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Circ-ABCB10 promotes proliferation and invasion of esophageal squamous cell carcinoma cells by modulating microRNA-670-3p

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Abstract. – OBJECTIVE: Circ-ABCB10 is a non-coding RNA newly discovered in recent years. It has been observed to serve as an oncogene in a variety of tumors, but its biological function in esophageal squamous cell carcinoma (ESCC) is still unknown. The purpose of this study was to investigate the circ-ABCB10 expression in ESCC and its possible molecular mechanism.

PATIENTS AND METHODS: Circ-ABCB10 expression in ESCC tissue samples and cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The impacts of circ-ABCB10 on the biological functions of ESCC cells were examined by cell counting kit-8 (CCK-8) and transwell assays. Moreover, bioinformatics analysis was used to determine the binding sites between miRNAs and circ-ABCB10, and the binding relationship was verified by qRT-PCR and Luciferase assay.

RESULTS: QRT-PCR analysis revealed that circ-ABCB10 expression in both ESCC tissues and cell lines was higher than that in the normal control group. Patients in high TNM stage exhibited a higher expression of circ-ABCB10 than those in low stage, and this high expression predicted a poor prognosis of ESCC patients. Inhibiting circ-ABCB10 expression remarkably inhibited the growth and metastasis of ESCC cells. In addition, it was demonstrated that circ-ABCB10 could bind to microRNA-670-3p and inhibit its expression. Downregulation of microRNA-670-3p partially reversed the inhibitory impact of low-expressing circ-ABCB10 on cell growth and migration rate.

CONCLUSIONS: Circ-ABCB10 accelerates the metastasis and proliferation of ESCC cells by binding to microRNA-670-3p. This circ-ABCB10 / microRNA-670-3p axis may become a potential therapeutic target for ESCC therapy.

Key Words:

ESCC, Circ-ABCB10, MicroRNA-670-3p, Cell proliferation, Cell invasion.

Introduction

Esophageal cancer (ECa) has been one of the common malignant tumors, with its incidence rate ranking the eighth in the world, especially in eastern Asia and southern Africa[1]. The common histopathological types of ECa include squamous cell carcinoma and adenocarcinoma. For inconspicuous early symptoms of ECa, most patients have been in the advanced stage at the time of consultation, accompanied by malnutrition. In addition, the surgical resection of some lesions is difficult, and the drug treatment is not effective[2]. Many large-scale clinical studies have shown that concurrent chemoradiotherapy is the standard treatment mode for patients with locally advanced ECa who cannot be treated with surgery[3]. However, there are currently no effective targeted therapies for patients with ECa intolerant to concurrent chemoradiotherapy, such as old age, severe cardiopulmonary complications or malnutrition[4,5]. Hence, it is still necessary to find potential molecular targets to guide the treatment for ECa. Circular RNA is a non-coding RNA that is expressed in large quantities in eukaryotic cells[6,7]. With the development of RNA sequencing technology, more and more circRNAs have been discovered. CircRNAs originate from exons, introns, or from both exons and introns[8,9]. The formation of circRNA is regulated by RNA-binding proteins. The splicing factor Mbl promotes the generation of circMbl by binding to exon flanking introns[8]. In the epithelial-mesenchymal transition pathway, quaking protein (QKI) can promote the formation of circRNA by linking flanking introns, which can shorten the cleavage of donor and acceptor[9]. DHX9 protein is an RNA nuclear helicase, which can bind to the reverse complementary sequence of flanking introns

