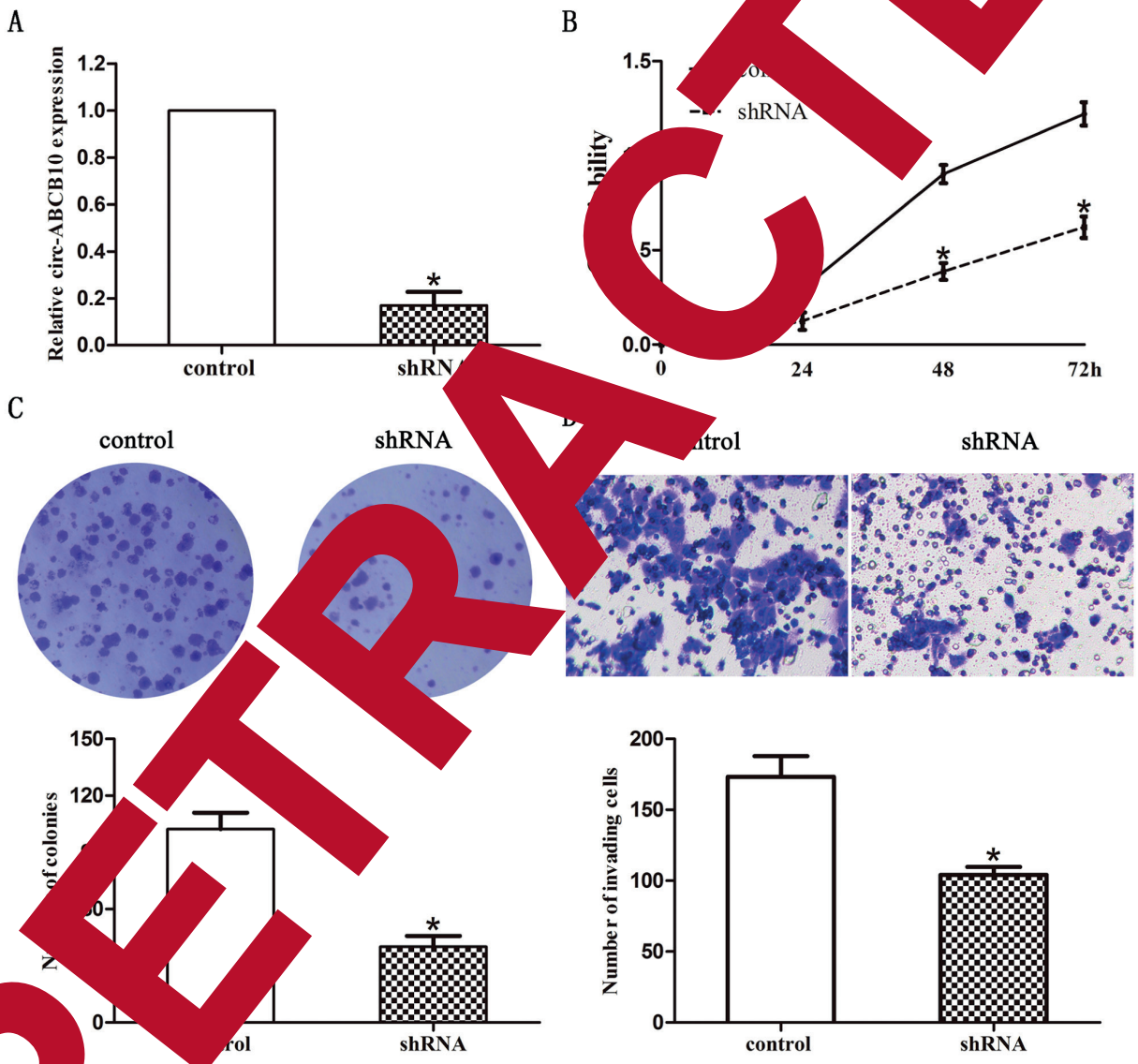


Fig f circ-ABCB10 inhibited thyroid cancer cell proliferation and invasion. **A,** Circ-ABCB10 expression in T cells o NA group and control group was detected by RT-qPCR. **B,** CCK-8 assay showed that silence of circ-ABCB10 ificantly repressed viability of TPC-1 cells. **C,** Colony formation assay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

