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Circular RNA circ-ABCB10 promotes non-small cell lung cancer proliferation and inhibits cell apoptosis through repressing KISS1

J.-P. ZHENG[1,2], Y.-M. DAI[1,2], Z. CHEN[1,2], Q. CHEN[1,2], Y. ZHENG[1,2], X. LIN[1,2], T.-J. CUI[1,2]

[1]Department of Oncology, Fujian Provincial Hospital, Fuzhou, China
[2]Provincial Clinical College of Fujian Medical University, Fuzhou, China

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Abstract. – OBJECTIVE: **Recent researches have proved that circular RNAs (circRNAs) act as an important role in many diseases. Our study aims to uncover the role of circ-ABCB10 in the progression of non-small cell lung cancer (NSCLC).**

PATIENTS AND METHODS: **Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to detect circ-ABCB10 expression in NSCLC patients. Then, we conducted Cell Counting Kit-8 (CCK-8) assay, colony formation assay, Ethynyl deoxyuridine (EdU) incorporation assay, cell cycle assay, and cell apoptosis assay in treated NSCLC cells. Besides, further experiments including RT-qPCR and Western blot assay were performed to explore the potential mechanism in vitro.**

RESULTS: **Circ-ABCB10 expression level was significantly higher in NSCLC samples compar- ing to that in adjacent tissues. Moreover tional assays showed that the cell growth lity of NSCLC cells was inhibited after circ-AB 10 was knocked down. In addition, the cell apo sis of NSCLC cells was promote circ-A CB10 was knocked down. Als e ressi of KISS1 was upregulated he dow of circ-ABCB10. Furtherm e, at KISS1 expression was gativel rrelated to the circ-ABCB10 expr n in NSC issues.**

CONCLUSIONS: **ove ind ated that circ-ABCB10 pro ted ce liferation and inhibited cell ap sis of NSC y suppressing KISS1, which gge d that c c-ABCB10 may be a potenti utic target in NSCLC.**

Key W
L no ding Circ-ABCB10, Non-small cell lu nce

Introduction

Lung cancer is the second most common cancer in both male and female which accounts for 14% of all newly diagnosed cancers in USA. Non-small cell lung cancer C) contributes to 85% of all lung er ca which is the major subtype of lu ncer. M ver, the morbidity and morta ty o CLC l be increasing for the ne everal he major characteristics SCL re c igration and tumor invasion[2]. r, the prognosis remains dismal, wi e 5- urv rate below 15%[3]. Despite ndous a s have been made in impro g or out me of NSCLC, the unde g mech n of NSCLC remains unclear.

a novel class noncoding RNAs, circular As (circRNAs) are formed by a junction of the d and 5' d. In the past few decades, the role has not been widely explored. As the opment of high-throughput sequencing chnology, circRNAs are indicated to participate e process of gene expression. CircRNAs function as important factors in tumorigenesis, cell apoptosis, proliferation, and migration in human carcinomas by modulating gene expressions as a molecular sponge or a ceRNA. CircRNA SMAD7 is reported to be overexpressed in esophageal squamous cell carcinoma and inhibit tumor proliferation and migration[4]. By regulating the expression of LATS1 and sponging miR-424-5p, circ-RNA_LARP4 suppresses the proliferation and invasion of gastric cancer cells[5]. Upregulation of circ-102004 enhances the proliferation of prostate cancer cell which may be a potential biomarker of prostate cancer[6]. Knockdown of circRNA CER restrains cell proliferation and cell migration in breast cancer *via* modulating the activity of miR-136/MMP13 signaling[7].

In this research, we found out that the expression of circ-ABCB10 was remarkably higher in NSCLC tissues. Besides, the. knockdown of circ-ABCB10 inhibited the proliferation of NS-

Corresponding Author: Tongjian Cui, MM; e-mail: fjslctj@163.com

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CLC cells, while knockdown of circ-ABCB10 promoted the cell apoptosis of NSCLC cells. Moreover, our further study explored the underlying mechanism of how circ-ABCB10 functioned in NSCLC development.

