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Circ-ABCB10 accelerates the malignant progression of oral squamous cell carcinoma by absorbing miRNA-145-5p

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Abstract. – OBJECTIVE: Previous studies have confirmed the carcinogenic role of circ-ABCB10 in certain types of tumors. However, the role of circ-ABCB10 in oral squamous cell carcinoma (OSCC) has not been reported yet. This report investigated the biological function of circ-ABCB10 in aggravating the progression of OSCC by absorbing microRNA-145-5p (miRNA-145-5p) as a ceRNA.

PATIENTS AND METHODS: Relative levels of circ-ABCB10 and miRNA-145-5p in OSCC tissues and cell lines were determined. The potential relation between circ-ABCB10 level and pathological indexes of OSCC patients was analyzed. Regulatory effects of circ-ABCB10 and miRNA-145-5p on proliferative and migratory capacities of CAL-27 and Tca8113 cells were assessed. The interaction between circ-ABCB10 and miRNA-145-5p was examined through dual-luciferase reporter gene assay and Chi-square test. At last, rescue experiments were carried out to uncover the role of the circ-ABCB10/miRNA-145-5p regulatory loop in regulating the progression of OSCC.

RESULTS: Circ-ABCB10 was upregulated in OSCC tissues and cells. OSCC patients expressing a high level of circ-ABCB10 presented worse tumor staging and a higher rate of distant metastasis relative to those with low level. Knockdown of circ-ABCB10 attenuated proliferative and migratory capacities in CAL-27 and Tca8113 cells. Besides, miRNA-145-5p was downregulated in OSCC tissues and cells. The knockdown of miRNA-145-5p accelerated OSCC cells to proliferate and migrate. Dual-luciferase reporter gene assay proved the binding between circ-ABCB10 and miRNA-145-5p. Moreover, the miRNA-145-5p level was negatively correlated to circ-ABCB10 level in OSCC tissues. Rescue experiments indicated that miRNA-145-5p knockdown could reverse the regulatory effects of circ-ABCB10 on viability, colony formation, and migratory capacity in OSCC cells.

CONCLUSIONS: Circ-ABCB10 is upregulated in OSCC, which is closely related to tumor staging and distant metastasis of OSCC patients. Circ-ABCB10 aggravates the progression of OSCC by absorbing miRNA-145-5p.

Key Words:
Circ-ABCB10, MiRNA-145-5p, Oral squamous cell carcinoma (OSCC), Malignant progression.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of malignant tumors originating in the maxillofacial region. The incidence of OSCC shows 50% elevation in the past decade[1,2]. The prognosis of OSCC is relatively poor due to the high malignancy, strong invasiveness to surrounding tissues, and cervical lymph nodes[3-5]. Individualized therapies combined with surgical resection, postoperative radiotherapy, and chemotherapy for OSCC have achieved great strides. However, the 5-year survival of OSCC is unsatisfactory and still lower than 50%[6,7]. It is significant to develop effective strategies for improving the prognosis of OSCC[8,9].

Circular RNA (circRNA) is a non-coding RNA with a special looped structure by ligating 5' and 3' ends of linear RNA precursors, which is related to transcriptional and post-transcriptional regulation on gene expressions[10,11]. In recent years, circRNAs have

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been extensively discovered in bacteria, fungi, plants, and mammals[12,13]. As a competitive endogenous RNA (ceRNA), a circRNA interacts with microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and proteins, thus exerting its biological functions. CircRNA is involved in a variety of diseases and tumor processes. Due to its abundance, unique stable structure and tissue-specificity, circRNAs are expected to provide new promising aspects in tumor progression[13-15]. The role of circRNAs in OSCC, however, is rarely reported[16]. Our previous findings illustrated that circ-ABCB10 was upregulated in OSCC through high-throughput sequencing and bioinformatics analysis. Multiple miRNA binding sites are distributed in the sequence of circ-ABCB10, which requires further explorations[17].

This study aims to examine the expression pattern and biological functions of circ-ABCB10 in OSCC. Circ-ABCB10 and its targeted miRNA were thoroughly analyzed in the malignant progression of OSCC.

Patients and Methods

Patients and OSCC Samples

Paired OSCC tissues and adjacent normal tissues were surgically resected from 34 OSCC patients. None of the enrolled OSCC patients received preoperative anti-tumor therapies. Their clinical indexes were collected for further analyses. Telephone follow-up or out-patient review were conducted and their data were recorded. Patients and their families in this work have been fully informed. This research was approved by the Ethics Committee of Peking University People's Hospital.