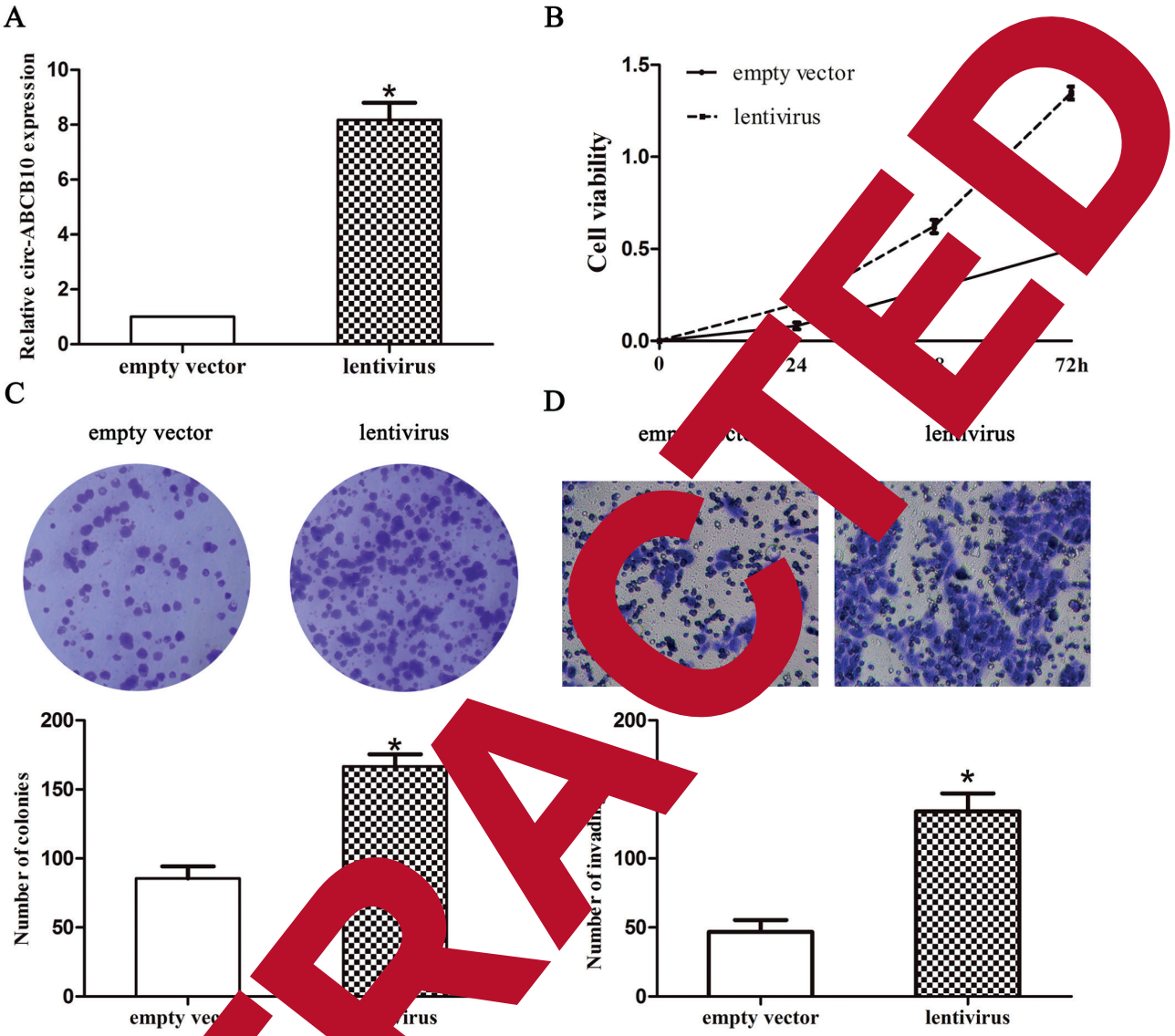


Figure 3. Overexpression of circ-ABCB10 enhanced thyroid cancer cell proliferation and invasion. **A,** Circ-ABCB10 expression in SW579 cells of lentivirus group and empty vector group was detected by RT-qPCR. **B,** CCK-8 assay showed that overexpression of circ-ABCB10 significantly promoted the viability 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×). **D,** Transwell assay showed that overexpression of circ-ABCB10 significantly promoted the invasion of SW579 cells (magnification: 40×). The results represented the average of three independent experiments (mean ± 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 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).