Patients and Methods

Tissue Samples

Before our study, a total of 50 NSCLC patients were obtained from NSCLC patients who underwent surgery at the Fujian Provincial Hospital. No radiotherapy or chemotherapy was performed before the surgery. All fresh tissues obtained from the surgery were maintained in liquid nitrogen. The Institutional Review Board of Fujian Provincial Hospital approved this research. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human NSCLC cell lines (A549, SPCA1, PC-9, and H1299) and normal human bronchial epithelial cell (16HBE) were purchased from American Type Culture Collection (ATCC, Man VA, USA). The culture medium consisted fetal bovine serum (FBS; Hyclone, South L UT, USA), Dulbecco's Modified Eagle's Med (DMEM; Hyclone, South Logan, UT, USA), well as penicillin. Besides, cells we ltured in an incubator containing 5%CO_2 a

Cell Transfection

The complementary de acids (cDNAs) oligonucleotides specie rgeting circ-ABCB10 (sh-circ-AB 0) were zed by GenePharma (Shangh hina) and inse nto the shRNA expressio GPH1/Neo. Then, sh-circ-ABCB10 we hen transfection in NSCLC cells. 48 h later, Rea antitative Polymerase Chai action (RT-qPC as used to monitor the tra ction fficiency.

RNA Extr a RT-q R

TRIzol rea rogen lsbad, CA, USA) was utilized to sep RNA from cultured NSCL d tissue gh the. reverse Transcrip KaRa Biotechnology Co., Ltd., Dalia ina), was reverse-transcribed to c Fol primers used for RT-qPCR: cir primers forward: 5′-CTAAGGAGTCA-CAGG TC-3′, reverse: 5′-GTAGAATCTCT-CAGACTC TG-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-CCAAAATCAGATGGGG GG-3′ and reverse 5′-TGATGGCAT AT-TCA-3′. The thermal cycle w follows: 30 s C, 5 s for 40 cycles at 95°C, 3 0°C.

Cell Proliferation Assay

Following the p ol of cell kit-8 (CCK-8) assay (D do L ratories, umamoto, Japan), the c growt bility f transfected cells in 96-wel ssesse 24, 48, and 72 h. A spect pho (The Fisher Scientific, Rockf IL, US zed to measure the absor e at 450 nm.

Colo Assay

H cells w d in a 6-well plate for 10 . Next, colon were treated with 10% aldehyde for 30 min and stained for 5 min 0.5% crystal violet. The Image-Pro Plus 6.0 dia Cyberne , Silver Springs, MD, USA) used for d nalysis.

Et xyuridine (EdU) Incorporation Assay

According to the manufacturer's manual, an Roche, Mannheim, Germany) was utilized to monitor the cell proliferation of transfected cells. Zeiss Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany) was performed to ke the representative images.

Cell Cycle Assay

2×10^5/mL cells were diluted by RNase A in 75% ice-cold ethanol overnight. These cells were stained with propidium iodide (PI; 50 mg/mL; MultiSciences Biotech Co., Ltd, Eugene, OR, USA) in the dark for 30 min at 4°C. Then, they were measured with a flow cytometer (FACScan, BD Bioscience, San Jose, CA, USA).

Cell Apoptosis Assay

Flow cytometry binding buffer (100 μL) was added after harvested cells were washed twice using ice-cold. A mixture containing 5 μL Annexin V/FICC and 5 μL PI (BD, Franklin Lakes, NJ, USA) was used for staining these cells for 15 min in the dark. Then, they were added with 400 microliters binding buffer. FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was performed to analyze cell apoptosis.

Western Blot Analysis

Cell samples were washed with precooled phosphate-buffered saline (PBS) and then lysed

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with cell lysis solution Radio Immunoprecipitation Assay (RIPA; Beyotime, Shanghai, China). Protein concentration was detected using bicinchoninic acid (BCA; Thermo Fisher Scientific, Waltham, MA, USA). The proteins were transferred on to a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), blocked in Tris-Buffered Saline and Tween-20 (TBST; 25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and incubated for 2 h. The proteins were incubated with the primary antibody of KISS1 and GAPDH (Abcam, Cambridge, MA, USA) and incubated at 4°C overnight. After being washed (3 × 10 min) with TBST, the secondary antibody was added and incubated at room temperature for 1 h. The results were analyzed by Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA). Student's *t*-test method was performed to analyse the data. Data were presented as mean ± SD (standard devi[illegible]. $p<0.05$ was considered of statistical signi[illegible].