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of circRNA to inhibit the production of circRNA[9]. Adenosine deaminase acting on RNA (ADAR1) can convert adenosine A into inosine I through deamination hydrolysis by binding to the double-stranded region, which promotes the opening of some circRNAs, thereby reducing the production of circRNA[10]. MiRNA, as a type of single-stranded microRNA with a length of about 22-23 nucleotides, can inhibit the translation of the target gene or mediate its degradation process by binding to the 3'UTR region of the target gene mRNA, thereby exerting the function of negatively regulating its expression. Furthermore, circRNA can serve as a "miRNA sponge" in combination with miRNA, reducing functional miRNAs, inhibiting miRNAs from negatively regulating the function of their target genes, causing abnormal expression of genes, and ultimately changing physiological and pathological processes. The circular RNA has been proven to be involved in the genesis and development of various tumors as a tumor-promoting gene. Circ-ABCB10 increases HMG20A expression through sponge binding to microRNA-670-3p, thereby promoting the progression of liver cancer[11]. CircABCB10 promotes the growth and migration of non-small cell lung cancer cells by modulating the microRNA-1252/ FOXR2 axis[12]. Circ-ABCB10 is associated with advanced clinicopathological features and poor prognosis of epithelial ovarian cancer[13]. However, the biological role of circ-ABCB10 in ESCC and its possible molecular mechanisms remain elusive.

Patients and Methods

Specimen Collection

A total of 45 ESCC tumor tissues and 45 normal control tissues were collected from ESCC patients who underwent surgery in the 7th Medical Center of PLA General Hospital from July 2017 to June 2019. All specimens were pathologically confirmed. ESCC tumor tissue pathological classification and staging criteria are performed in accordance with the Union for International Cancer Control (UICC) staging criteria. All subjects did not accept radiotherapy, chemotherapy and other adjuvant treatments before surgery and signed informed consent. This investigation was approved by the Ethics Committee of the 7th Medical Center of PLA General Hospital.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using PrimeScript™ RT kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was carried out with the SYBR Green kit, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 as an internal reference. Primer sequences were as follows: circ-ABCB10 Forward: 5'-CTTATCCACTTGGCCGGAG-3', Reverse: 5'-CGCGTAGATCTCAGGGG-3'; microRNA-670-3p Forward: 5'-CTGATCGTGAGGAGAGAGTGT-3', Reverse: 5'-GGTCTTCGACATCGGGGCGG-3'; GAPDH Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; U6 Forward: 5'-GCTGAGGTGACGGTCTCAAA-3', Reverse: 5'-GCCTCCCAGTTTCATGGACA-3'.

Cell Culture

The human normal esophageal cell line HEEC (Het-1A) was provided by American Type Culture Collection (ATCC, Manassas, VA, USA), while the ESCC cell lines TE1, KYSE30, KYSE70 and KYSE150 were from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells grew in Roswell Park Memorial Institute-1640 (RPMI-1640) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin and placed in a 37°C, 5% CO_2 incubator.

Cell Transfection

For transient transfection, Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with circ-ABCB10 siRNA, microRNA-670-3p mimics, or microRNA-670-3p inhibitor (GenePharma, Shanghai, China) and then added into cells when cell density reached to 50%.

Cell Counting Kit-8 (CCK-8) Experiment

Cells were plated in 96-well plates (2×10^3 cells/well) in 100 mL of culture medium. CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) was performed according to the manufacturer's protocol.

Transwell Experiment

At 24 h after transfection, cells were prepared into cell suspensions and seeded in the upper transwell chamber (10,000 cells/well) supplemented with serum-free medium, and then

10% fetal bovine serum medium was added to the lower compartment. After that, the migrated cells were counted and observed after stained by crystal violet under a microscope (Olympus, Tokyo, Japan), and 5 fields of view were randomly selected.

Dual-Luciferase Assay

The bioinformatics website was used to predict the binding sites of microRNA-670-3p and circ-ABCB103. The circ-ABCB10 Luciferase reporter vector containing microRNA-670-3p binding site was constructed by GenePharma (Shanghai, China), and the relative fluorescence value after plasmid transfection was measured based on standardized methods.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism (GraphPad Software, Inc., Armonk, NY, USA) were used for data analysis and mapping. Measurement data are expressed as mean ± SD (standard deviation). The difference was statistically significant when p was less than 0.05.

