CircZNF236 facilitates malignant progression in oral squamous cell carcinoma by sequestering miR-145-5p

Qing Lu1 · Hongze Che2 · Yanhai Che3 · Min Hu4,5

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Abstract
Background  A number of non-coding circular RNAs (circRNAs) have recently been implicated in the modulation of gene expression in cancer models. We therefore sought to explore if circZNF236 has a role in oral squamous cell carcinoma (OSCC).

Methods  We first examined circZNF236 expression in 32 pairs of OSCC and noncancerous tissues. We then investigated a functional role for circZNF236 using knockdown and overexpression approaches in OSCC cancer cell lines. Cell counting kit-8, wound healing, Transwell, and flow cytometry were employed to assess circZNF236 function in vitro. The association between circZNF236 and miR-145-5p, or that between miR-145-5p and malignant brain tumor domain containing 1 (MBTD1) was predicted by bioinformatics and demonstrated by dual-luciferase reporter assays, RNA pull-down assays as well as RNA immunoprecipitation (RIP) assays. A mouse OSCC xenograft model was employed to demonstrate the impacts of circZNF236 inhibition on tumor development in vivo.

Results  OSCC tissues and cells had higher levels of circZNF236 expression compared with normal controls. Furthermore, high circZNF236 levels in patients with OSCC correlated with a poor prognosis. CircZNF236 silencing decreased the malignant properties of OSCC cells and suppressed OSCC tumor formation in the mouse model. We then noticed that miR-145-5p can be regulated by circZNF236, and that circZNF236 promoted OSCC development by absorbing miR-145-5p and consequently upregulating MBTD1 expression.

Conclusion  CircZNF236 modulates OSCC via the miR-145-5p/MBTD1 axis. These results support the potential of circZNF236 as a treatment target for OSCC.

Keywords  Circular RNAs · Oral squamous cell cancer · circZNF236 · miR-145-5p · MBTD1

Introduction

Oral squamous cell carcinoma (OSCC) possesses a high incidence and makes up 95% of head and neck cancer cases [1–3]. Despite its prevalence, there has been little improvement in OSCC outcomes over the last 30 years [4, 5]. Hence, there is an urgent need for new therapies to improve survival in patients with OSCC.

Circular RNAs (circRNAs), which do not encode proteins, are classified by a closed-loop structure [6–8]. CircRNAs have been implicated in proliferation, autophagy, apoptosis, as well as tumorigenesis and metastasis of cancers [9–12]. Indeed, a growing body of evidence has linked abnormal expression of circRNAs to the occurrence and development of many carcinomas. In osteosarcoma, high expression of circLRP6 promotes carcinogenesis by binding with LSD1 and EZH2 to suppress KLF2 and
APC gene expression [13]. Specifically, tumor growth in breast cancer has been correlated with the regulation of DNA methylase expression by circFECR1 [14]. A typical mechanism of action of circRNAs is to sponge microRNAs (miRNAs) [15–18]. In gastric cancer, circRNA_100782 downregulation is correlated with a decrease in a tumor suppressor gene, RB, via interaction with miR-574-3p, resulting in increased tumor cell proliferation and invasion [19]. Similarly, circ-RanGAP1 has been shown to promote gastric cancer invasion and metastasis by absorbing miR-877-3p [20]. In OSCC, circ_0000140 has been found to regulate the expression of CDC73 via miR-182-5p, and thereby inhibiting the proliferation, migration, and glycolytic metabolism of tumor cells [21]. We screened differentially expressed circRNAs in OSCC from DataSets (GSE131182) and found that circZNF236 was significantly overexpressed in OSCC cells. However, little is known regarding the functions of circZNF236 and no studies to date have examined the implication of circZNF236 in OSCC.

We explored the role of circZNF236 in OSCC. To this end, we performed tissue expression analysis, employed knockdown and overexpression cell models, and tested the role of circZNF236 in a xenograft model of OSCC. We demonstrate that circZNF236 expression is upregulated in OSCC tissues relative to paired normal tissues and is correlated with a worse OSCC patient prognosis. Furthermore, we found that circZNF236 promoted OSCC progression via binding and absorbing miR-145-5p, thereby enhancing the transcript level of malignant brain tumor domain containing 1 (MBTD1).

