CircRNA_002178 promotes the proliferation and migration of oral squamous cell carcinoma cells by activating the Akt/mTOR pathway

Y. YANG[1], H.-S. CI[2], Y.-L. MAO[1], J.-W. LI[1], J.-H. ZUO[1]

[1]Department of Oral and Maxillofacial Surgery, Binzhou Medical University Hospital, Binzhou, China
[2]Department of Stomatology, Binzhou Medical University Hospital, Binzhou, China

Abstract. – OBJECTIVE: We aimed to investigate the biological effects of circRNA_002178 in oral squamous cell carcinoma (OSCC) tissues and to analyze its potential mechanism.

PATIENTS AND METHODS: CircRNA_002178 expression in 50 pairs of OSCC tissues and adjacent ones was studied by quantitative polymerase chain reaction (qPCR) analysis, and the correlations of circRNA_002178 with clinicopathological indicators, as wells as prognosis of OSCC patients were analyzed. qPCR was used to verify circRNA_002178 expression in OSCC cell lines. Subsequently, circRNA_002178 knockdown models were constructed using lentivirus in OSCC cell lines, and the impacts of circRNA_002178 on the function of OSCC cells were assessed by cell counting kit-8 (CCK-8) test, plate cloning experiment, transwell assay and nude mouse tumor formation experiments. Finally, rescue experiments *in vitro* were used to explore the potential mechanism of circRNA_002178 activating Akt/mTOR pathway.

RESULTS: Our data showed that circRNA_002178 expression in OSCC tissue specimens was remarkably higher than that in adjacent ones. In comparison to patients in low circRNA_002178 expression group, patients in high expression group showed higher incidences of advanced pathological stage and distant metastasis, and a lower overall survival rate. Cell functional experiments revealed that knockdown of circRNA_002178 markedly attenuated the proliferation and migration ability of OSCC cells compared to the sh-NC group, and the consistent results were observed in the nude mouse experiment. In addition, Western blot suggested that the expression of key proteins in Akt/mTOR signaling pathway was remarkably reduced after downregulation of circRNA_002178 in OSCC cells. Meanwhile, Akt activator SC79 reversed the inhibitory effect of circRNA_002178 on the metastasis and proliferation of OSCC cells.

CONCLUSIONS: CircRNA_002178, over-expressed in OSCC tissues and cell lines, may promote the malignant progression of OSCC through activating the Akt/mTOR signaling pathway.

Key Words:
CircRNA_002178, Akt/mTOR pathway, OSCC, Proliferation, Migration.

Introduction

Oral squamous cell carcinoma (OSCC), a common type of oral cancer[1,2], has attracted great attention from oral researchers at home and abroad for its increasing incidence[1,2]. Although surgical and adjuvant treatments for OSCC have been remarkably improved with the development of medical and health technology, the overall prognosis is still poor compared with that of other malignant tumors[2-4]. So far, high incidence of metastasis and recurrence are still one of the key factors for its high mortality[4-6]. Therefore, finding new markers that can be used to predict the risk of OSCC and elucidating the signaling pathways related to tumor cell invasion and metastasis are very critical steps for the research and development of rational drugs for the treatment of advanced OSCC[7-10].

CircRNA is a class of circular RNA molecules without free 5 ‘and 3’ ends formed by reverse splicing of pre-mRNA. Reverse splicing is a completely different splicing method from classical splicing, and is composed of covalent bonds formed between the splicing sites at the downstream 5 ‘end and the upstream 3’ end[7-9]. At first, people did not understand the function of circRNA, and regarded it as the “noise” in gene transcription[11,12]. With the rapid development of bioinformatics technology and

Corresponding Author: Haosu Ci, MM; e-mail: bychs2010@126.com

high-throughput chip technology, circRNA has really entered this field of vision[12]. CircRNA is abundant in human cells, whose expression is dozens of times higher than that of linear isomers. CircRNA expression varies greatly in different tissues, different developmental stages, and different diseases, with specificity in tissue, developmental stage, disease and sex[13,14]. Considering the above characteristics, circRNA has great potential to become a new type of clinical diagnostic marker or therapeutic target[15]. Recently, circRNA has been found to be closely associated with various tumors[16-18]. However, as of now, there are not many reports on the relationship between circRNA and OSCC.