Results

Circ-ABCB10 Expression was Increased In ESCC

QRT-PCR results showed that circ-ABCB10 expression in ESCC tissue samples was remarkably higher than that in normal control tissue samples (Figure 1A). Meanwhile, the analysis showed that circ-ABCB10 expression in the tissues of ESCC patients in stage III + IV was markedly higher than those in stage I + II (Figure 1B). According to the median expression of circ-ABCB10, patients were divided into circ-ABCB10 high-expression and low-expression group. Through clinical information analysis, it was found that the overall survival in the former was significantly lower than that in the latter (p = 0.0416) (Figure 1C). Consistently, circ-ABCB10 was highly expressed in ESCC cell lines as compared with the normal control

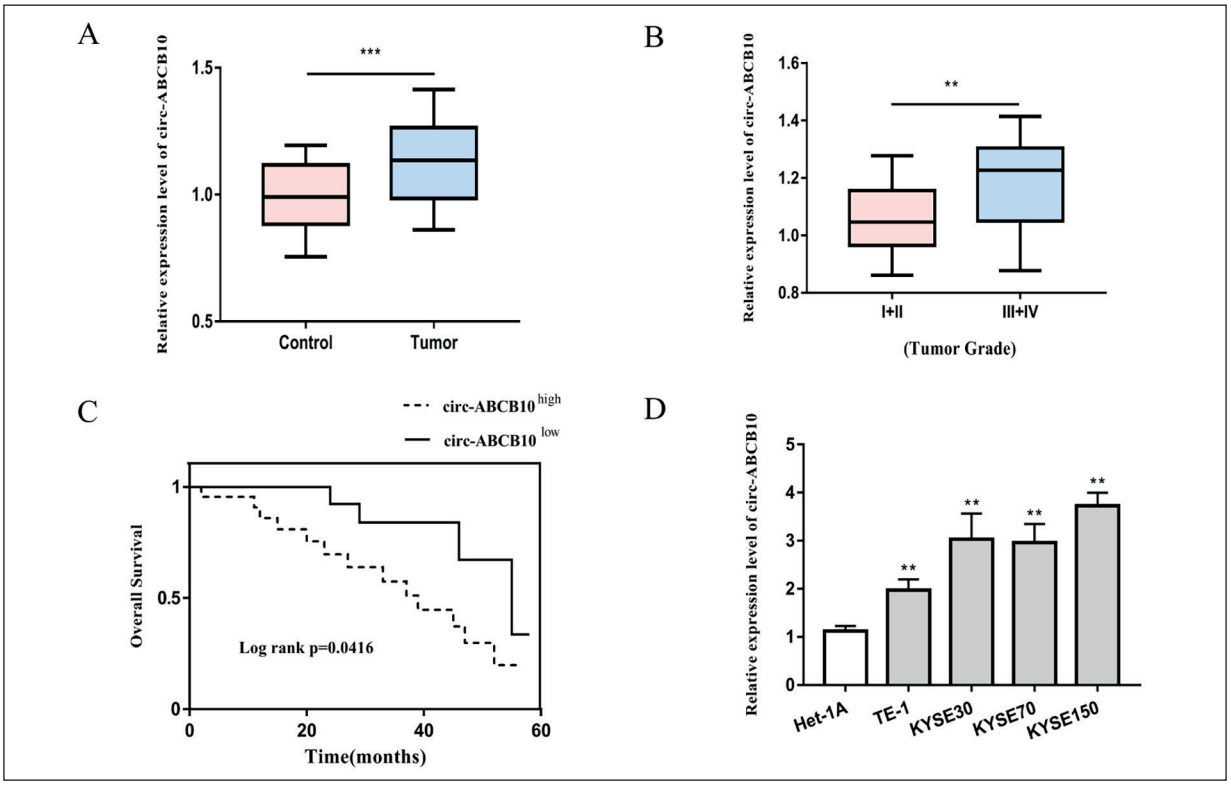

Figure 1. Circ-ABCB10 expression was significantly increased in ESCC. **A,** The relative expression level of circ-ABCB10 in ESCC tissues and normal control tissues was detected by qRT-PCR. **B,** The relative expression level of circ-ABCB10 in ESCC tissues with different TNM stages was detected by qRT-PCR. **C,** Highly expressed circ-ABCB10 was predictive of poor prognosis in ESCC patients. **D,** The expression of circ-ABCB10 in ESCC cell lines was detected by qRT-PCR. **p<0.01, ***p<0.001.

cell line Het-1A (Figure 1D). The above results suggest that circ-ABCB10 may serve as a cancer-promoting gene in ESCC.