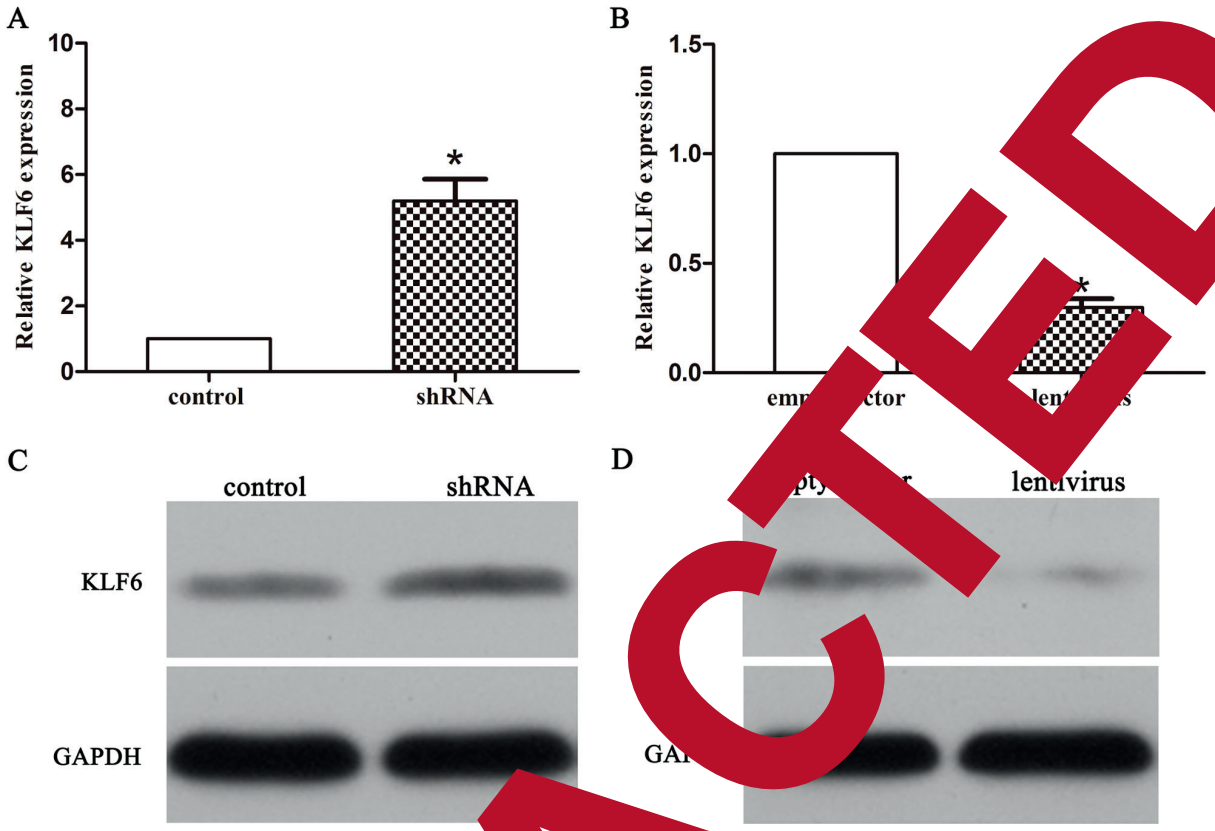


Figure 4. Interaction between circ-ABCB10 and K… **A,** R1-… showed that KLF6 expression increased signifi-cantly in shRNA group compared with control group … PC-… s. **B,** …-qPCR results showed that KLF6 expression de-creased remarkably in lentivirus group compared with e… r group in SW579 cells. **C,** Western blot results showed that the protein level of KLF6 was up-regulated in shRNA grou… pared with control group in TPC-1 cells. **D,** Western blot results showed that the protein level of KLF6 … n-regulate… ntivirus group compared with empty vector group in SW579 cells. The results represented the ave… ndepender… riments (mean ± standard error of the mean). *p<0.05.

Dis… ss…

Thyroid cancer … e of the mos… mon ma-lignancies in th… Currently, the … urrence rate is remar… hi… patients with thyroid cancer. Eff… tive targete… pies for thyroid cancer de… ds on a better … tanding of the molecu… echanism of the pro…eration and me-tasta… thyroi… ncer. Recently, circRNAs have be… orted … regulat… in thyroid cancer and involv… origene… Bi et al[11] observed that …RNA_… 1 fun… s as an oncogene in PTC … hance… …pment of thyroid cancer *via* acti… g the β-… in pathway. Circ_0025033 is up-r… ated in PTC and promotes cell invasion b… -1231 and miR-1304[12]. Our pre-… study has demonstrated that circ-ABCB10 … -regulated in thyroid cancer. The aim of this w… s to uncover the role of circ-ABCB10 in the p… eration and invasion of thyroid cancer.

Circ-ABCB10 is 724 length in gene symbol ABCB10, which is located at chr1:229665945-229678118. Liang et al[13] 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 overexpression *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, Krueppel-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[14-16]. 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 [illegible] remarkably up-regulated in thyroid cancer patients. Meanwhile, it facilitate[illegible] liferation and invasion of thyroid can[illegible] cells *in vitro* by targeting KLF6. All our f[illegible]ngs sug[illegible]ed that circ-ABCB10/KLF6 axi[illegible]d be[illegible] therapeutic target for t[illegible]roi[illegible]atients.

Conflict of Int[illegible]

The Authors de[illegible] that t[illegible] no conflict of interests.