Hence, the aim of our study is to provide experimental evidence for the regulatory mechanism of circRNA_002178 in OSCC development, so as to provide new methods for the early diagnosis and treatment of this cancer.

Patients and Methods

Patients and OSCC Samples

50 patients with OSCC who underwent surgical treatment in our hospital were randomly collected. Inclusion criteria: each patient was diagnosed by pathological examination, no radiotherapy or chemotherapy was performed before surgery, and patients had no other major diseases or history of other tumors. Besides, important biochemical indicators of the body were in the normal range, which could be treated with surgery. Exclusion criteria: patients complicated with other malignancies, those with mental disease, those complicated with heart failure or other chronic diseases or those previously exposed to radioactive rays. Immediately after all tissue samples were placed in cryopreservation tubes, they were stored in liquid nitrogen for future use. Tumor staging was assessed based on the guideline proposed by the Union for International Cancer Control (UICC). In addition, this study was in line with the declaration of Helsinki and good clinical practice guidelines. This investigation was approved by the Ethics Committee of Binzhou Medical University Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Human OSCC cells (Fadu, SCC-25, CAL-27, Tca8113) and a normal human oral epithelial cell (Hs 680.Tg) purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C in an incubator with 5% CO_2.

Transfection

Lentiviral transfection was performed with sh-circRNA_002178 (GenePharma, Shanghai, China) when cell density reached 30%-50% according to the manufacturer's instructions. Cells were harvested 48 hours later for cell experiments.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) was performed based on the manufacturer's protocol.

Plate Formation Test

200 cells were seeded in each well of a 6-well plate and cultured with complete medium for 2 weeks. The medium was changed after one week and then twice a week. After 2 weeks, the cells were cloned and then fixed in 2 mL of methanol for 20 minutes. After the methanol was aspirated, the cells were stained with crystal violet and counted under a light-selective environment.

Transwell Assay

Cell migration was tested using a 24-well plate cell pre-coated with matrix gel according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ, USA).

Quantitative Polymerase Chain Reaction (qPCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissue samples. Complementary deoxyribose nucleic acid (cDNA) synthesis was accomplished using AMV reverse transcription kit. Next, qPCR was performed according to the instructions of SYBR® Premix Ex Taq ™ kit (TaKaRa, Otsu, Shiga, Japan), with β-actin as internal reference. The primers used in the qPCR were as follows: circRNA_002178 forward: 5'-CACTCCACTCCCATGTCCC-3', reverse: 5'-GCCTCTGGCCCTAGTCTCA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3', reverse: 5'-GTACTCAGCGGCCAGCATCG-3'.

Western Blot

OSCC cells were centrifuged at 4°C, 14000 × g for 15 minutes. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Finally, Western blot was carried out based on standard procedures.

Dual-Luciferase Assay

OSCC cell lines were co-transfected with reporter vector and the transcription factor expression plasmid to be tested. The Luciferase activity (Promega, Madison, WI, USA) of each group was measured after 48 h of transfection.

In Vivo Xenograft Model

This study was approved by the Animal Ethics Committee of Binzhou Medical University Animal Center. Nine 8-week-old male nude mice were purchased from the Animal Center and randomly divided into 3 groups (3 in each group). OSCC cells transfected with the circRNA_002178 knockdown vector and administered with AKT activator SC79 were injected subcutaneously into the armpits of mice, and tumor size was monitored every 7 days; then mice were sacrificed 6 weeks later. Tumor volume = $(\text{width})^2 \times \text{length} / 2$.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison among multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). The data are mean ± standard deviation, and $p<0.05$ was considered statistically significant.