Inhibition of Circ-ABCB10 Could Inhibit the Proliferation, Migration and Invasion of ESCC Cells

ESCC cell lines KYSE30 and KYSE150 with high circ-ABCB10 expression were selected for *in vitro* experiments. First, circ-ABCB10 was downregulated in above two cell lines through transfection with circ-ABCB10 siRNA, and the efficiency was verified by qRT-PCR test (Figure 2A). Afterwards, CCK-8 and transwell experiments were carried out to reveal that knockdown of circ-ABCB10 remarkably suppressed the proliferative capacity, as well as invasiveness of ESCC cells (Figure 2B-2D), suggesting that circ-ABCB10 is capable of enhancing ESCC cell invasion and proliferation capabilities.

Circ-ABCB10 Exerted Its Biological Function by Adsorbing MicroRNA-670-3p

Studies have shown that circular RNA regulates the expression of downstream genes mainly by adsorbing miRNAs, thus exerting biological effects. Hence, it was predicted that miRNAs can bind to circ-ABCB10 through the search of bioinformatics website (Figure 3A). It was found that circ-ABCB10 had potential binding sites with a set of miRNAs, among which microRNA-183-5p, microRNA-541-5p and microRNA-670-3p had higher binding scores. Hence, the expression levels of these miRNAs were detected by qRT-PCR after reducing circ-ABCB10 level in KYSE30 and KYSE150 cells. The results showed that inhibiting circ-ABCB10 expression remarkably increased the expressions of above three miRNAs, especially that of microRNA-670-3p (Figure 3B). Then, the circ-ABCB10 wild-type plasmid (circ-ABCB10-WT) and the mutant plasmid (circ-ABCB10-MUT) (Figure 3C) were designed and constructed, and Luciferase assay was performed to verify the binding correlation between circ-ABCB10 and microRNA-670-3p. Figure 3D showed that microRNA-670-3p mimics effectively attenuated the Luciferase activity of circ-ABCB10-WT without remarkably affecting that of circ-ABCB10-MUT group, indicating that microRNA-670-3p can directly target circ-ABCB10. Subsequent qRT-PCR tests revealed an under expressed microRNA-670-3p level in ESCC (Figure 3E). Moreover, Spearman rank correlation analysis demonstrated a significant negative correlation between circ-ABCB10 and microRNA-670-3p ($R = -0.5663$, $p < 0.001$) (Figure 3F).

Inhibition of MicroRNA-670-3p In ESCC Cells Reversed the Function of Circ-ABCB10 With Low Expression

In KYSE30 and KYSE150 cells, the expressions of circ-ABCB10 and microRNA-670-3p were downregulated simultaneously, and cell growth and metastasis were tested by *in vitro* cell experiments. QRT-PCR revealed that microRNA-670-3p inhibitor partially reversed the promotion effect of circ-ABCB10 siRNA on microRNA-670-3p expression, which further indicated the negative regulation of circ-ABCB10 on microRNA-670-3p (Figure 4A). Subsequently, CCK-8 and transwell experiments demonstrated that downregulation of circ-ABCB10 markedly suppressed the proliferation and metastasis abilities of NPC cells, which could be reversed by simultaneous transfection of microRNA-670-3p inhibitor (Figure 4B-4D). Taken together, the above data suggest that circ-ABCB10 enhances cell proliferative ability and invasiveness *via* modulation of microRNA-670-3p.

Discussion

The early signs of ESCC patients are not evident, and the current gold standard for diagnosis is endoscopy and biopsy, so the patients have poor examination experience, which results in a clinical status quo that more patients with ECa have been in the late stage, accompanied by lymph node metastasis. ESCC metastasis is a complex process involving genetic and epigenetic changes, and the specific molecular mechanism is still unclear[14,15]. Therefore, it is particularly urgent to explore the internal molecular mechanism of ESCC occurrence and development, deepen the understanding of ESCC disease progression, and study effective molecular targets for ECa treatment and prognosis improvement.