Results

Circ-ABCB10 Expression Le[illegible] NSCLC Tissues and Cells

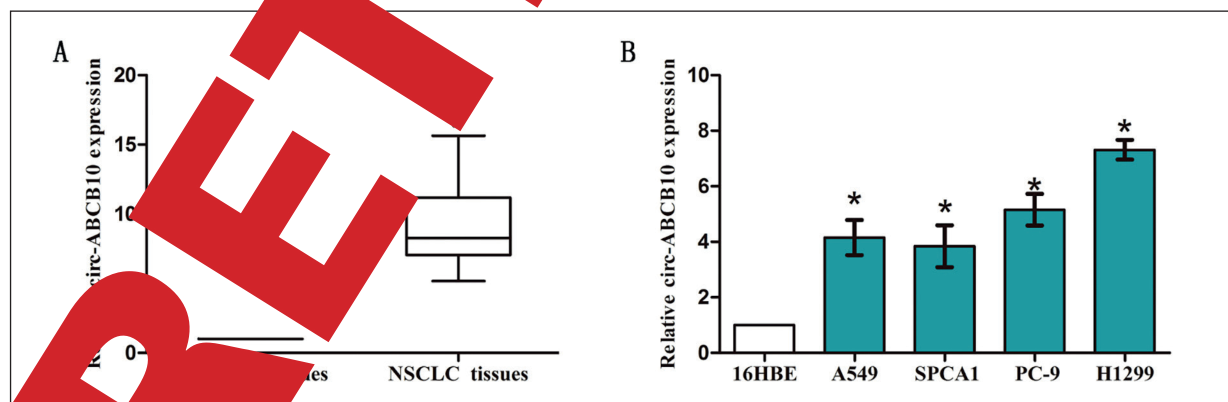
Circ-ABCB10 expression [illegible] det[illegible] RT-qPCR in 50 NSCLC pa[illegible] and [illegible] NSCLC cell lines. Results [illegible]howe[illegible] circ-ABCB10 was significantly high[illegible] in tumor tissue samples than th[illegible] adjac[illegible]ues (Figure 1A). Moreover, [illegible] expression [illegible]our human NSCLC cell li[illegible]d normal [illegible]an bronchial epithelial cell ([illegible]as als[illegible]onitored. Compared wit[illegible] the exp[illegible]HBE, circ-ABCB10 expr[illegible]on level w[illegible]arkably higher in NSCLC[illegible]ls (Fi[illegible]e 1B). [illegible]e results suggested that c[illegible]ABC[illegible] might participate in the process of [illegible]ressio[illegible].

Knockd[illegible] of Circ-AB[illegible] Suppressed Cell P[illegible]on of NSCLC Cells

We [illegible]ose[illegible] NSCLC cell line for the sile[illegible] of circ-[illegible]. Then, circ-ABCB10 ex[illegible]sion was dete[illegible] by RT-qPCR (Figure [illegible]. As shown in Figure 2B, the results of CCK-[illegible]ssay showed that silence of circ-ABCB10 [illegible]ibited cell gr[illegible] ability of NSCLC cells. As [illegible]n in Figu[illegible]C, results of colony formation a[illegible]at the number of colonies was rem[illegible]creased after circ-ABCB10 was silenced in NSCLC cells. As shown in Figure [illegible], results of EdU incorporation assay also [illegible]hat the percentage of EdU positive [illegible]s [illegible]uced after the silence of circ-ABCB10 [illegible] H1299 cells.

Knockdown of Circ-ABCB10 Promoted [illegible]ell Apoptosis and Regulated Cell Cycle of NSCLC Cells

To detect the function of circ-ABCB10 in NSCLC apoptosis, cell apoptosis assay was performed. Results showed that knockdown of



Figur[illegible] [illegible]ion levels of circ-ABCB10 were increased in NSCLC tissues and cell lines. **A,** Circ-ABCB10 expression was significant[illegible] in the NSCLC tissues compared with adjacent tissues. **B,** Expression levels of circ-ABCB10 relative to GAPDH were d[illegible]ned in the human NSCLC cell lines and 16HBE (normal human bronchial epithelial cell) by RT-qPCR. Data are presented [illegible] the mean ± standard error of the mean. *$p<0.05$.