Results

CircRNA_002178 Is Highly Expressed In OSCC

QPCR results indicated that circRNA_002178 showed a significantly higher expression in collected OSCC tissue samples than in paracancerous normal ones (Figure 1A). Meanwhile, *in vitro* cell qPCR detection also revealed an increased expression of circRNA_002178 in OSCC cells, especially in CAL-27 and SCC-25 cell lines, when compared to that in Hs 680.Tg (Figure 1B).

CircRNA_002178 Expression Is Closely Linked to the Pathological Stage, Distant Metastasis Incidence and Prognosis of OSCC Patients

We divided the 50 pairs of tissue samples collected from OSCC patients into high circRNA_002178 expression group and low expression group, and further explored the associations of circRNA_002178 with clinicopathological parameters and prognosis of OSCC patients by using Chi-square test. As shown in Table I, circRNA_002178 expression had correlations with distant metastasis incidence and pathological stage, but not with age, gender, and lymph node metastasis (Figure 1C). In addition, Kaplan-Meier survival curve showed that highly expressed circRNA_002178 exhibited great relevance to the poor prognosis of OSCC patients ($p<0.05$; Figure 1D). In addition, ROC curves were depicted for assessing the specificity and sensitivity of circRNA_002178 in OSCC. The calculated AUC indicated the prognostic value of circRNA_002178 on OSCC (AUC=0.873, 95%CI=0.793-0.976), revealing that circRNA_002178 is valuable in the diagnosis of OSCC.

Knockdown of CircRNA_002178 Remarkably Reduces the Proliferation and Migration of OSCC Cells

To explore the impact of circRNA_002178 on the proliferation rate and migration capacity of OSCC cells, we first constructed circRNA_002178 lentiviral knockdown vector in OSCC cells, and verified the transfection efficiency by qPCR (Figure 1E). Subsequently, both CCK-8 test (Figure 2A) and plate cloning experiments (Figure 2B) showed that knockdown of circRNA_002178 attenuated the proliferation ability of OSCC cells. Meanwhile, the migration ability of OSCC cells was also reduced after knocking down circRNA_002178, according to transwell assay (Figure 2C).