CircRNA, as a special type of noncoding RNA (ncRNA), has a structure different from that of traditional linear RNA. It has a closed ring structure and exists in a large number of eukaryotic transcriptomes. Most circRNAs are composed of exon sequences that are highly conserved in different species, with expression specificity in

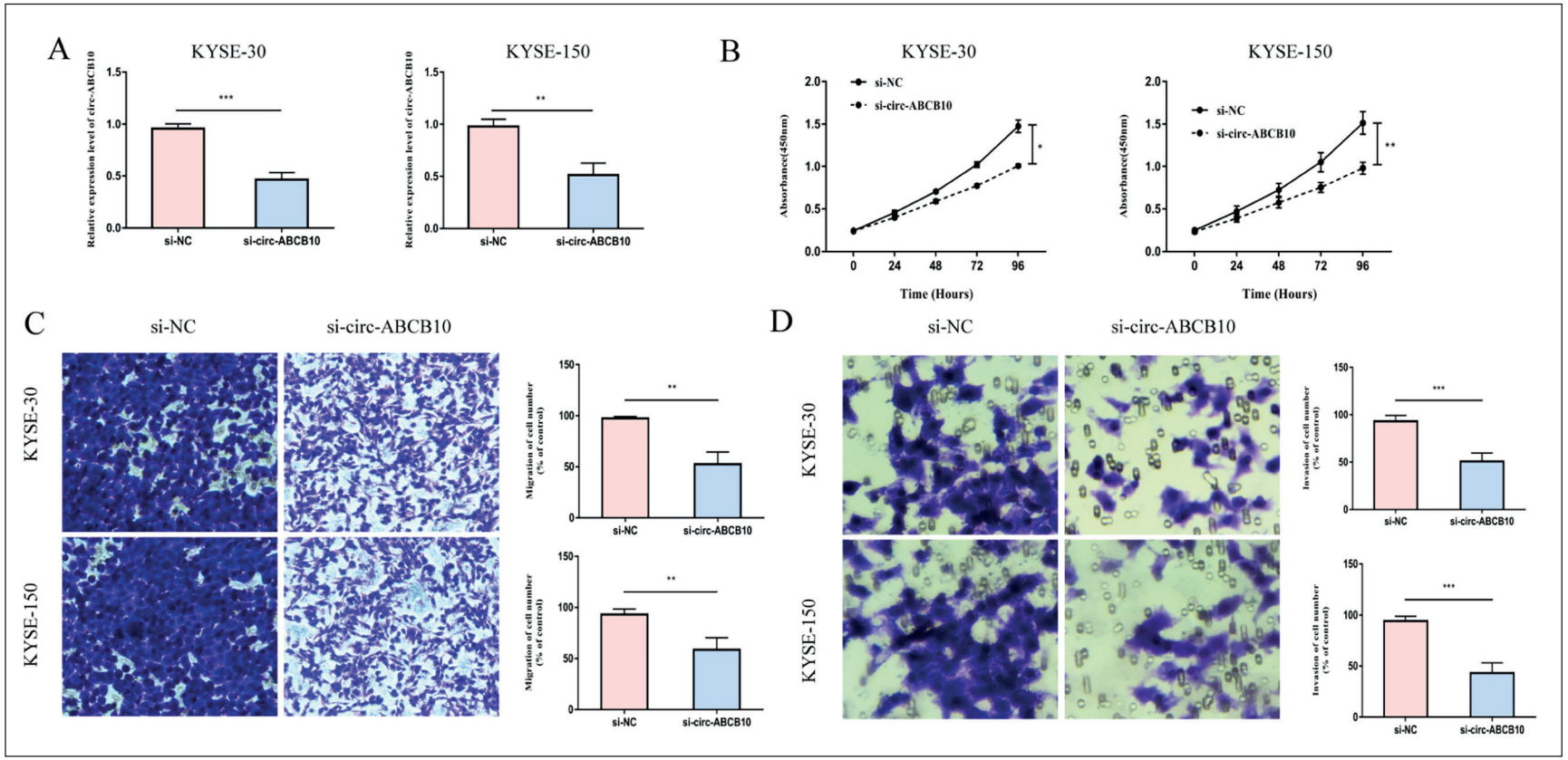

Figure 2. Down-regulation of circ-ABCB10 could inhibit the proliferation, migration and invasion of ESCC cells. **A,** qRT-PCR was used to detect the expression of circ-ABCB10 in KYSE30 and KYSE150 cells transfected with si-NC or si-circ-ABCB10. **B,** The effect of circ-ABCB10 on the proliferation of KYSE30 and KYSE150 cells was tested by CCK-8 assay. **C,** The effect of circ-ABCB10 on the migration of KYSE30 and KYSE150 cells was detected by the transwell migration experiment, (magnification: 200×). **D,** The effect of circ-ABCB10 on KYSE30 and KYSE150 cell invasion was tested by transwell invasion assay, (magnification: 200×). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