**Materials and methods**

**Clinical specimens**

Thirty-two pairs of tumorous and control tissues were collected from OSCC patients subjected to surgical resection in the Stomatological Hospital of Jilin University. Patients complied with the following criteria: (1) a pathologic diagnosis of OSCC; (2) no any other treatment before surgery; (3) no previous history of cancer and (4) no chemotherapy or radiotherapy. All samples were confirmed using histopathological examination. Immediately after removal, the tissue was flash frozen in liquid nitrogen and kept in a −80 °C freezer until being used in subsequent experiments. Our research was permitted and supervised by the Ethics Committee of Stomatological Hospital of Jilin University. The OSCC patients were treated in line with the Declaration of Helsinki and granted signed informed consent.

**Cell culturing and transfection**

The OSCC cell lines (namely SCC-9, HSC-3, SCC-15, CAL-27, and SCC-25) and human oral keratinocytes (HOK) were provided by the Shanghai Cell Bank (Chinese Academy of Sciences, China). All the OSCC cells were grown in Dulbecco’s Modified Eagle’s Medium containing fetal calf serum (10%) and working concentrations of penicillin and streptomycin under 37 °C, 5% CO2 conditions in a moisturized incubator. ShRNAs targeting circZNF236 (sh-circZNF236-1 and sh-circZNF236-2), miR-145-5p mimics and inhibitors, circZNF236-overexpression and MBTD1-overexpression plasmids and their negative controls were bought from GenePharma (China). For transient transfections, lipofectamine 2000 (Invitrogen, USA) was used as per the provided protocols.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

For isolation of total RNA and separation of nuclear and cytoplasmic RNA, the TRIzol reagent (TaKaRa, Japan) and the PARIS Kit (Invitrogen, USA) were employed, respectively. Reverse transcription was applied to obtain cDNA using the HiScript III RT SuperMix kit (Vazyme Biotech, China). PCRs were conducted with the BeyoFast SYBR Green qPCR Mix kit (Beyotime) in accordance with the manufacturer’s protocols on an ABI 7300 platform (Thermo Fisher Scientific, USA). Relative expression levels of target genes were computed with the $2^{-\Delta\Delta C_T}$ algorithm, with GAPDH and U6 genes serving as internal references. The sequences of the primers are listed in Table 1.

**RNase R digestion and actinomycin D treatment**

We used 2 mg/mL actinomycin D for transcription inhibition and an equal volume of dimethyl sulfoxide (Sigma-Aldrich, USA) for control. The RNase R digestion assay was performed as per the manufacturer’s protocol (GenePharma, China). The results of the RNase R digestion assay were analyzed by qRT-PCR.

| Name        | Sequence (5′→3′)           |
|-------------|---------------------------|
| circZNF236  | Forward: 5'-GGAAAGCCCGGAGAAACTGG-3'  |
|             | Reverse: 5'-AGCCGATATTGTCACCCGG-3'  |
| miR-145-5p  | Forward: 5'-CTGATGGTGGAGGCTCACA-3'  |
|             | Reverse: 5'-GTGCGGCCGAGGCTCACA-3'  |
| MBTD1       | Forward: 5'-CTACGCTCAGCCCAGATCA-3'  |
|             | Reverse: 5'-GAGGCGAGGCTCACA-3'      |
| GAPDH       | Forward: 5'-CTCAGCTCAGCCCAGATCA-3'  |
|             | Reverse: 5'-AGGCGAGGCTCACA-3'       |
| U6          | Forward: 5'-CTCAGCTCAGCCCAGATCA-3'  |
|             | Reverse: 5'-AGGCGAGGCTCACA-3'       |

Table 1 Sequence of primers in this study
USA) as the negative control. Total RNA extracted from CAL-27 and SCC-15 cell lines was incubated with 4 U/mg RNase R as per the manufacturer’s recommendations and analyzed by qRT-PCR assays.