Cell Culture

OSCC cell lines (Fadu, SCC-25, CAL-27, and Tca8113) and *homo sapiens* tongue normal cells Hs 680. Thyroglobulin (Tg) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. At 80-90% confluence, the cell passage was conducted using 1×trypsin + EDTA (ethylenediaminetetraacetic acid).

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells were pre-seeded in the 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 70% confluence. At 48 h, cells were harvested for verifying transfection efficacy and subsequent functional experiments.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 2×10^3 cells per well. At the established time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were seeded in a 6-well plate (200 cells/well) and incubated for 10-14 days. Visible colonies were washed with phosphate-buffered saline (PBS) twice, fixed in 4% paraformaldehyde and dyed with Giemsa solution for 30 min. Colonies were captured and those containing over 50 cells were counted.

Transwell Migration Assay

Cells were adjusted to a dose of 5.0×10^5/mL. 200 μL/well suspension and 700 μL of medium containing 10% FBS were applied in the upper and bottom side of transwell chamber (Millipore, Billerica, MA, USA), respectively. After 48 h of incubation, cells migrated to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. The number of migratory cells was counted in 5 randomly selected fields per sample (magnification 10×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Each sample was performed

in triplicate. The relative level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer 5.0 was used for designing qRT-PCR primers. Primer sequences used in this study were as follows: Circ-ABCB10, F: 5'-GCAGTTCACCGTACTCACATC-3', R: 5'-CGGTAGGGCGTCTCCGCGAGA-3'; miR-145-5p, F: 5'-ACGCTTGTGTAACATCCTCGCCTG-3', R: 5'-GATTGCGTCCGTAAAGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

CAL-27 and Tca8113 cells were co-transfected with miRNA-145-5p mimics/NC and pmirGLO-circ-ABCB10-WT/pmirGLO-circ-ABCB10-MUT using Lipofectamine 2000, respectively. 24 h later, co-transfected cells were harvested for determining luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. Kaplan-Meier curves were introduced for survival analysis. Chi-square test was performed to evaluate the relation between expression levels of the two genes. $p<0.05$ was considered as statistically significant.

Results

Circ-ABCB10 Was Upregulated in OSCC

A total of 34 paired OSCC and adjacent normal tissues were collected. Compared with adjacent normal tissues, circ-ABCB10 was upregulated in OSCC tissues (Figure 1A). Similarly, circ-ABCB10 was highly expressed in OSCC cells relative to *homo sapiens* tongue normal cells Hs 680. Tg (Figure 1B). CAL-27 and Tca8113 cells expressed the highest level of circ-ABCB10 among the four selected OSCC cell lines, which were chosen to establish the *in vitro* downregulation model of circ-ABCB10.

Circ-ABCB10 Expression Was Correlated to Pathological Staging and Distant Metastasis in OSCC Patients

Based on the median level of circ-ABCB10, enrolled OSCC patients were divided into high-level and low-level groups. Their clinical data were collected, including age, gender, pathological staging, lymphatic metastasis, and distant metastasis statue. It is shown that circ-ABCB10 level was correlated to pathological staging and distant metastasis of OSCC patients, rather than other indexes (Table I). Moreover, the miRNA-145-5p level was identified to correlate to pathological staging and distant metastasis of OSCC patients.

Downregulation of Circ-ABCB10 Inhibited Cell Growth and Migration

Three shRNAs targeting circ-ABCB10 were constructed, namely sh-circ-ABCB10#1, sh-circ-

