Circ_0014359 promotes oral squamous cell carcinoma progression by sponging miR-149

Xubin Chen1, Jingxin Chen1✉, Liangbin Fu, Jimin Chen2, Yang Chen2 and Feng Liu3

1Department of Oral and Maxillofacial Surgery, Hainan General Hospital (Hainan Affiliated Hospital of Hainan Medical University), Haikou, Hainan, China; 2Departments of Pathology, Hainan Province People’s Hospital, Haikou, Hainan, China; 3Department of Stomatology, Hunan Province People’s Hospital, Changsha, Hunan, China

This study aimed to explore the role and mechanism of circ_0014359 in the OSCC. We firstly investigated the expression levels of circ_0014359 in OSCC tissues and cell lines. Then, the effects of knocking down circ_0014359 on cellular viability, apoptosis, migration, and invasion of OSCC cell lines were observed by cell counting kit-8 assay, flow cytometry, and transwell assay. Xenografts mouse model was established to explore the in vivo effect of circ_0014359 on the tumor volume and size of OSCC. We found that circ_0014359 was highly expressed in the OSCC tissues and cell lines compared to the normal controls (P<0.05). The expression of circ_0014359 was associated with the survival of patients (P<0.05). For the OSCC cell lines, circ_0014359 knock down induced apoptosis and inhibited migration, invasion, and epithelial-mesenchymal transition of OSCC cells (P<0.001). In vivo, silencing the circ_0014359 blocked the growth of OSCC tumors. The circ_0014359 can directly interact with the micro-RNA-149 (miR-149). Inhibition of miR-149 can rescue the inhibitory effects of knockdown or overexpression of circ_0014359 on the proliferation, migration, invasion, and epithelial-mesenchymal transition of OSCC cells. The circ_0014359-miR-149 pathway may be a novel target for developing strategies for the diagnosis and treatment of OSCC.

Key words: circ_0014359, micro-RNA-149, oral squamous cell carcinoma, proliferation, migration

INTRODUCTION

Head and neck cancer is one of the leading causes of cancer-related deaths worldwide. It was estimated that more than 450,000 patients died due to head and neck cancer every year (Ferlay et al., 2019). About 90% of the head and neck cancers are squamous cell carcinomas (Wyss et al., 2013), which originate from the oral, pharyngeal, or laryngeal mucosal epithelium. Oral squamous cell carcinoma (OSCC) is characterized by strong local invasion ability, prone to neck lymphatic metastasis, and high recurrence rate, of which patients have poor clinical prognosis (Zhang et al., 2018). So far, strategies for the treatment of OSCC include surgical resection, radiotherapy, chemotherapy, as well as targeted therapy (Cramer et al., 2019). Although the last few years witnessed the advances of multidisciplinary treatment for the OSCC, the long-term survival of patients is still low (Holsinger et al., 2015; Nor, et al., 2018). The main obstacle hampering the development of satisfied strategy to OSCC is the limited understanding of the complex mechanisms of tumor genesis and metastasis. Therefore, it is in urgent need to clarify the genetic and molecular mechanisms underlying the proliferation, invasion, and metastasis of these cancer cells.

Circular RNA (CircRNA) is a class of newly discovered non-coding RNA generated by back-splicing of pre-mRNA (Chen 2020). The absence of 5'-cap and 3'-poly A tail results in increased stability of these RNAs, in which 3', 5'-phosphodiester bond was formed between the spliced exons (Meng et al., 2017). Accumulating evidences indicated the importance of circRNA in the generation and progression of malignant tumors (Cheng et al., 2021). The abnormal expression of circRNA was considered to be related to many cancers and may be used as important target molecules and promising biomarkers for the diagnosis and treatment of various cancers (Wei et al., 2021). Recently, a novel circRNA, circ_0014359, was found to be highly expressed in glioma (Shi et al., 2019). The circ_0014359 was a micro-RNA (miRNA) -153 sponge and promoted the tumor progression through phosphoinositol 3 kinase (PI3K) pathway. However, the role and mechanisms of circ_0014359 in other cancers including OSCC are largely unknown.

In order to clarify the regulatory role and mechanisms of circ_0014359 in OSCC, we used in vitro and in vivo methods to systemically investigate the effects of knockdown or overexpression of circ_0014359 on the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of OSCC cell lines. We hope to provide evidence for exploring the potential biomarker of targeted therapy of OSCC.