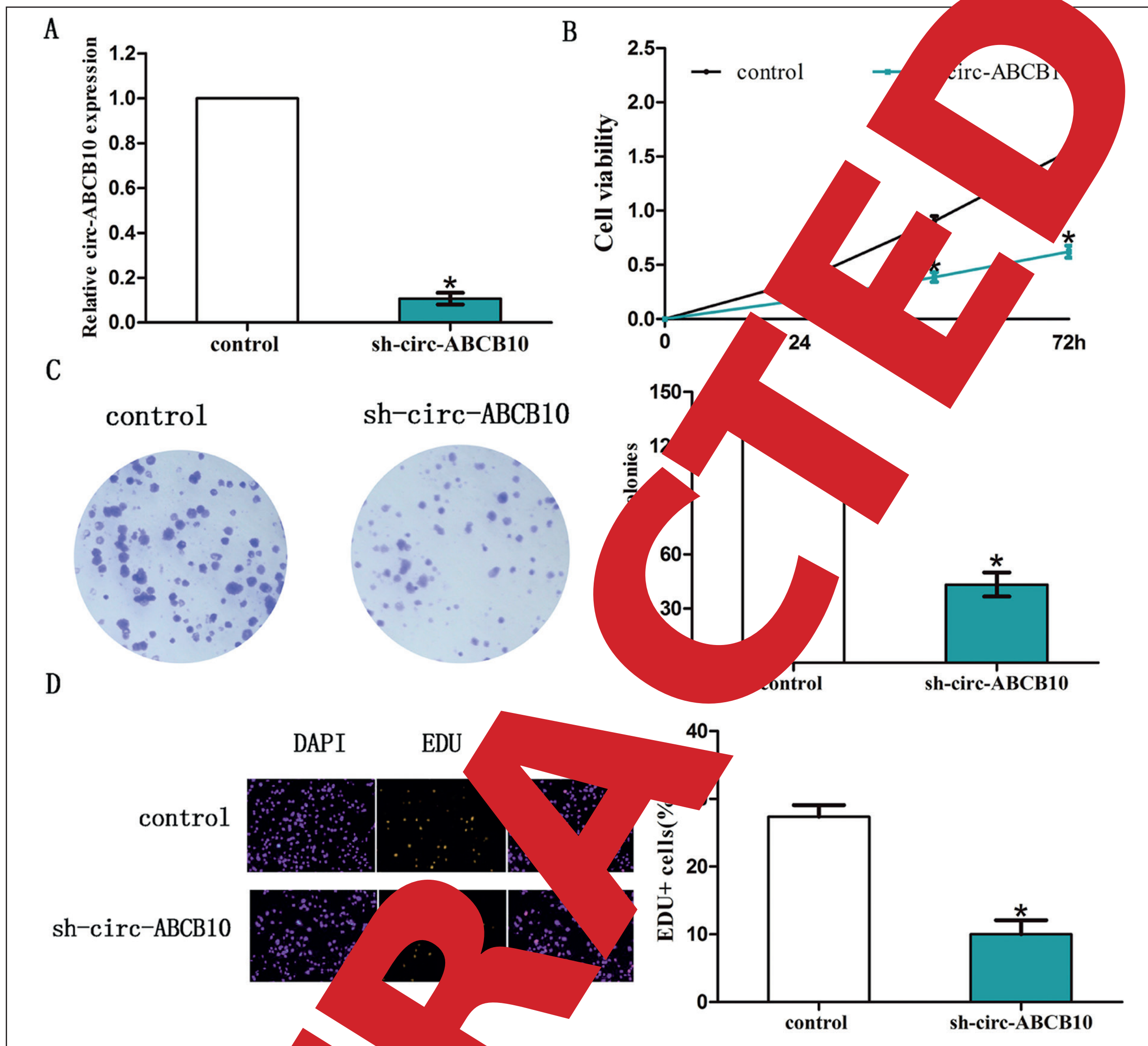


Figure 2. Knockdown of BCB10 represse LC cell proliferation. **A,** Circ-ABCB10 expression in NSCLC cells transfected with sh-circ-A control vector was detected by RT-qPCR. GAPDH was used as an internal control. **B,** CCK8 assay showed tha circ-ABCB10 significantly repressed cell proliferation in NSCLC cells. **C,** Colony formation assay showe numb es was significantly decreased *via* knockdown of circ-ABCB10 in NSCLC cells (magnification: 10×). **D,** EdU incorpor showed that number of EdU positive cells was significantly decreased *via* knockdown of circ B10 in NSCLC c (magnification: 40×). The results represent the average of three independent experiments (mea tandard error of the mean). *p<0.05, as compared with the control cells.