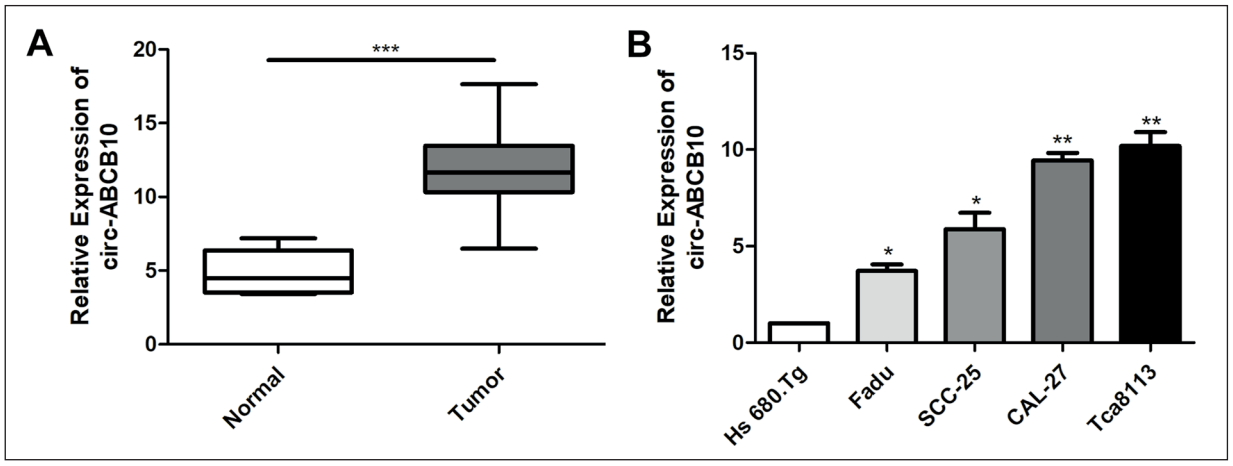

Figure 1. Circ-ABCB10 was upregulated in OSCC. **A,** Relative level of circ-ABCB10 in OSCC tissues and adjacent normal tissues. **B,** Relative level of circ-ABCB10 in OSCC cell lines (Fadu, SCC-25, CAL-27, and Tca8113) and homo sapiens tongue normal cells Hs 680. Tg.

Table I. Association of circ-ABCB10 and miR-145-5p expression with clinicopathologic characteristics of oral squamous cell carcinoma.

Parameters	Number of cases	circ-ABCB10 expression		*p*-value	miR-145-5p expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.151			0.354
< 60	12	8	4		3	9	
≥ 60	22	9	13		9	13	
Gender				0.303			0.473
Male	17	10	7		5	12	
Female	17	7	10		7	10	
T stage				0.004			0.005
T1-T2	22	15	7		4	18	
T3-T4	12	2	10		8	4	
Lymph node metastasis				0.052			0.138
No	25	15	10		7	18	
Yes	9	2	7		5	4	
Distance metastasis				0.004			0.038
No	22	15	7		5	17	
Yes	12	2	10		7	5	

ABCB10#2, and sh-circ-ABCB10#3. All of them showed effective transfection efficacy in CAL-27 and Tca8113 cells and the former one was selected due to its best transfection efficacy among the three shRNAs (Figure 2A). After transfection of sh-circ-ABCB10#1, the viabilities in CAL-27, and Tca8113 cells were markedly reduced (Figure 2B). The relative colony number decreased after the transfection of sh-circ-ABCB10#1, confirming the attenuated proliferative capacity (Figure 2C). Additionally, transwell assay showed the reduced number of migratory cells after knockdown of circ-ABCB10 in CAL-27 and Tca8113 cells (Figure 2D).

MiRNA-145-5p Was Downregulated in OSCC

Relative to adjacent normal tissues, miRNA-145-5p was downregulated in OSCC tissues (Figure 3A). Similarly, miRNA-145-5p was downregulated in OSCC cells as well (Figure 3B). Chi-square test showed a negative correlation between the miRNA-145-5p level and circ-ABCB10 level in OSCC tissues (Figure 3C).

MiRNA-145-5p Was a Direct Target of Circ-ABCB10

To further validate the targeting of miRNA-145-5p to circ-ABCB10, circ-ABCB10 sequences were cloned into the luciferase reporter plasmid pmirGLO. The mutation vector pmirGLO-circ-ABCB10-MUT was constructed as well. After the co-transfection of pmirGLO-circ-ABCB10-WT and miRNA-145-5p mimics in CAL-27 and Tca8113 cells, a significant reduction in the luciferase activity was observed, verifying the binding between miRNA-145-5p and circ-ABCB10 (Figure 3D).

Downregulation of MiRNA-145-5p Promoted Cell Growth and Migration

Transfection of miRNA-145-5p inhibitor in CAL-27 and Tca8113 cells significantly downregulated miRNA-145-5p level (Figure 4A). The CCK-8 assay showed that transfection of miRNA-145-5p inhibitor enhanced the viability of OSCC cells (Figure 4B). Colony formation assay revealed the accelerated colony formation ability after knockdown of miRNA-145-5p, suggesting the stimulated proliferation (Figure 4C). Migratory ability of OSCC cells was stimulated by the transfection of miRNA-145-5p inhibitor (Figure 4D).