**Fluorescence in situ hybridization (FISH)**

The probe of circZNF236 were designed and synthesized by RiboBio (China). The analysis was conducted with a FISH Kit (GENESEED, China) in accordance with the manufacturer’s protocols. The photographs were obtained under the Thermo Fisher microscope (Thermo Fisher Scientific, USA).

**Cell proliferation assay**

Proliferation of OSCC cell lines was analyzed with Cell Counting Kit-8 (CCK-8, Beyotime, China) following the included protocol. First, 2 × 10^4 cells were cultured for the indicated durations in each well of 96-well culture plates. Next, the cells were grown for 2 h in the presence of CCK-8 solution. The 450 nm optical density was recorded for each well using a microplate reader.

**Wound healing assay**

For wound healing assays, the OSCC cells were grown to 100% confluency in 6-well plates and scraped with the tip of a 200 μL pipette. The wells were imaged at 0 and 48 h after the scratching wound was created.

**Transwell invasion assay**

To investigate the invasive ability of the OSCC cells, a Matrigel Transwell system (Corning, USA) was applied. Briefly, the cells were grown in 200 μL of serum-free medium in the upper chambers. The lower chambers of the Matrigel Transwell system were added with 750 μL of DMEM containing 10% FBS. After 24 h, the number of cells that invaded into the lower chambers was determined by a light microscope (Olympus, Japan).

**Apoptosis assay**

A commercial kit from BD Pharmingen (BD Pharmingen, USA) was employed for evaluation of cell apoptosis. Briefly, cells were harvested 48 h after transient transfections and were resuspended in 200 μL binding buffer added with 5 μL each of Annexin V and PI. The mixture was incubated at ambient temperature for 15 min and subjected to flow cytometry analyses using a flow cytometer (FACSCalibur, BD Biosciences, USA).

**Dual luciferase assay**

Before transfection, 1 × 10^5 cells were inoculated in each well of 24-well plates. After culturing for 24 h, the cells were treated with miR-145-5p mimics and circZNF236-Wild Type (WT), circZNF236-Mutant (MUT), MBTD1-WT, or MBTD1-MUT reporter plasmid. Forty-eight hours after the transfections, the firefly and renilla luciferase activities were detected by applying the Dual-Luciferase® Reporter Assay System (Promega, USA).

**Xenograft model**

The experiments with mice were permitted and supervised by the Ethics Committee of the First Hospital of Jilin University. For establishment of xenograft mouse models, six 6-week-old nude BALB/c mice were evenly and randomly grouped. Three mice in one group were subcutaneously injected (right flank) with 1 × 10^6 CAL-27 cells expressing the negative control shRNA (sh-NC) or the shRNA against circ-ZNF236 (sh-circZNF236). The volumes of xenograft tumors, which were determined using the formula of tumor volume = 1/2 [length (mm) × width^2 (mm^2)], were measured once weekly for 5 weeks. Tumor weight was determined after the mice were sacrificed.

**RNA pulldown assay**

Biotin-labeled circZNF236 probe was allowed to bind with streptavidin-conjugated agarose beads for 2 h at 4 °C to obtain probe-coated beads. SCC-15 and CAL-27 cell lysates incubated with the probe-coated beads. The binding between miR-145-5p and the probe was evaluated by qRT-PCR.

**RNA immunoprecipitation (RIP) assay**

For RIP assays, we utilized a commercial kit from Millipore (Millipore, MA). Briefly, OSCC cell lysates were incubated with anti-IgG or anti-Ago2 antibody-labelled magnetic beads (Millipore, USA). The amounts of circZNF236 and miR-145-5p in the immunoprecipitates were finally quantified by qRT-PCR.