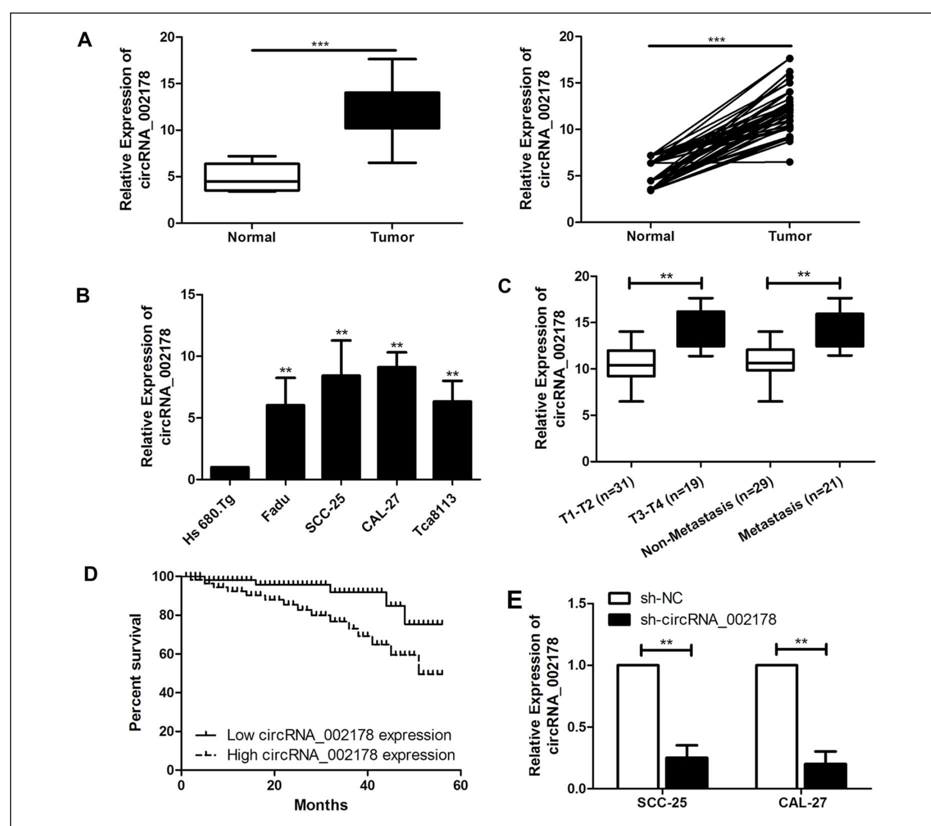

Figure 1. CircRNA_002178 is highly expressed in oral squamous cell carcinoma tissues and cell lines. **A,** Expression of circRNA_002178 in oral squamous cell carcinoma tissues and adjacent tissues detected via qPCR; **B,** Expression of circRNA_002178 in oral squamous cell carcinoma lines detected via qPCR; **C,** Different expressions of circRNA_002178 in oral squamous cell carcinoma tissues of patients with different pathological stages and distant metastases detected via qPCR; **D,** Kaplan Meier survival curve of oral squamous cell carcinoma patients based on circRNA_002178 expression; patients with a high expression had significantly worse conditions than those with a low expression; **E,** Interference efficiency of circRNA_002178 after transfection of circRNA_002178 knockdown vector in oral squamous cell carcinoma cell lines SCC-25 and CAL-27 verified *via* qPCR. Data are presented as average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table I. Association of circRNA_002178 expression with clinicopathologic characteristics of oral squamous cell carcinoma

Parameters	No. of cases	circRNA_002178 expression		*p*-value
		Low (%)	High (%)	
Age (years)				1.000
< 60	20	10	10	
≥ 60	30	15	15	
Gender				0.396
Male	25	14	11	
Female	25	11	14	
T stage				0.045
T1-T2	31	19	12	
T3-T4	19	6	13	
Lymph node metastasis				0.136
No	33	19	14	
Yes	17	6	11	
Distance metastasis				0.010
No	29	19	10	
Yes	21	6	15	