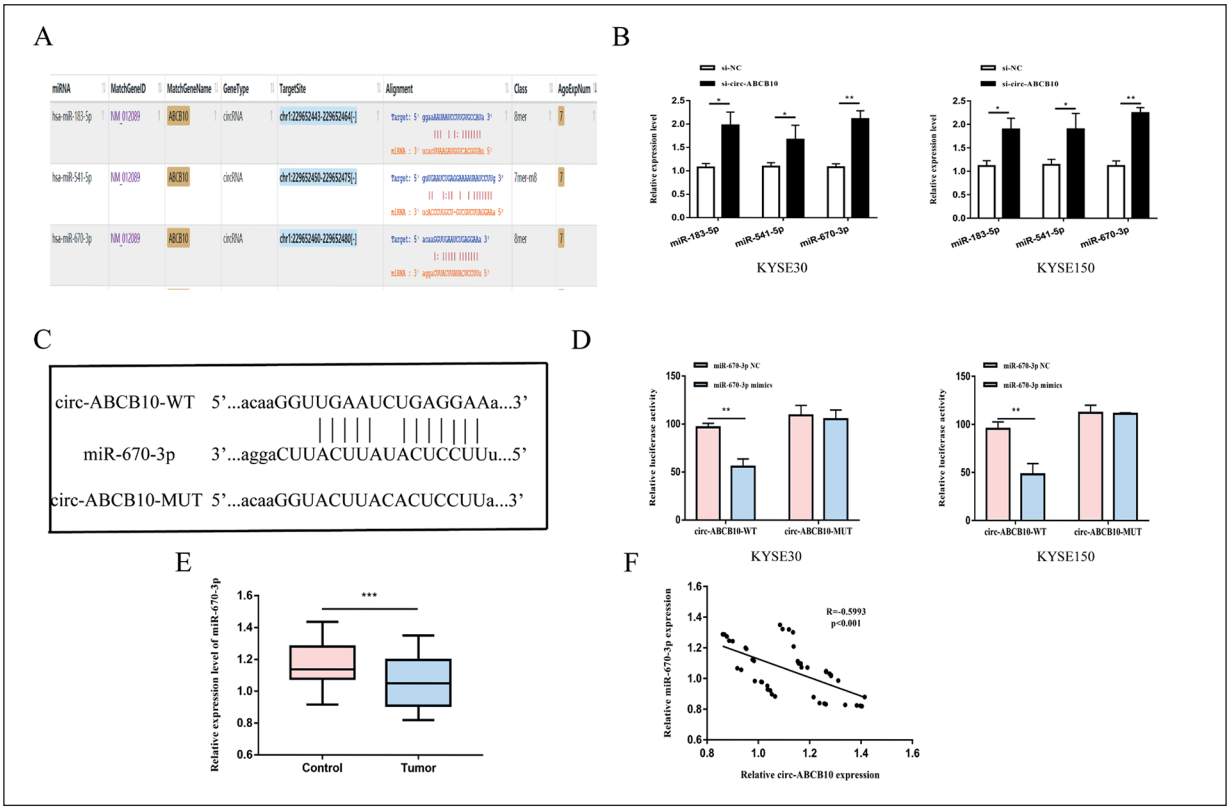

Figure 3. Circ-ABCB10 was able to adsorb miR-670-3p. **A,** Bioinformatics website (http://starbase.sysu.edu.cn) predicted miRNAs with binding sites to circ-ABCB10, and miRNAs with higher binding scores were selected. **B,** The expressions of miR-183-5p, miR-541-5p, and miR-670-3p were detected by qRT-PCR after KYSE30 and KYSE150 were transfected with siNC or circ-ABCB10 siRNA. **C,** Circ-ABCB10 wild-type and mutant plasmids were designed and constructed. **D,** After co-transfection of miR-670-3p mimics and Luciferase reporter genes containing circ-ABCB10-WT or circ-ABCB10-MUT in KYSE30 and KYSE150 cells, the reporter gene activity was detected. **E,** Detection of miR-670-3p expression in ESCC tissues and adjacent normal tissues by qRT-PCR. **F,** Analysis of the correlation between circ-ABCB10 and miR-670-3p expression in ESCC tissues by Spearman's rank correlation analysis. **p*<0.05, ***p*<0.01, ****p*<0.001.

different tissues and developmental stages[16]. Due to its highly conserved circular structure and low sensitivity to ribozyme, circRNAs are more stable than linear RNAs, giving circRNAs significant advantages in the development and application prospects becoming new clinical diagnostic markers and therapeutic targets[16]. CircRNAs can regulate transcription, shearing, and gene