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad, USA). Data are expressed as mean ± standard deviation (SD) of at least three experimental replicates. For comparison of data, Student’s t test (for data from two groups) or one-way analysis of variance (for data from more than two groups) was implemented. *P* values
of less than 0.05 were indicative of statistical significance. Kaplan–Meier testing was performed to establish overall survival curves.

**Results**

**CircZNF236 was upregulated in OSCC and correlated with clinical outcomes**

Using qRT-PCR, it was found that circZNF236 was upregulated in OSCC relative to the paired noncancerous tissues (Fig. 1A). Increased levels of circZNF236 were also observed in the five investigated OSCC cell lines as compared with HOK (Fig. 1B). Among the five investigated OSCC cell lines, SCC-15 and CAL-27 were chosen for...
subsequent analyses as they exhibited the highest levels of circZNF236. To confirm the stability of circZNF236, OSCC cells were cultured with actinomycin D for 24 h to inhibit transcription. After the treatment, relative ZNF236 expression was reduced, while there was almost no decrease in circZNF236 expression (Fig. 1C, D). To confirm the circular nature of circZNF236, total RNA from OSCC cell lines was treated with RNase R. CircZNF236 expression was not significantly altered after the treatment, whereas ZNF236 expression was dramatically decreased relative to the mock treatment group (Fig. 1E). FISH and PCR analysis of circZNF236 distribution within the OSCC cell lines suggested that circZNF236 mostly localized to the cytoplasm (Fig. 1F, G). Kaplan–Meier analysis verified that higher circZNF236 expression was correlated with worse survival outcomes in OSCC patients (Fig. 1H). Overall, our findings indicated that upregulated circZNF236 is related to worse clinical outcomes in OSCC.

CircZNF236 knockdown suppressed malignant biological behaviors

Next, circZNF236’s role in OSCC was investigated using cell function assays. The efficiency of knockdown or overexpression of circZNF236 was verified in transfected OSCC cells by qRT-PCR (Fig. 2A, B). The results of cell proliferation assays suggested that knockdown of circZNF236 significantly decreased the proliferative capacity of OSCC expression was correlated with worse survival outcomes in OSCC patients (Fig. 1H). Overall, our findings indicated that upregulated circZNF236 is related to worse clinical outcomes in OSCC.

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cells (Fig. 2C). OSCC cell migration was also suppressed by downregulation of circZNF236 (Fig. 2D). Furthermore, Transwell assays suggested that circZNF236 downregulation resulted in a lower invasive ability in CAL-27 cell line (Fig. 2E). Flow cytometry analysis also exhibited that circZNF236 knockdown increased OSCC cell apoptosis (Fig. 2F). Consistent with these findings, circZNF236 overexpression promoted the proliferative, migration and invasive properties of the OSCC cells and significantly reduced apoptosis in SCC-15 cells (Fig. 2C–F). Taken together, our findings indicate that modulation of circZNF236 is able to regulate the malignant properties of OSCC cells.

CircZNF236 directly absorbed miR-145-5p in OSCC cells

The location of circZNF236 indicated that circZNF236 might act as a “miRNA sponge”, a mechanism of action identified for many circRNAs. We predicted that miR-145-5p is a target of circZNF236 using Starbase, circInteractome and circbank (Fig. 3A). The levels of miR-145-5p in the OSCC tissues and five OSCC cell lines were notably lower than that in adjacent normal tissues and HOK (Fig. 3B, C). Pearson correlation analysis indicated that the expression levels of circZNF236 and miR-145-5p were

![Image](https://example.com/image.png)