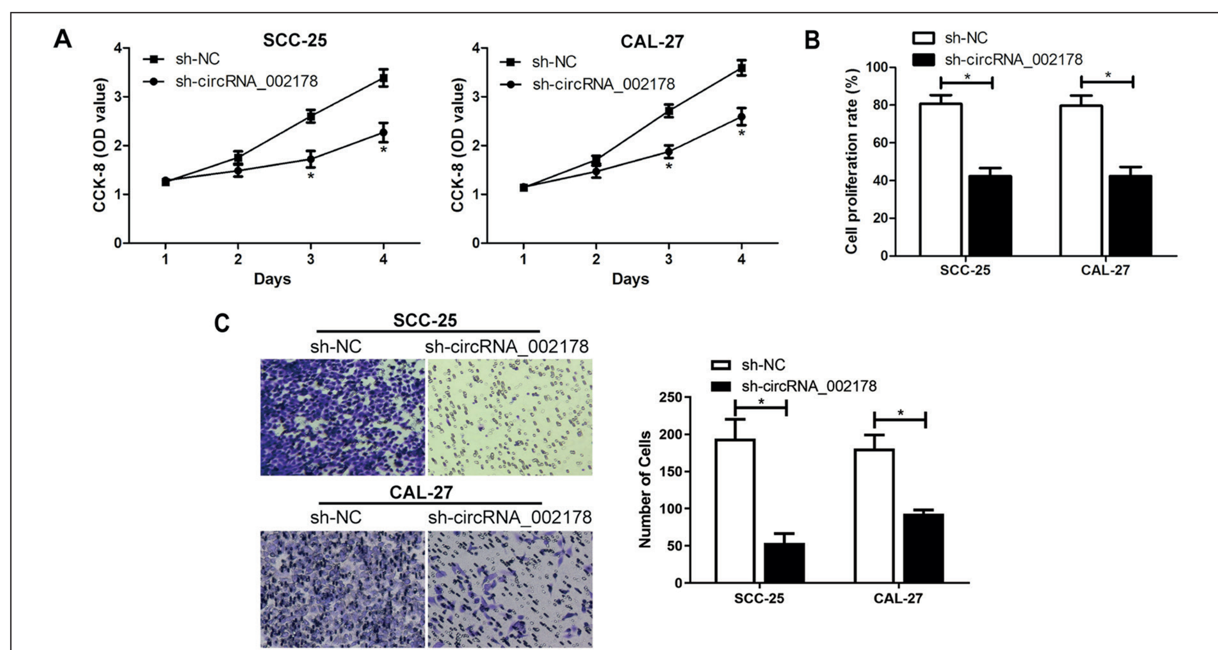

Figure 2. Silencing circRNA_002178 can inhibit the proliferation of oral squamous cell carcinoma. **A,** Proliferation rate of oral squamous cell carcinoma cells after transfecting circRNA_002178 knockdown vector in oral squamous cell carcinoma cell lines SCC-25 and CAL-27 detected via CCK-8 cell proliferation assay; **B,** Proliferation of oral squamous cell carcinoma cells after transfecting circRNA_002178 knockdown vector in oral squamous cell carcinoma cell lines SCC-25 and CAL-27 detected via Plate cloning assay; **C,** Migration ability of oral squamous cell carcinoma cells after transfecting circRNA_002178 knockdown vector in oral squamous cell carcinoma cell lines SCC-25 and CAL-27 detected *via* transwell migration assay (magnification: 40 ×). Data are presented as average ± SD, * $p<0.05$.

Knockdown of CircRNA_002178 Significantly Deactivates Akt Signaling Pathway In OSCC Cells

Western blot assay was implemented to examine the influence of circRNA_002178 on the expression of proteins in Akt signaling pathway. The results suggested that the protein expression of p-AKT in OSCC cells after knockdown of circRNA_002178 was markedly reduced (Figure 3).

AKT Activator Reverses the Inhibitory Effect of sh-circRNA_002178 on the Malignant Progression of OSCC

To test the interaction between circRNA_002178 and AKT signaling pathway in OSCC cell lines, AKT activator SC79 was added in cells transfected with circRNA_002178 knockdown vector. Western blot revealed that administration of SC79 markedly enhanced

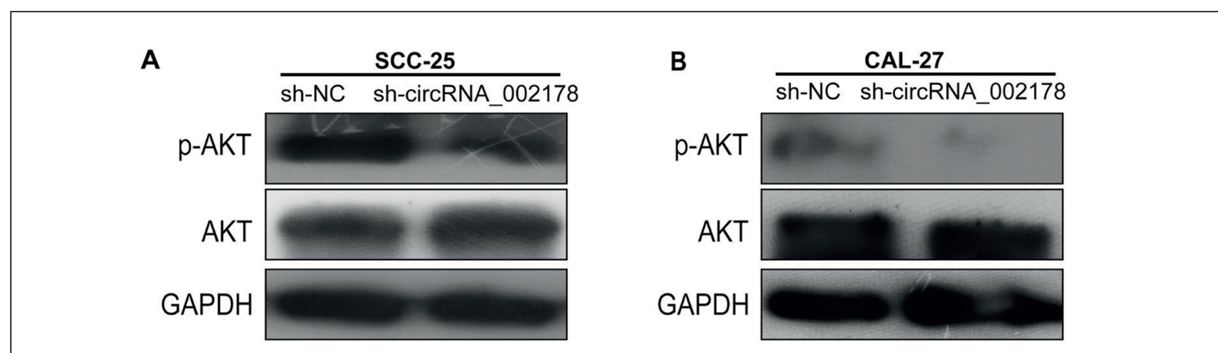

Figure 3. The Akt/mTOR signaling pathway in oral squamous carcinoma cells is significantly reduced after silencing circRNA_002178. The Western blot test results showed that the expression of p-AKT Akt/mTOR signaling pathway in oral squamous cell carcinoma cells was significantly reduced after circRNA_002178 was silenced.