References

1) [illegible]Y, Xin[illegible] Recent [illegible]cidences and differential [illegible]oid can[illegible] the USA. Endocr Relat Ca[illegible]6; 23: 3[illegible]22.

[illegible]ccare[illegible] [illegible]ehi S, Bray F, Wild CP, Plum[illegible]M, Dal[illegible] Worldwide thyroid-cancer epi[illegible]ic? The in[illegible]asing impact of overdiagnosis. N [illegible] J Med 2016; 375: 614-617.

[illegible], Copland JA. Anaplastic thyroid carcinoma: pathogenesis and emerging therapies. Clin Oncol (R Coll Radiol) 2010; 22: 486-497.

[illegible]Y, Dou P, Hu M, Xu J, Xia W, Sun H. CircRNAC[illegible]mediates malignant progression of breast cancer through targeting the miR136/MMP13 axis. Mol Med Rep 2019; 19: 3314-33[illegible]

5) Zhang J, Liu H, Hou L, Wang G, Z[illegible] Y, Chen X, Zhu J. Circular RNA [illegible]RP4 inhibit[illegible] cell proliferation and invasion o[illegible]tric cancer by sponging miR-424-5p and re[illegible] LATS1 expression. Mol Cancer 2017; 16: 1[illegible]

6) Luan W, Shi Y, Zhou [illegible]ia Y, W[illegible] Ci[illegible] cRNA_0084043 pron[illegible] malignant [illegible] progression via miR[illegible]3p/Sn[illegible] axis. Bio[illegible] Biophys Res Com[illegible]2018; 5[illegible]2-29.

7) Chen J, Li Y, Zhe[illegible]ao C[illegible]J, Chen[illegible]yu D, Zheng B, Xu Y[illegible]ong[illegible] Zhu H,[illegible] Y, He X, Shi Y, Hu[illegible]. Circ[illegible]NA pr[illegible]dentifies circPVT1[illegible] proliferativ[illegible] prognostic marker i[illegible]tric cancer. Ca[illegible]t 2017; 388: 208-2[illegible]

8) Fu L[illegible]n Q, [illegible]T, Ying S, Hu Y, Guo J. Hsa_circ_0005986 in[illegible]arcinogenesis by acting [illegible]R-129-5p spo[illegible]d is used as a novel [illegible]ker for hepatocel[illegible]r carcinoma. Oncotarget 2017; 8: 43878-43888.

Chen Q, Zhan[illegible]He Y, Wang Y. Hsa_circ_0061140 knockdown [illegible]erses FOXM1-mediated cell growth and m[illegible]stasis in ovarian cancer through [illegible]-370 sp[illegible] activity. Mol Ther Nucleic Acids [illegible].

10) Hu C, [illegible]ng Y, Li A, Zhang J, Xue F, Zhu L. Overexpressed circ_0067934 acts as an oncogene to facil[illegible]te cervical cancer progression via the miR-545/[illegible]axis. J Cell Physiol 2019; 234: 9225-9232.

[illegible] Bi W, Huang J, Nie C, Liu B, He G, Han J, Pang R, Ding Z, Xu J, Zhang J. CircRNA circRNA_102171 promotes papillary thyroid cancer progression through modulating CTNNBIP1-dependent activation of beta-catenin pathway. J Exp Clin Cancer Res 2018; 37: 275.

12) Pan Y, Xu T, Liu Y, Li W, Zhang W. Upregulated circular RNA circ_0025033 promotes papillary thyroid cancer cell proliferation and invasion via sponging miR-1231 and miR-1304. Biochem Biophys Res Commun 2019; 510: 334-338.

13) Liang HF, Zhang XZ, Liu BG, Jia GT, Li WL. Circular RNA circ-ABCB10 promotes breast cancer proliferation and progression through sponging miR-1271. Am J Cancer Res 2017; 7: 1566-1576.

14) Hsu LS, Huang RH, Lai HW, Hsu HT, Sung WW, Hsieh MJ, Wu CY, Lin YM, Chen MK, Lo YS, Chen CJ. KLF6 inhibited oral cancer migration and invasion via downregulation of mesenchymal markers and inhibition of MMP-9 activities. Int J Med Sci 2017; 14: 530-535.

15) Zhang S, Zhang JY, Lu LJ, Wang CH, Wang LH. MiR-630 promotes epithelial ovarian cancer proliferation and invasion via targeting KLF6. Eur Rev Med Pharmacol Sci 2017; 21: 4542-4547.

16) He AD, Xie W, Song W, Ma YY, Liu G, Liang ML, Da XW, Yao GQ, Zhang BX, Gao CJ, Xiang JZ, Ming ZY. Platelet releasates promote the proliferation of hepatocellular carcinoma cells by suppressing the expression of KLF6. Sci Rep 2017; 7: 3989.