expression processes by acting as miRNA sponges, shear or transcriptional regulators and RNA-binding proteins. A large number of circRNAs are involved in regulating multiple biological functions such as proliferation, metastasis, invasion, apoptosis and drug resistance of tumor cells[17,18]. CircRNA hsa_circ_0000654 promotes the progression of ESCC by regulating the microRNA-149-5p/il-6 / STAT3 pathway[19]. Up-regulation of circ-smad7 inhibits tumor proliferation and migration in ESCC[20]. Hsa_circ_0006948 enhances tumor progression and epithelial mesenchymal transformation in ESCC by microRNA-490-3p/HMGA2 axis[21]. CiRS-7 inhibits autophagy in ESCC cells by acting as a microRNA-1299 sponge to target EGFR signaling[22].

In this study, it was found that circ-ABCB10 expression in ESCC tissue samples was remarkably higher than that in the normal control group, which predicted a poor prognosis of ESCC patients. Consistently, *in vitro* cell experiments also revealed that downregulation of circ-ABCB10 in ESCC cell lines markedly attenuated cell invasiveness and proliferation ability. In addition, it was also demonstrated that circ-ABCB10 could bind to microRNA-670-3p and inhibit its expression. Meanwhile, it was observed that down-regulation of microRNA-670-3p could partially reverse the inhibitory effect of low-expression circ-ABCB10 on