AKT expression in comparison to sh-circRNA_002178 group (Figure 4A). Subsequently, CCK-8 (Figure 4B) and plate cloning experiment (Figure 4C) indicated that SC79 treatment significantly enhanced proliferation capacity of OSCC cells with circRNA_002178 knockdown; consistently, their migration capacity was also increased (Figure 4D).

CircRNA_002178 Promotes the Proliferation of OSCC Through Regulating AKT In Vivo

In vivo, OSCC cells transfected with the circRNA_002178 knockdown vector and administered with AKT activator SC79 were inoculated *in situ* into each nude mouse through injection into the left armpit. As expected, both tumor volume (Figure 5A) and tumor weight (Figure 5B) increased after administration of SC79 in OSCC cells with circRNA_002178 knockdown. Subsequently, the tumorigenic tissue protein and RNA of nude mice were extracted, and circRNA_002178 expression showed a significant elevation after treatment of SC79 (Figure 5C). At the same time, immunohistochemical experiments indicated that SC79 enhanced Ki-67 expression in tumorigenic tissue of nude mice significantly *in vivo* (Figure 5D).

Discussion

Tumor proliferation and metastasis are complex processes involving multiple signal transduction pathways with multiple steps, which are regulated by a variety of factors[19,20]. The potential of tumor proliferation and metastasis depends on the interaction between tumor cells and internal environmental factors that promote the growth, survival, angiogenesis, and metastasis of tumor cells[20,21]. OSCC is one of the most common malignant oral and maxillofacial head and neck tumors, with biological behaviors such as strong invasion, fast growth and easy metastasis to cervical lymph nodes[1-4]. The mechanisms of the occurrence and development of oral squamous cell carcinoma remain unknown to a great extent, making biological treatments including gene