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circ-A increas apoptosis rate of NSC (Figure 3A). To detect the effect of -ABC SCLC cell cycle, cell cycle a as he outcome of cell cycle ass vealed that the percentage of G0/G1 cells wa ed and the percentage of S cells was reduced nockdown of circ-ABCB10 in H1299 cells (Figure 3B).

The Interaction Between KISS1 and Circ-ABCB10 in NSCLC

Starbase v2.0 was used to predict the target proteins of circ-ABCB10, among which KISS1 was selected for our following experiments. The RT-qPCR results showed that the expression level of KISS1 in NSCLC cells was remarkably higher in sh-circ-ABCB10 group compared with that in

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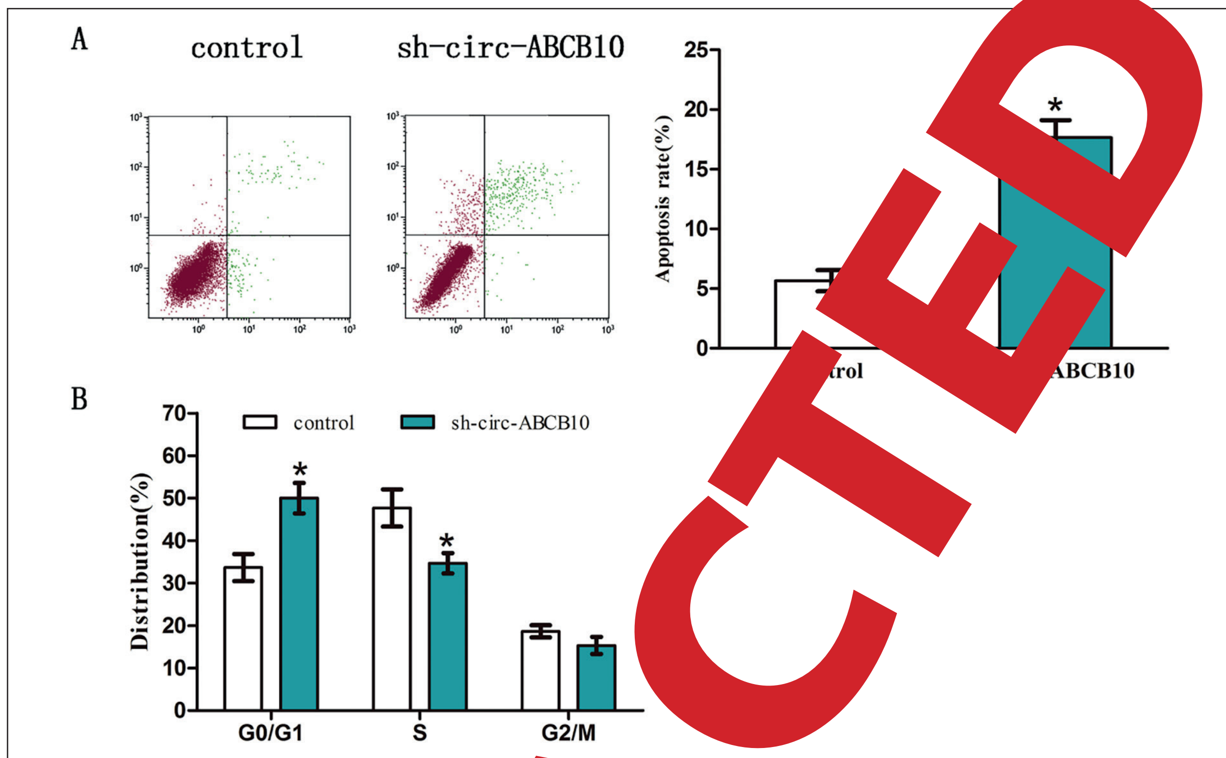