Circ-ABCB10 Modulated OSCC Cells by Targeting MiRNA-145-5p

To uncover the role of the circ-ABCB10/miRNA-145-5p regulatory loop in the progression of OSCC, a series of rescue experiments were conducted. The downregulated level of circ-ABCB10 in CAL-27 and Tca8113 cells transfected with sh-circ-ABCB10#1 was partially reversed after the co-transfection of miRNA-145-5p inhibitor (Figure 5A). Of note, a decreased viability in OSCC cells with circ-ABCB10 knockdown was enhanced to some extent after the knockdown

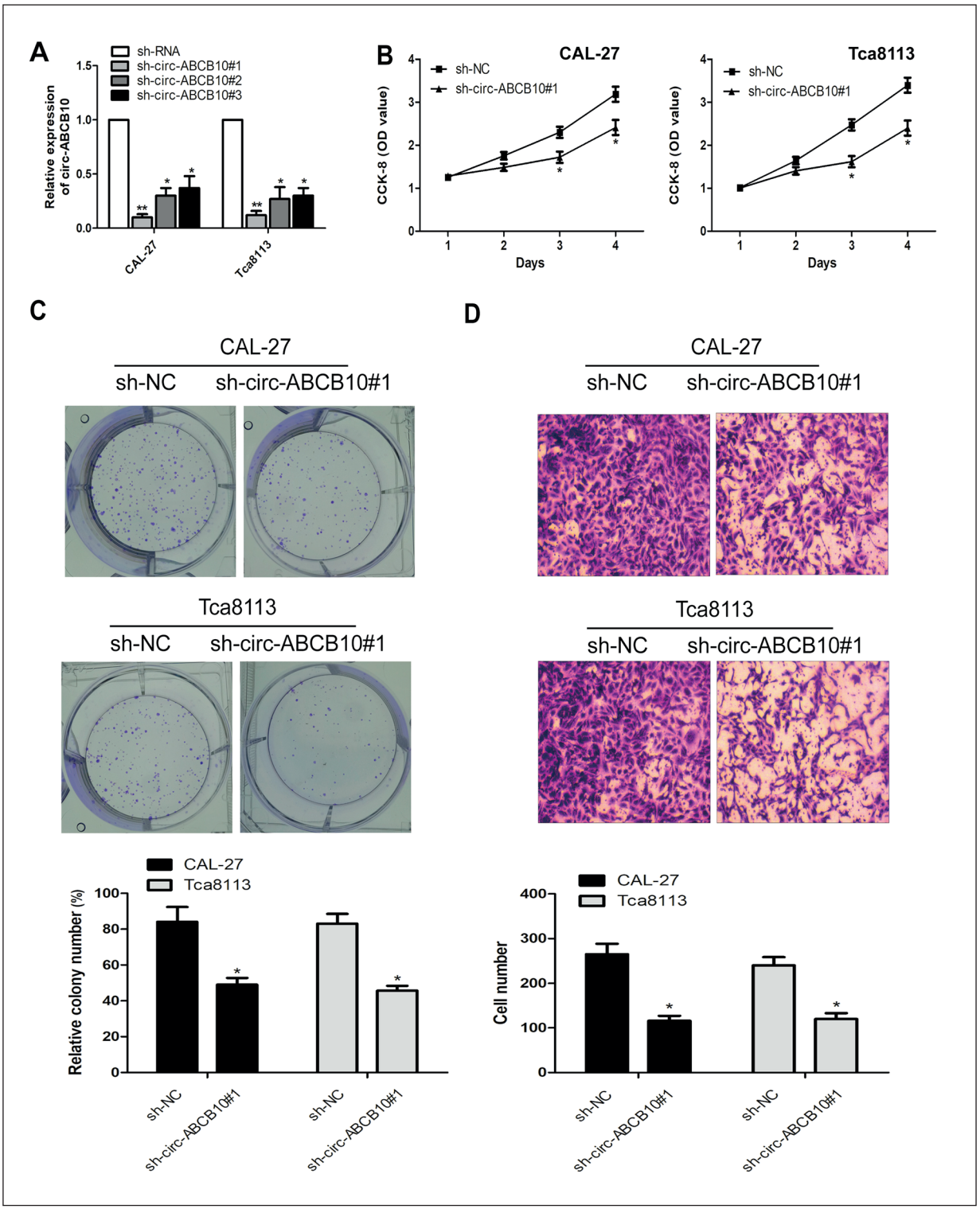

Figure 2. Downregulation of circ-ABCB10 inhibited cell growth and migration. **A,** Transfection efficacy of sh-circ-ABCB10#1, sh-circ-ABCB10#2, and sh-circ-ABCB10#3 in CAL-27 and Tca8113 cells. **B,** CCK-8 assay showed viability in CAL-27 and Tca8113 cells transfected with sh-NC or sh-circ-ABCB10#1. **C,** Colony formation assay showed the colony number in CAL-27 and Tca8113 cells transfected with sh-NC or sh-circ-ABCB10#1 (magnification 10×). **D,** Transwell assay showed the migratory cell number in CAL-27 and Tca8113 cells transfected with sh-NC or sh-circ-ABCB10#1 (magnification 10×).