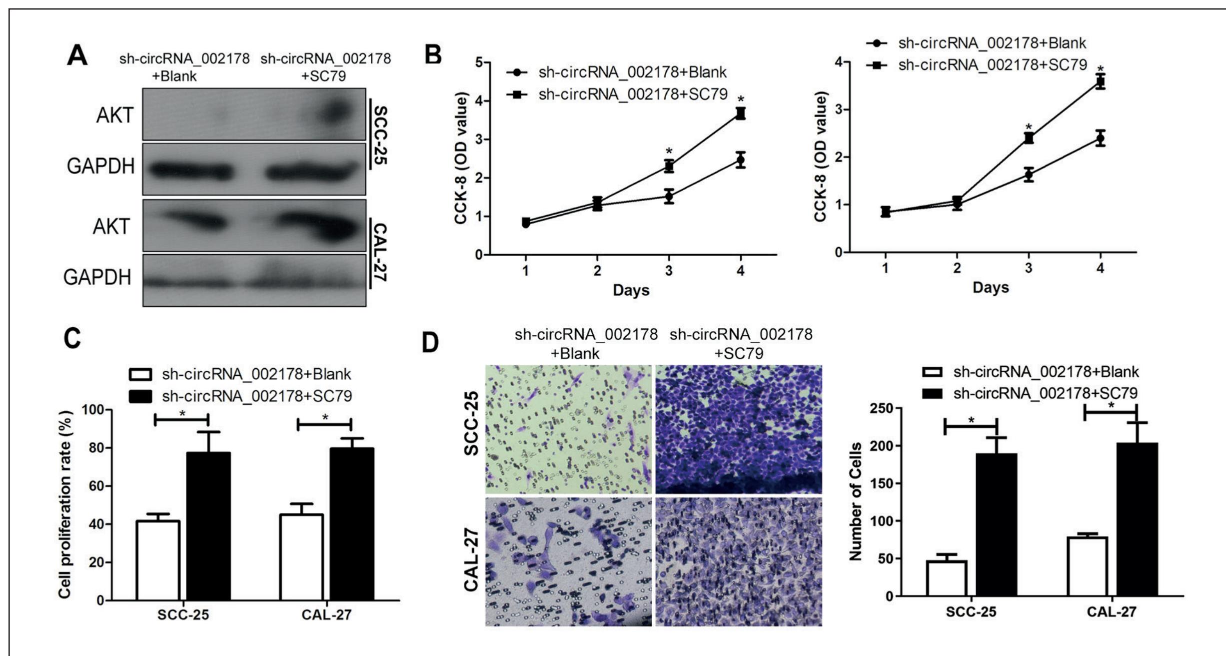

Figure 4. AKT activator can reverse the inhibitory effect of silenced circRNA_002178 on the malignant progression of oral squamous cell carcinoma. **A,** Expression of AKT after transfection of circRNA_002178 knockdown vector and administration of AKT activator SC79 in SCC-25 and CAL-27 validated via Western blot; **B,** Effects of circRNA_002178 knockdown vector and AKT activator SC79 on the proliferation rate of oral squamous cell carcinoma cells detected via CCK-8 cell proliferation assay; **C,** Effects of circRNA_002178 knockdown vector and AKT activator SC79 on the proliferation of oral squamous cell carcinoma cells detected via plate cloning test; **D,** Effects of circRNA_002178 knockdown vector and AKT activator SC79 on the migration ability of oral squamous cell carcinoma cells *via* detected transwell migration assay, (magnification: 20×). Data are presented as average ± SD, * $p<0.05$.