Figure 3. Knockdown of circ-ABCB10 promoted [illegible] and regulated cell cycle. **A,** Cell apoptosis assay showed that knockdown of circ-ABCB10 significa[illegible] promote[illegible] in NSCLC cells. **B,** Percentage of G0/G1 cells was increased and the percentage of S cells was redu[illegible] after kn[illegible] circ-ABCB10. The results represent the average of three independent experiments (mean ± standard er[illegible]). *$p<0.05$, as compared with the control cells.

control group (Figure 4A). We[illegible] assay found out that after circ-ABC[illegible] was [illegible] down, KISS1 could be upr[illegible]ated [illegible] level (Figure 4B). Besides, w[illegible] KISS1 expression in NSCLC cell[illegible] was s[illegible]tly lower when compared wit[illegible]6HBE ([illegible]C). Furthermore, we foun[illegible]at KISS1 exp[illegible]ion in NSCLC tissues w[illegible]ably lower when compared with th[illegible] of a[illegible] tissues (Figure 4D). Correlation analysis [illegible]ated that KISS1 expressi[illegible]evel was negat[illegible]y correlated to circ-AB[illegible]0 ex[illegible]ession in cancer tissues (Figure 4E).

[illegible]

In[illegible]vidence has suggested that circR[illegible]s are [illegible]lators in carcinogenes[illegible] NS[illegible]tently, circRNA 100146 fu[illegible] an oncogene and enhances the cell prolifer[illegible] cell in NSCLC by binding to miR-615-5p[illegible]R-361-3p directly[8]. Enhanced expression of [illegible]rcular RNA hsa_circ_000984 [illegible]omotes cells proliferation and metastasis in non-small cell lung cancer by modulating Wnt/beta-catenin pathway[9]. As a miR-1252 sponge, hsa_circ_0043256 inhibits cell proliferation and induces cell apoptosis in NSCLC[10]. By sponging miR-338-5p and miR-331-3p, circ_0001649 inhibits the progression of NSCLC which may serve as a prognostic biomarker[11].

Circ-ABCB10, also known as hsa_circ_0008717, is 724 length in gene symbol ABCB10 which is located at chr1:229665945-229678118. In breast cancer, circ-ABCB10 facilitates cell proliferation and tumorigenesis by sponging miR-1271[12]. In this study, we found that circ-ABCB10 was upregulated in NSCLC samples. Besides, the silence of circ-ABCB10 repressed cell proliferation of NSCLC cells. The cell apoptosis of NSCLC cells was promoted through the knockdown of circ-ABCB10. The above results indicated that circ-ABCB10 participated in cell proliferation, cell cycle, and NSCLC apoptosis and might act as an oncogene.

To further identify the underlying mechanism of how circ-ABCB10 affects NSCLC, we used Starbase v2.0 to predict the target proteins of