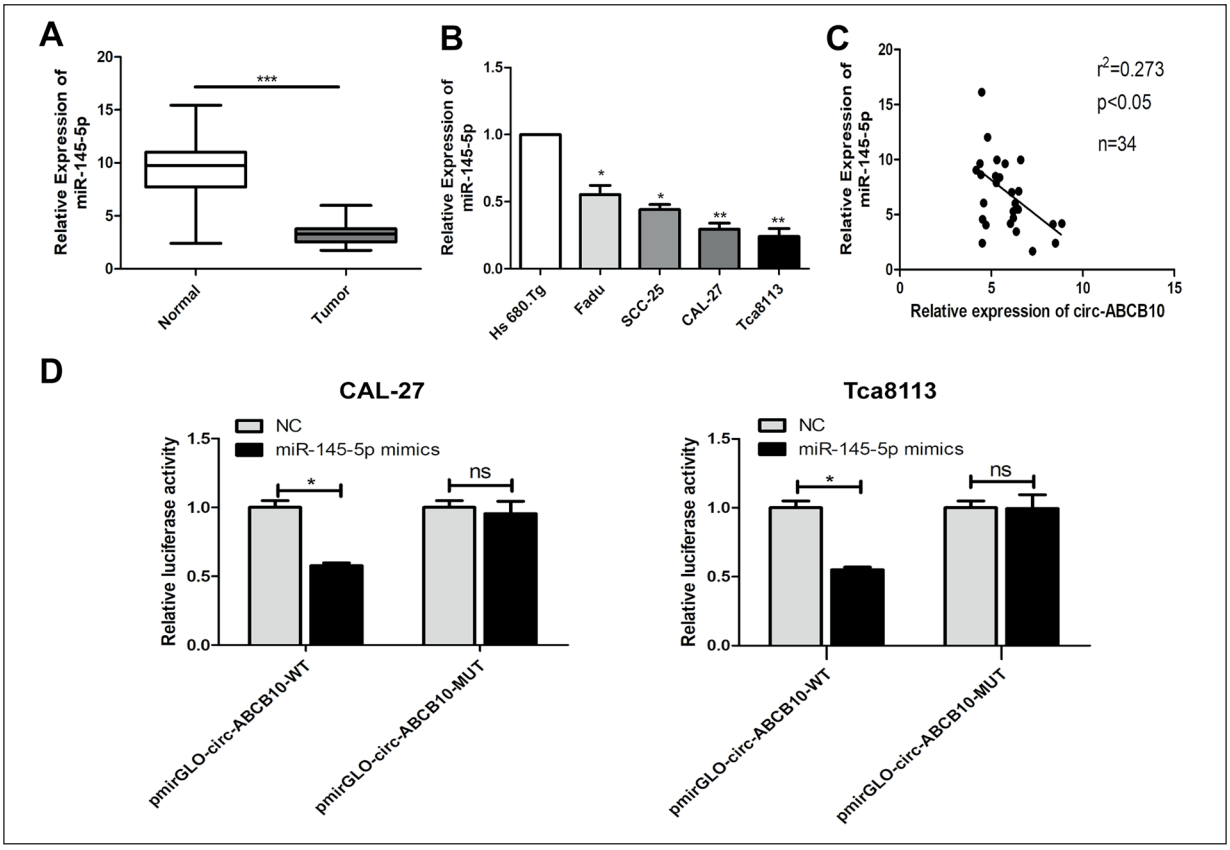

Figure 3. MiR-145-5p was the target of circ-ABCB10. **A,** Relative level of miR-145-5p in OSCC tissues and adjacent normal tissues. **B,** Relative level of miR-145-5p in OSCC cell lines (Fadu, SCC-25, CAL-27 and Tca8113) and homo sapiens tongue normal cells Hs 680. Tg. **C,** A negative correlation between expression levels of circ-ABCB10 and miR-145-5p. **D,** Dual-luciferase reporter gene assay showed relative luciferase activity in CAL-27 and Tca8113 cells co-transfected with miR-145-5p mimics/NC and pmirGLO-circ-ABCB10-WT/pmirGLO-circ-ABCB10-MUT.

of miRNA-145-5p (Figure 5B). Relative colony number and migratory cell number were reduced after the transfection of sh-circ-ABCB10#1, which were partially reversed by the miRNA-145-5p knockdown (Figures 5C, 5D).

Discussion

OSCC is a highly prevalent malignancy in the oral and maxillofacial region. It usually affects the tongue, cheek, gingiva, sacral, and maxillary *sinus*. Pathologically, OSCC is characterized as high malignancy, invasive rate, and lymphatic metastasis rate[1-3]. Due to malignant phenotypes, the prognosis of OSCC patients is unsatisfactory[4-6]. Therefore, it is urgent to develop effective therapeutic and prognostic approaches for OSCC[7]. Abundant RNAs related to RNA transcription are distributed in cells, including microRNAs, lncRNAs, piRNAs, siRNAs, scRNAs, etc. With in-depth researches, these functional RNAs are considered to be ceRNAs to interact with other molecules[8,9].

CircRNA derives from the cyclization of a precursor mRNA and therefore has many extraordinary properties compared to those of a linear RNA. First, it is ubiquitously expressed in nature and highly conserved. Second, circRNA is resistant to RNase due to the lacking poly tail, showing pronounced stability. Third, circRNA is deferentially expressed in tissues, exhibiting the tissue-specificity. Lastly, circRNA is highly enriched in exosomes, which have been verified in the previous experiments[10-13]. Due to the above characteristics, circRNA is a promising hallmark in the diagnosis and treatment of tumors[14,15]. In this paper, circ-ABCB10 was upregulated in OSCC tissues and cells. A higher level of circ-ABCB10 predicted worse tumor staging