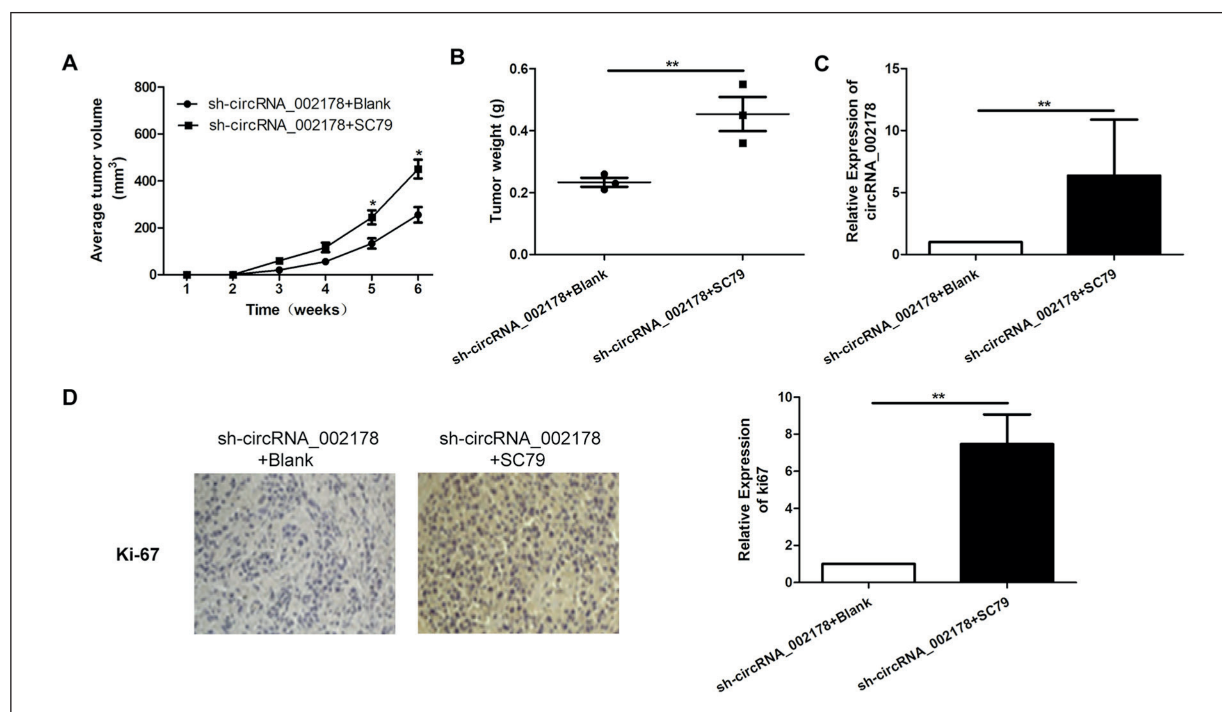

Figure 5. CircRNA_002178 promotes the proliferation of oral squamous cell carcinoma *in vivo via* regulating AKT. **A,** Tumor volume growth curves in different nude mouse groups after injection of circRNA_002178 knockdown vector and administration of AKT activator SC79; **B,** Tumor weights in different nude mouse groups after injection of circRNA_002178 knockdown vector and administration of AKT activator SC79. **C,** Level of circRNA_002178 in nude mice with oral squamous cell carcinoma after injection of circRNA_002178 knockdown vector and administration of AKT activator SC79 detected via qPCR; **D,** Level of Ki-67 in nude mice with oral squamous cell carcinoma after injection of circRNA_002178 knockdown vector and administration of AKT activator SC79 detected *via* immunohistochemical experiments, (magnification: 20×). Data are presented as average ± SD, * $p<0.05$, ** $p<0.01$.

therapy and targeted therapy still in the initial stage[4-6]. The formation of malignant tumors is often not caused by gene mutation itself, but is closely related to the process of gene expression modulation[5]. To study the regulatory mechanism of related genes in OSCC is of great significance to elucidate the pathogenesis of OSCC, and has clinical significance for the treatment and prognosis of OSCC[7-9].

CircRNA is a newly recognized special class of RNA molecules, most of which are formed by reverse splicing of more than one exon, and it is abundant in eukaryotic cells[10-13]. According to its source, it can be divided into exon-derived circRNA and intron-derived circRNA[14,15]. CircRNA is not easily degraded by nucleic acid exonuclease for its strong structural stability, so it can participate in the regulation of gene expression and thus plays an essential part in a variety of diseases[13-15]. The relationship between circRNA and disease is a major research focus at present. Specific circRNA is specifically highly or poorly expressed in specific tumors, and its expression level is directly related to the proliferation, migration, invasion and metastasis of tumor cells[16-18]. The oncogenic role of circRNA_002178 has been previously reported. However, the relationship between circRNA_002178 and OSCC is not clear. Therefore, the objective of this study was first to elucidate the oncogenic role of circRNA_002178 in the progression of OSCC, and its specific mechanism. In this study, circRNA_002178 expression was found remarkably up-regulated in OSCC tissue samples and cell lines, which showed close associations with distant metastasis incidence and pathological stage, suggesting that circRNA_002178 may serve an oncogene in the progression of OSCC. In addition, functional investigations *in vitro* revealed that downregulation of circRNA_002178 was able to enhance the migration ability and proliferation rate of OSCC cells. However, the exact molecular mechanism still remains unknown.

As we know, the function of ATK/mTOR signaling pathway is mainly to promote cell proliferation and suppress cell apoptosis, which plays a critical role in the prognosis of human malignant tumors[22]. ATK/mTOR signaling pathway is an important way to promote cell survival, proliferation, anti-apoptosis, angiogenesis and chemotherapy tolerance; it is involved in the development and angiogenesis of various tumors and is also considered as the primary pathway for cancer cell survival[22,23]. This study suggested that the expressions of p-AKT proteins in the Akt/mTOR signaling pathway in OSCC cells were markedly reduced after circRNA_002178 was downregulated. CircRNA_002178 is usually abnormally activated by the Akt/mTOR pathway, which plays a pivotal role in the progression of OSCC. To further explore the interaction between circRNA_002178 and Akt/mTOR signaling pathway, AKT activator SC79 was utilized to treat OSCC cells transfected with sh-circRNA_002178, which significantly enhanced AKT expression. Subsequently, the migration and proliferation capacity of OSCC cells was also enhanced after the administration of SC79. In addition, Ki-67 expression was remarkably increased in tumor-forming tissues of nude mice after SC79 was administered *in vivo*, indicating a significantly enhanced cell proliferation. The above findings suggest that circRNA_002178 may accelerate malignant progression of OSCC through ATK/mTOR signaling pathway.

Conclusions

In summary, circRNA_002178, overexpressed in the pathological OSCC tissues, promotes the metastasis and proliferation of OSCC cells by activating Akt/mTOR pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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