MATERIALS AND METHODS

Specimen Source

A total of 30 pairs of tumor tissues and the adjacent normal tissues were collected from our hospital and stored at −80°C. All patients did not undergo chemotherapy or radiotherapy before the operation. They were followed up after surgery and the follow up period was from 3 to 54 months with a median of 29.5 months. This study was approved by the Ethics Committee of Hainan Provincial People’s Hospital (No.20-05-12). The informed consents were obtained from the patients. All procedures were in accordance with the Helsinki declaration.
Cell Culture

Human OSCC cell lines (PE/CA-PJ41, SCC15, SCC25, and HSC-4) and oral mucosa epithelium cell line (NHOK) were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/mL penicillin/streptomycin (Beyotime, China) at 37°C in an atmosphere humidified with 5% CO₂.

Cell Transfection

miR-149 mimic, miR-149 inhibitor, and their negative controls were provided by GenePharma (Shanghai, China). The sequence for silencing circ_0014359 (si-circ) and negative control (si-NC) were synthesized by General biocompany (Hefei, Anhui). Cells were transfected with Lipofectamine2000 according to manufacturer’s instructions. After 6 to 8 h of culture, changed the medium to complete medium, and continued to culture for 48 h for subsequent experiments.

CCK-8 assay

Cells were cultured in 96-well plates. Then, cell viability was assessed at 48 h by adding 10 μL of Cell Counting Kit-8 (CCK-8) solution (C0037, Beyotime, Haimen, China) to each well and quantifying the absorbance at 450 nm. After 2 h incubation, a microplate reader from Thermo Fisher Scientific (formerly Fermentas, Schwerte, Germany) was used to monitor the results.

Apoptosis assay

Cell apoptosis was detected by using an Annexin V-fluorescein isothiocyanate propidium iodide (FITC/PI) apoptosis detection kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Cells were harvested and washed with 1x phosphate buffered saline twice. Cells were then incubated with Annexin V_FITC and PI at room temperature for 15 minutes in the dark. The cell apoptosis was analyzed by a FACSscan flow cytometer (BD Biosciences, Franklin Lakes, USA).

Transwell assay

First, the upper chamber was pre-coated with or without matrigel (BD, USA). After the matrigel was solidified, transfected PECAPJ41 and HSC-4 cells were seeded on the upper chamber (Millipore, MA, USA) and cultured in 200 μL serum-free DMEM. DMEM containing 12% FBS was added to the lower chamber. Cells were incubated at 37°C. 24 h later, cells migrated or invaded to the lower surface were fixed with methanol, followed by staining with 0.1% crystal violet at room temperature for 20 min. Finally, the migrated or invaded cells were imaged under an optical microscope (Nikon, Japan).

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

After extracting total RNA with Trizol reagent (Invitrogen, USA), 5 μg of RNA was reverse transcribed into cDNA with a reverse transcription kit (Takara, Dalian, China) according to the instructions. qRT-PCR reaction was carried out with Power SYBR-Green PCR Master mix (Thermo Fisher Scientific, USA) on a FAST7500 realtime-PCR system (ABI, USA). The PCR was performed under the following reaction conditions: pre-denaturation at 96°C for 5min, 30 cycles of denaturation at 96°C for 30s, annealing at 54°C for 30s, extension at 72°C for 30s; finally, extension at 72°C was 10min long and stored at 4°C. The relative expression levels were calculated using the 2−ΔΔCt method. GAPDH and U6 were used as the internal control for the mRNA and miRNAs, respectively.

Western blot analysis

Protein Extraction kit (catalog no. NP2P-37853; Novus Biologicals LLC, Littleton, CO, USA) was used to extract total protein from the in vitro cultured cells. A bicinchoninic acid Protein Quantiﬁcation kit (catalog no. ab102536; Abcam, Cambridge, UK) was used to determine protein concentrations with all steps performed in strict accordance with the manufacturer’s protocol. Protein samples were denatured and subjected to SDS-PAGE. Following protein transfer onto polyvinylidene difluoride membranes, membranes were blocked in 5% fat-free milk in PBS at room temperature for 2 h. The primary antibodies E-cadherin (ab40772, 1:10000), Snail (ab216347, 1:1000), Vimentin (ab92547, 1:1000) and GAPDH (ab8245, 1:5000) were purchased from Abcom Company and the incubation was performed at 4°C for 15 h. A secondary incubation was performed at 24°C for 2 h using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (ab6721, 1:5000). An ECL™ Western Blotting Analysis system (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to develop signals. Finally, ImageJ software (version 146; National Institutes of Health, Bethesda, MD, USA) was used to normalize the data.