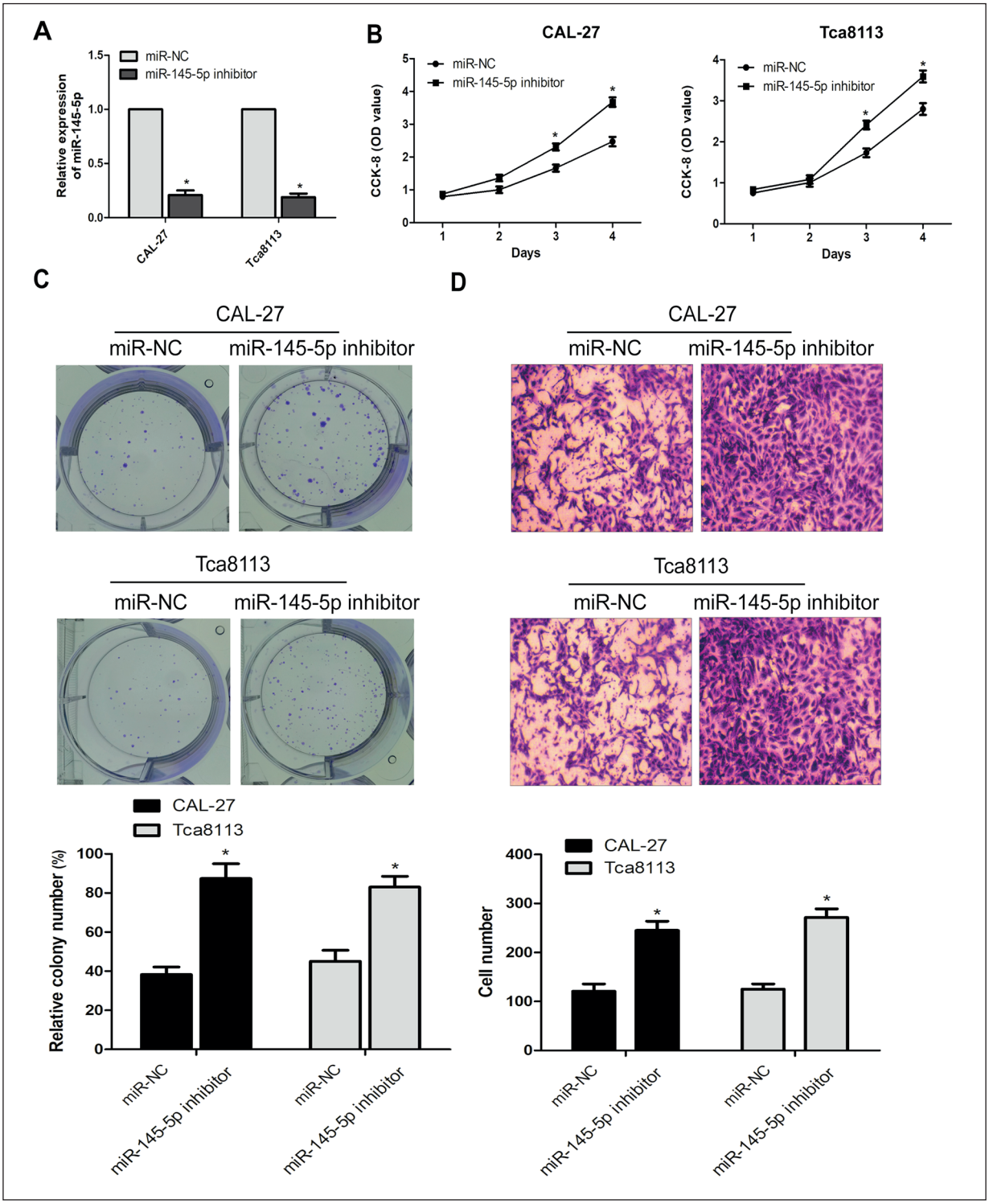

Figure 4. Downregulation of miR-145-5p promoted cell growth and migration. **A,** Transfection efficacy of miR-145-5p inhibitor in CAL-27 and Tca8113 cells. **B,** CCK-8 assay showed viability in CAL-27 and Tca8113 cells transfected with miR-NC or miR-145-5p inhibitor. **C,** Colony formation assay showed the colony number in CAL-27 and Tca8113 cells transfected with miR-NC or miR-145-5p inhibitor (magnification 10×). **D,** Transwell assay showed the migratory cell number in CAL-27 and Tca8113 cells transfected with miR-NC or miR-145-5p inhibitor (magnification 10×).