**Fig. 3** CircZNF236 directly absorbed miR-145-5p. **A** MiR-145-5p interacted with circZNF236 were predicted by using Starbase, circInteractome and circbank. **B, C** MiR-145-5p expression levels in OSCC tissues and OSCC cell lines. **D** Pearson correlation analyses between the expression levels of circZNF236 and miR-145-5p. **E** Expression levels of miR-145-5p in OSCC cell lines after transfection with circZNF236 or sh-circZNF236. **F** Putative interaction sites between circZNF236 and miR-145-5p. **G** Luciferase activity assays in OSCC cells that expressed circZNF236-WT or circZNF236-MUT and miR-145-5p mimics. **H, I** RNA pulldown and RIP assays to verify the interaction between circZNF236 and miR-145-5p in OSCC cell lines. *p* values < 0.05, < 0.01 and < 0.001 are represented by *, **, and ***, respectively.
inversely correlated in OSCC tissue samples (Fig. 3D). Furthermore, miR-145-5p expression was enhanced upon circZNF236 silencing, while circZNF236 overexpression repressed miR-145-5p levels in OSCC cell lines (Fig. 3E). The putative binding sites between circZNF236-WT or circZNF236-MUT 3'UTR and miR-145-5p are exhibited by Fig. 3F. This interaction was probed using a dual luciferase reporter assay. We observed a reduced luciferase activity in the circZNF236-WT + miR-145-5p mimics group, while no obvious effect was observed in circZNF236-MUT + miR-145-5p mimics group (Fig. 3G). In addition, RNA pull down using circZNF236 revealed its interaction with miR-145-5p that was not present in the control group (Fig. 3H). This interaction was also confirmed using an anti-Ago2 antibody to capture Ago2 protein and its bound RNAs, where circZNF236 co-precipitated with miR-145-5p (Fig. 3I). Collectively, we demonstrated that circZNF236 was able to bind miR-145-5p in SCC-15 and CAL-27 cells.

CircZNF236 regulated OSCC progression by absorbing miR-145-5p

To evaluate if circZNF236 modulated OSCC progression by absorbing miR-145-5p, we combined knockdown of circZNF236 with miR-145-5p inhibitors. In the presence of miR-145-5p inhibitors, circZNF236 knockdown-mediated effects on proliferation, invasion and migration of OSCC cells were significantly increased, while that on apoptosis was significantly inhibited (Fig. 4A–D).

MBTD1 acted as a molecular target of miR-145-5p

The targets of miR-145-5p were predicted through four online tools and 10 mRNAs (CSRNP2, FSCN1, ABHD17C, MYO5A, FLI1, ABRACL, MBTD1, BLOC1S6, SWAP70, TPT1) were picked out (Fig. 5A). RNA pull-down assay results showed that MBTD1 was
pulled down by miR-145-5p (Fig. 5B). We performed luciferase reporter assays and our data revealed that miR-145-5p mimics decreased the luciferase activity of MBTD1-WT, but did not significantly affect that of MBTD1-MUT (Fig. 5C, D). Furthermore, the expression of MBTD1 was reduced by miR-145-5p mimics but enhanced by anti-miR-145-5p (Fig. 5E). Results from qRT-PCR verified the increased MBTD1 expression in OSCC tissues and cells (Fig. 5F, G). In addition, expression of MBTD1 was inversely correlated with that of miR-145-5p (Fig. 5H). These findings revealed that miR-145-5p targeted MBTD1 in OSCC tissues and cells.

CircZNF236 promoted OSCC progression via the miR-145-5p/MBTD1 axis

Next, we examined whether circZNF236 promoted the malignant behaviors of OSCC cells via regulating MBTD1. We found that circZNF236 knockdown decreased MBTD1 transcript abundance; however, miR-145-5p inhibitors and MBTD1 overexpression rescued these effects (Fig. 6A).
addition, circZNF236 expression level was positively associated with that of MBTD1 in OSCC tissues (Fig. 6B). Furthermore, MBTD1 overexpression rescued the inhibitory effects of circZNF236 knockdown on OSCC cell proliferation (Fig. 6C). Overexpression of MBTD1 was also able to partially rescue migration and invasion inhibition effects caused by circZNF236 knockdown (Fig. 6D, E). MBTD1 overexpression also reversed the higher level of apoptosis in the circZNF236-silencing group (Fig. 6F).