Dual Luciferase Reporter Gene Experiment

Bioinformatics software was used to predict the target genes of circ_0014359. The wild-type and mutant sequence containing the binding region of miR-149 were synthesized and cloned into the pGL3.1 reporter plasmid. The wild-type and mutant plasmid along with miR-149 mimic and its negative control were transfected into OSCC cells. After 48 h, the luciferase activity was determined by a dual luciferase detection kit (Promega, USA). Firefly luciferase activity was normalized to Renilla.

RNA Pull Down

Biotin labeled miR-149 probe, or the negative control were obtained from Sangon Biotech (CA, USA). Cells were transfected with specific probes for 48 h. The transfected cells were harvested and lysed. Then, the lysates were incubated with pre-coated beads overnight. RNA enrichment was evaluated by qPCR assay.

Xenografts Model

Six-week-old male nude mice (20 to 25g) used for the in vivo study were provided by Shanghai SLRC Experimental Animal Center (Shanghai, China). The mice were injected subcutaneously with 5×10⁶ OSCC cells per mouse. Tumor size was measured every 3 days, volume=length (mm)×width (mm)²×0.52. At the end of the experiment, the mice were sacrificed, and the tumors were removed, imaged, and weighed. The present study was approved by our hospital.

Statistical analysis

Data were analyzed by GraphPad Prism 6 (La Jolla, CA, USA). Data were subjected to normality test before further analysis. Data with normal distribution were
shown as mean ± SD. One way or two-way ANOVA as well as Student’s t-test were used to analyze differences between groups, followed by Post-Hoc Test (Least Significant Difference, LSD). Significant difference was defined as \( P < 0.05 \).

RESULTS

Circ_0014359 is highly expressed in the OSCC tissues and cell lines and relates to the survival of patients

In order to clarify the role of circ_0014359 in OSCC, we firstly compared the expression levels of circ_0014359 in OSCC tissues and adjacent normal tissues, as well as OSCC cell lines SCC15, SCC25, PECAJ41, HSC-4, and mucosa epithelium cell line NHOK. The qRT-PCR results showed that the expression of circ_0014359 was significantly higher in the OSCC tissues than that of normal tissues \((P<0.001, \text{Fig. 1A})\). We further divided the OSCC patients into two groups according to the median expression levels of circ_0014359 and compared the survival of patients between the two groups. Consistently, the survival of patients with low circ_0014359 expression was significantly shorter than those with high circ_0014359 levels \((P<0.05, \text{Fig. 1B})\). In addition, the OSCC cell lines SCC15, SCC25, PECAJ41, and HSC-4 were shown with significantly higher expressions of circ_0014359 compared with that of NHOK cells \((P<0.001, \text{Fig. 1C})\).

Circ_001439 knock down induces apoptosis and inhibited migration, invasion and EMT of OSCC cells

Given the abnormal expression of circ_0014359 in OSCC tissues and cell lines, we further investigated the effects of circ_0014359 knock down on the OSCC cell lines. Transfection of vectors carrying si-circ_0014359 successfully inhibited the expression of circ_0014359 in the PECAJ41 cells and HSC-4 cells \((P<0.001, \text{Fig. 2A})\). Interestingly, the viability of OSCC cells was significantly decreased after knock down of circ_0014359 \((P<0.001, \text{Fig. 2B})\). Results of flow cytometry confirmed that the cell apoptosis rates of PECAJ41 and HSC-4 cells were significantly increased in the si-circ_0014359 group compared with that of si-NC group and control group \((P<0.001, \text{Fig. 2C})\). To further explore whether the circ_0014359 is involved in the migration and invasion of OSCC cells, we applied the transwell test. The results showed that the ability of migration and invasion of OSCC cells were significantly decreased after silencing the circ_0014359 \((P<0.001, \text{Fig. 2D and E})\). Since EMT plays an important role in the tumor development, we further investigated the changes in protein markers of EMT after circ_0014359 knock down. The expressions of E-cadherin were increased while the expressions of snail and vimentin were decreased \((P<0.01, \text{Fig. 2F and G})\).
snail and vimentin were decreased in the group of cells with circ_0014359 silencing (P<0.001, Fig. 2F and G), indicating an increase of EMT.