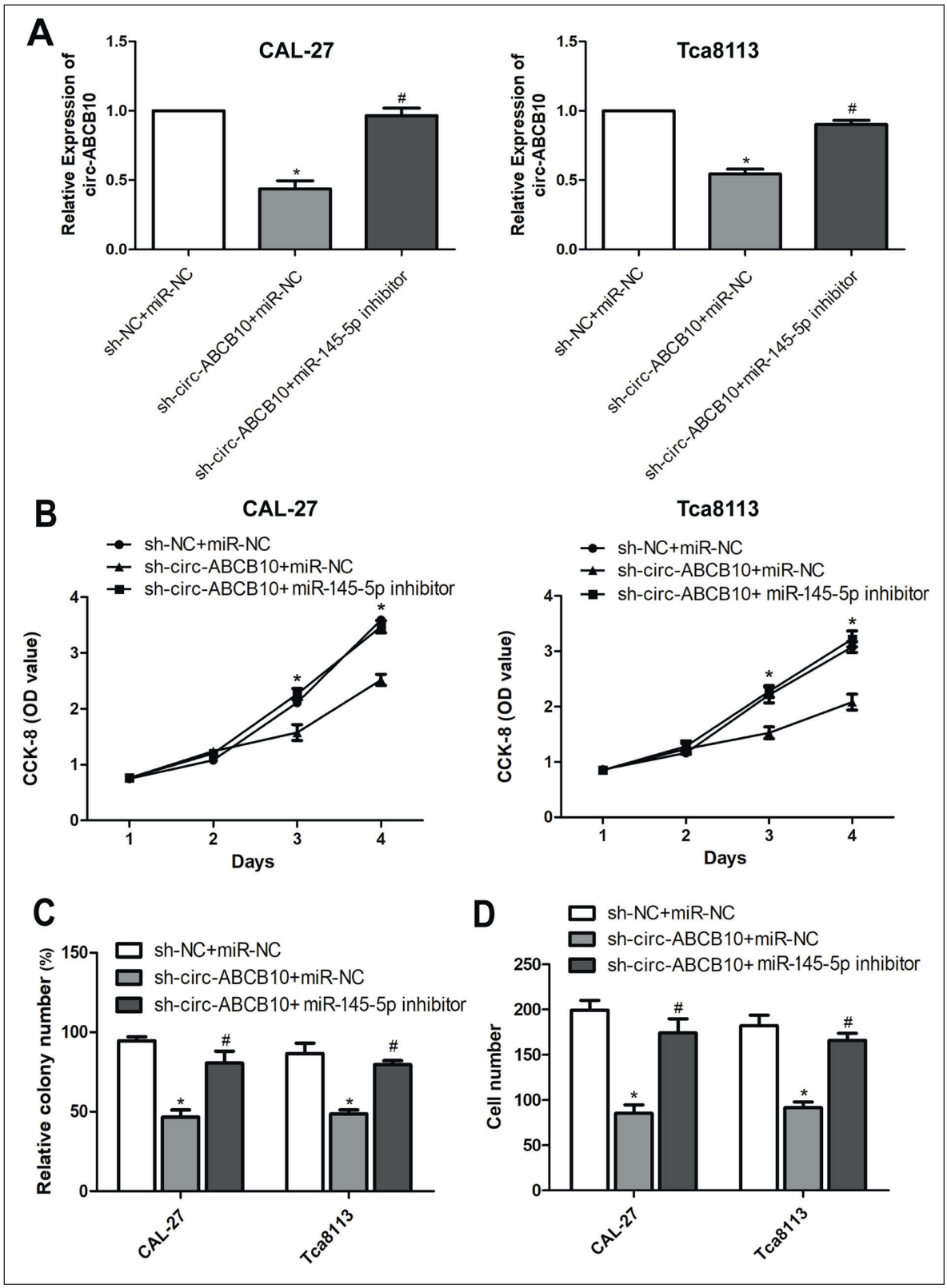

Figure 5. Circ-ABCB10 modulated OSCC cells by targeting miR-145-5p. CAL-27 and Tca8113 cells transfected with sh-NC + miR-NC, sh-circ-ABCB10#1 + miR-NC or sh-circ-ABCB10#1 + miR-145-5p inhibitor. **A,** Relative level of circ-ABCB10. **B,** CCK-8 assay showed the viability. **C,** Colony formation assay showed the colony number. **D,** Transwell assay showed the migratory cell number.

and a higher rate of distant metastasis in OSCC patients. Knockdown of circ-ABCB10 attenuated proliferative and migratory capacities if CAL-27 and Tca8113 cells.

CircRNA serves as a miRNA sponge to achieve its biological functions. In prostate cancer, circTCF25 influences the tumor progression *via* targeting miR-103a-3p/miR-107/CDK6 axis[18]. Upregulated circPVT1 in gastric cancer accelerates the proliferative rate of cancer cells by absorbing miR-125[19]. By sponging miR-145, has-circ001569 stimulates colorectal cancer cells to proliferate and invade by regulating the downstream genes E2F5, BAG4, and FMNL2[20]. Our study verified the direct binding in the promoter region of circ-ABCB10 and miRNA-145-5p through dual-luciferase reporter gene assay. Subsequently, a negative correlation between expression levels of them was identified as Chi-square test uncovered. Consistent with circ-ABCB10, miRNA-145-5p was downregulated in OSCC tissues and cell lines. Notably, the miRNA-145-5p knockdown could reverse the regulatory effects of circ-ABCB10 on viability, colony number, and migratory cell number of OSCC cells. As a result, a positive regulatory loop circ-ABCB10/miRNA-145-5p was determined, which aggravated the progression of OSCC.

Conclusions

Briefly, circ-ABCB10 was upregulated in OSCC and closely regulated to tumor staging and distant metastasis of OSCC patients. Circ-ABCB10 aggravated the progression of OSCC by absorbing miRNA-145-5p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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