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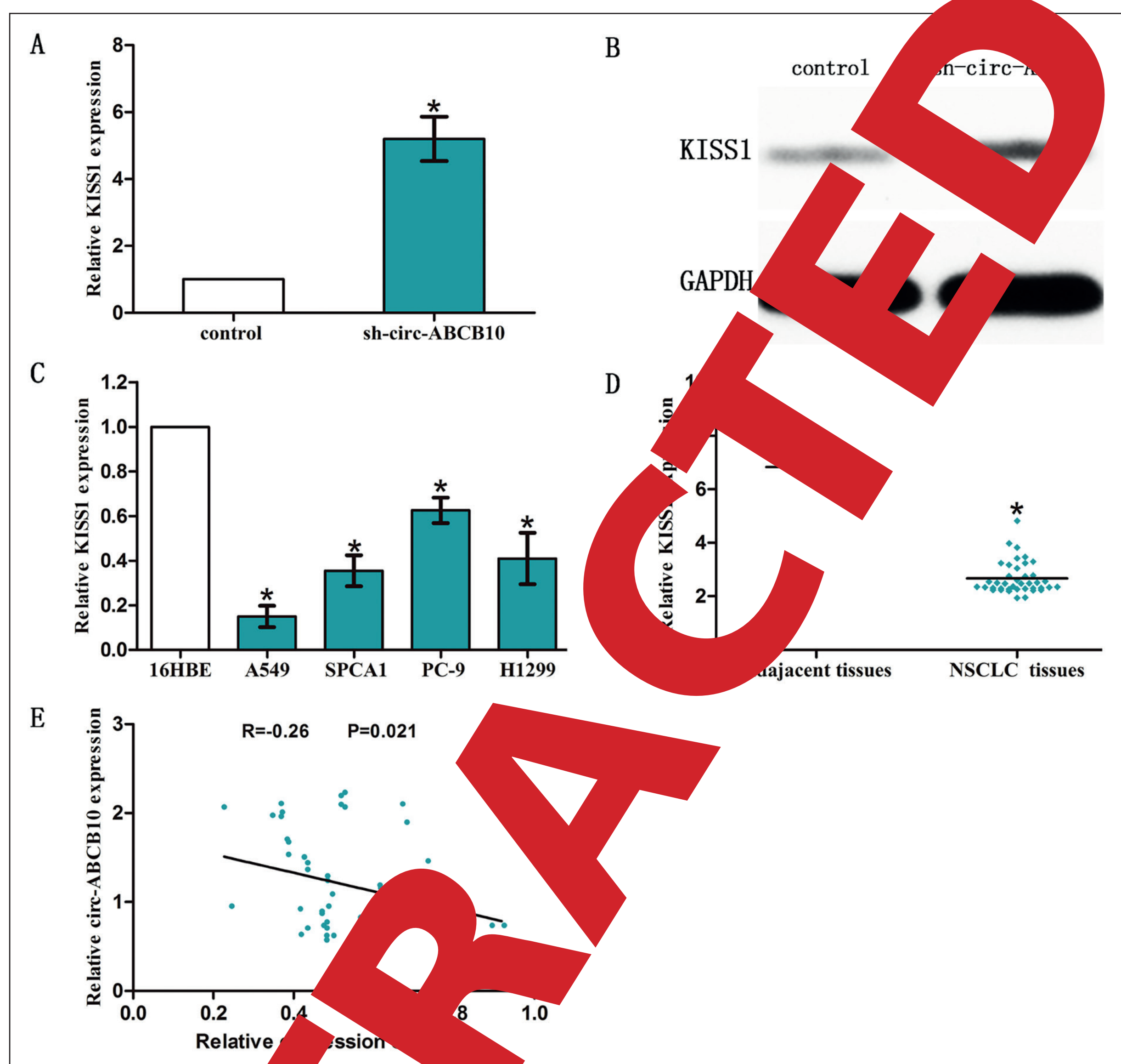

Figure 4. Interaction bet[illegible]ABCB10 and KI[illegible]1. **A,** RT-qPCR results showed that KISS1 expression was increased in sh-circ-ABCB10 compar[illegible]ol group. **B,** Western blot results showed that KISS1 expression was increased in sh-circ-ABCB10 compared [illegible] the c[illegible] **C,** Expression levels of KISS1 relative to GAPDH were determined in the human NSCLC cell lines and 16HBE by RT-qP[illegible]S1 was significantly downregulated in NSCLC tissues compared with adjacent tissues. **E,** Linear c[illegible]tion between the ex[illegible]sion level of KISS1 and circ-ABCB10 in NSCLC tissues. The results represent the average of thre[illegible]ependent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05.

circ-ABCB1[illegible]ed K[illegible] as the potential targets of circ-AB[illegible]ISS1 gene encodes KISS1 [illegible] has re[illegible]een reported to exhibit [illegible]static and [illegible]nti-tumoral roles in a va[illegible] of c[illegible] KISS1 expression level i[illegible]ed [illegible]malignant transformation of t[illegible] mucosa and upregulation of KISS1 is asso[illegible]ith worse prognosis in colorectal cancer[13]. A[illegible]S1 expression was repressed, cell proliferation was promoted and cell apoptosis was inhibited in clear cell renal cell carcinoma[14]. KISS1 functions as a tumor suppressor and restricts breast cancer brain metastases which also sensitizes oncolytic virotherapy[15]. In the present study, we first discovered the interaction between KISS1 and circ-ABCB10. The KISS1 could be upregulated after knockdown of circ-ABCB10. Besides, KISS1 expression was lower in NSCLC cell lines. Furthermore, KISS1 expression in NSCLC tissues was negatively related to circ-AB-

CB10 expression. All these results suggested that circ-ABCB10 might promote tumorigenesis of NSCLC by downregulating KISS1.

Conclusions

The data above reported demonstrated that circ-ABCB10 could facilitate tumorigenesis of NSCLC by downregulating KISS1, which provides a candidate target for NSCLC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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