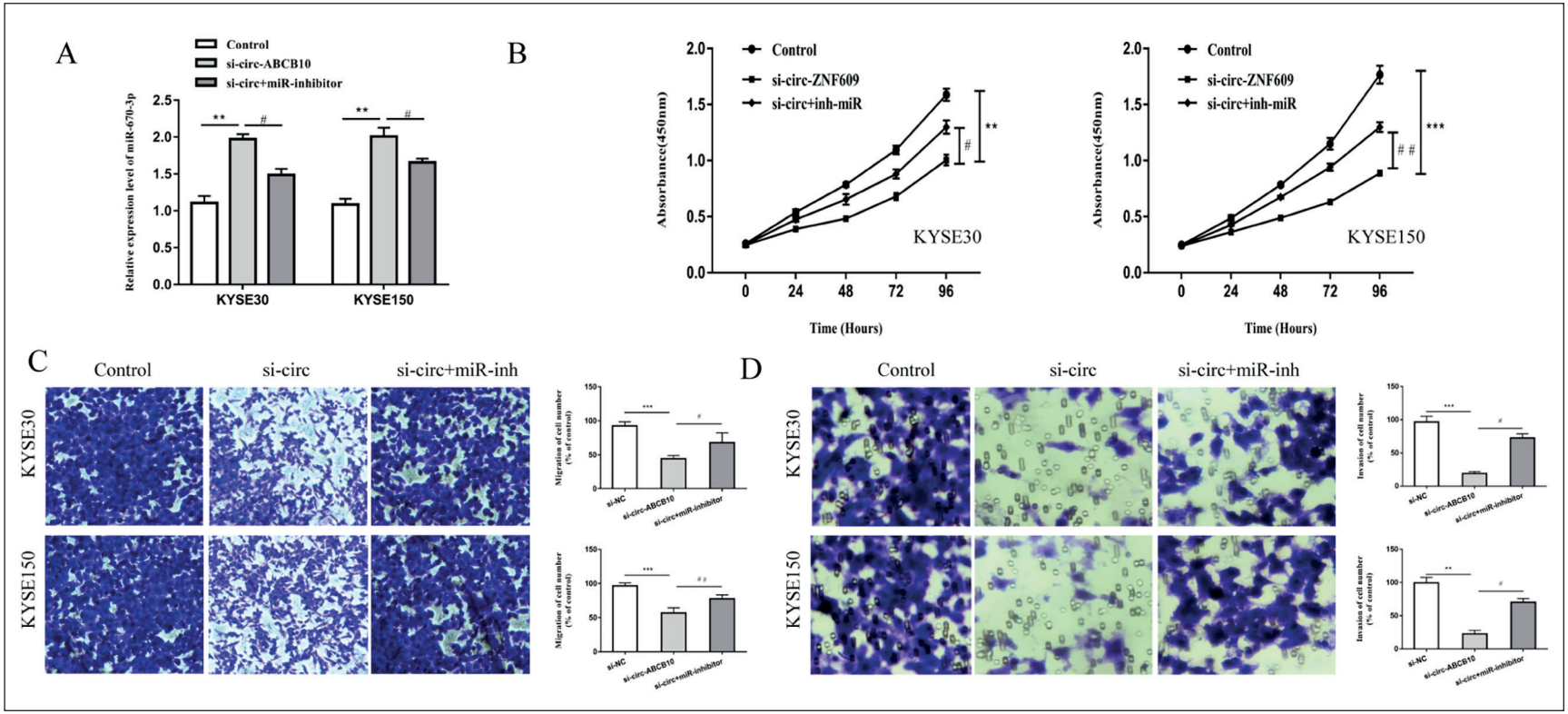

Figure 4. Inhibition of miR-670-3p could partially reverse the inhibitory effect of circ-ABCB10. **A,** miR-670-3p expression was detected by qRT-PCR after circ-ABCB10 and miR-670-3p were simultaneously down-regulated in KYSE30 and KYSE150 cells. **B,** The effect of circ-ABCB10/miR-670-3p on cell proliferation was tested by CCK-8 assay. **C,** The effect of circ-ABCB10/miR-670-3p on cell migration ability was detected by transwell migration assay, (magnification: 200×). **D,** The effect of circ-ABCB10/miR-670-3p on cell invasion was tested by transwell invasion assay, (magnification: 200×). *p<0.05, **p<0.01, ***p<0.001, $^{\#}p$<0.05, $^{\#\#}p$<0.01.

cell migration and proliferation. Taken together, the above results suggested that circ-ABCB10 may be engaged in the progression of ESCC by directly binding to microRNA-670-3p and inhibiting its expression. However, there are still shortcomings in this study. First, the downstream genes of microRNA-670-3p have not been explored and studied in this study. Second, this study was only verified *in vitro* cells, without *in vivo* animal verification. Finally, whether circ-ABCB10 can combine with other miRNAs to regulate downstream genes should be further explored. For the first time we demonstrated that circ-ABCB10 was highly expressed in ESCC tissues and cell lines, and its increased expression is correlated with malignant clinicopathological features. Furthermore, it was detected that circ-ABCB10 promoted proliferation, migration and invasion of ESCC cells probably by directly targeting microRNA-670-3p. This finding will improve understanding of the mechanism involved in cancer progression and provide novel targets for the molecular treatment of ESCC.

Conclusions

Briefly, circ-ABCB10 is highly expressed in ESCC, which may combine with microRNA-670-3p to participate in ESCC progression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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