**Knockdown of circZNF236 suppressed OSCC tumorigenesis in vivo**

The impacts of circZNF236 inhibition on the formation of OSCC xenograft tumor were further explored. OSCC cells that expressed sh-circZNF236 or sh-NC were injected subcutaneously into BALB/c mice. Five weeks later, the OSCC tissues were collected. We found that the size and weight of xenograft tumors were decreased by circZNF236 silencing (Fig. 7A–C). In addition, circZNF236 silencing could induce miR-145-5p expression and decrease that of MBTD1 in vivo (Fig. 7D). These results revealed that circZNF236 silencing could restrain OSCC tumor growth in vivo.

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**Fig. 6** CircZNF236 promoted OSCC progression through the miR-145-5p/MBTD1 axis. **A** The transcript abundance of MBTD1 in CAL-27 expressing anti-miR-145-5p or pcDNA3.1-MBTD1 plasmids. **B** The correlation between the expression levels of circZNF236 and MBTD1 in OSCC tissues. **C** Proliferation of OSCC cell lines co-transfected with sh-circZNF236 or pcDNA3.1-MBTD1. **D** Wound healing and **E** Transwell experiments. **F** Effects of circZNF236 knockdown and MBTD1 overexpression on apoptosis of the OSCC cell lines as revealed by our flow cytometry analyses. p values <0.05, <0.01 and <0.001 are shown by *, **, and ***, respectively.
Discussion

As a newly identified type of ncRNA, circRNAs have been implicated in the development of various cancers including OSCC. For instance, circMEMO1 can regulate hepatocellular carcinoma progression through modulating the promoter methylation and gene expression of TCF21 [22]. Circ_0021205 binds miR-204-5p to regulate the expression of RAB22A to stimulate the proliferation, invasion, and migration of cholangiocarcinoma cells [23]. In this study, we found that circZNF236 was expressed at a higher level in OSCC tissues relative to paired normal tissues. Further, our in vitro and in vivo findings indicated that circZNF236 functioned as a pro-oncogene in OSCC.

It has been demonstrated that miR-145-5p functions as a tumor inhibiting factor in multiple types of cancers. According to previous research, miR-145-5p can suppress the invasion, migration, and metastasis of hepatocellular carcinoma cells via regulating ARF6 [24]. In gastric cancer cells, miR-145-5p overexpression led to restrained viability, proliferation, and promoted apoptosis [25]. Furthermore, miR-145-5p can restrain malignant behaviors of esophageal cancer cells via regulating ABRACL [26]. In this study, Starbase, circInteractome and circbank were applied to identify the interaction sites between miR-145-5p and circZNF236. Our luciferase assays showed that miR-145-5p targeted the 3′-UTR of circZNF236, and increased expression of miR-145-5p could partially attenuate the malignant behaviors observed in OSCC cell lines.
Furthermore, we found that circZNF236 was able to absorb miR-145-5p to increase the expression of MBTD1. In line with this observation, MBTD1 expression has been shown to be enhanced in prostate cancer tissues and is associated with aggressive clinicopathological characteristics [27]. Indeed, we found that increased expression of MBTD1 could rescue the inhibitory effects of circZNF236 silencing on malignant properties of OSCC cells. Moreover, MBTD1 could rescue the inhibitory effects of circZNF236 silencing [27]. Indeed, we found that increased expression of MBTD1 associated with aggressive clinicopathological characteristics.

Taken together, our results indicate that circZNF236 upregulation in OSCC facilitates tumorigenesis through dysregulation of the miR-145-5p/MBTD1 axis (Fig. 8). These findings suggest that circZNF236 can be a novel target for OSCC therapy.

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Author contributions QL and MH conceived the experiments. YC and HC performed the experiments. QL wrote the article. All authors made contributions to the study and approved publication of this work.

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Data availability Additional data can be obtained from the corresponding author.

Declarations

Conflict of interest We declare no conflicting interests.

Ethical approval Our research was permitted and supervised by the Ethics Committee of Stomatological Hospital of Jilin University. The OSCC patients were treated in line with the Declaration of Helsinki and granted signed informed consent. The experiments with mice were permitted and supervised by the Ethics Committee of the First Hospital of Jilin University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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