**Circ_0014359 targets to miR-149**

In order to further clarify the target of circ_0014359, we firstly used bioinformatics software (https://circinteractome.nia.nih.gov) to predict the binding sites of miRNA. It was showed that circ_0014359 can bind to the miRNA-149 (Fig. 3A). Compared with the control group or mimic control group, simultaneous transfection of circ_0014359 and miR-149 mimic resulted in significant decline of luciferase activity in the PECAPJ41 cells (P<0.01, Fig. 3B) or HSC-4 cells (P<0.01, Fig. 3C). In addition, mutation of circ_001439 did not change the luciferase activity. These results suggested a direct interaction between circ_0014359 and miR-149. Furthermore, in the RNA-pull down assay, the circ_0014359 was significantly enriched by the miR-149 probe compared with the control probe (P<0.001, Fig. 3D). Since miR-149 was confirmed to be a target of circ_0014359, we further investigated whether the circ_0014359-miR-149 axis was involved in the OSCC. Consistent with our hypothesis, the expression of miR-149 was significantly decreased in the tumor tissues compared with the adjacent normal tissues (P<0.001, Fig. 3E). In addition, a negative correlation was found between the expressions of miR-149 and circ_0014359 (P<0.001, R^2=0.5141, Fig. 3F). In the OSCC cells, knock down of circ_0014359 increased the expression of miR-149. **P<0.01. ***P<0.001.

**MiR-149 inhibition rescues the effects of circ_0014359 silencing on OSCC cells**

To further confirm the role of circ_0014359-miR-149 pathway in the OSCC, we investigated the effects of miR-149 inhibition on OSCC cells after knock down of circ_0014359. Firstly, transfection of miR-149 inhibitor successfully decreased the expression of miR-149 in the PECAPJ41 cells and HSC-4 cells (P<0.01, Fig. 4A). Secondly, inhibition of miR-149 partially rescued the decline of cell viability induced by circ_0014359 silencing (P<0.01, Fig. 4B). Compared with the si-circ+miR-NC group, the cell apoptosis was also decreased in the OSCC cell lines (P<0.001, Fig. 4C). Thirdly, the migration rate (P<0.05, Fig. 4D), invasive rate (P<0.05, Fig. 4E), as well as ex-
expressions of E-cadherin, snail, and vimentin (P<0.05, Fig. 4F and G), were all reversed after inhibition of miR-149 in the OSCC cells with circ_0014359 knock down. ***P<0.001.

**Circ_0014359 knock down inhibits the tumor growth**

Finally, we conducted in vivo experiments to confirm the role of circ_0014359 in tumor growth. We established a xenograft mouse model using the HSC-4 cells with stable expression of si-circ_0014359 or circ-NC (Fig. 5A). The results showed that the tumor volume and weight were significantly lower in the mouse model with knock down of circ_0014359 compared with that of control group (P<0.001, Fig. 5B and C), further suggesting that inhibition of circ_0014359 blocked the growth of OSCC.

**DISCUSSION**

The present study provided new insights into the mechanisms of OSCC. We found that circ_0014359 was an essential molecule in the progression of OSCC. By sponging miR-149, the circ_0014359 enhanced the ability of migration, invasion, and EMT of OSCC cells, thereby promoting the OSCC development. The circ_0014359-miR-149 pathway may be a novel target for improving the strategies of treatment and prognosis of OSCC patients in clinical practices.

As mentioned above, head and neck squamous cell carcinomas brought great burden to the global health (Johnson et al., 2020). Treatment strategies for head and neck squamous cell carcinomas include surgery, radiotherapy, chemotherapy, systemic therapy, targeted therapy, as well as immunotherapy (Cramer et al., 2019). Even though there were rapid advances on the diversity of treatment methods for OSCC in recent years, the clinical outcomes of patients were unsatisfactory (Muzaffer et al., 2021). In order to develop novel effective therapeutic strategies for OSCC, it is of great importance to clarify the mechanisms underlying the generation and progression of OSCC at molecular and cellular levels.

In recent years, the role of circular RNA (a subset of non-coding RNA) in cancers has attracted the attention of researchers, including the field of OSCC (Fan et al., 2020). CircRNAs are evolutionarily conserved genetic molecules, which are widely expressed in various species, including human tissues (Dong et al., 2017; Rybak-Wolf et al., 2015). At present, about 15 000 kinds of circRNAs have been discovered expressing curve patterns with spatio-temporal specificity (Lei et al., 2019). It has been well established that abnormal expression of circRNA was associated with the generation, progression, and metastasis, which was considered as a stable biomarker of different tumors (Kulcheski et al., 2016; Zhang et al., 2018). As for OSCC, dozens of circRNAs were found to be differentially expressed in tumor tissues or cell lines (Zhao et al., 2018). Previous studies demonstrated that the expressions of circRNA_006740 (Wang et al., 2018), hsa_circ_0079755 (Wang et al., 2019) and hsa_circ_0008309 (Li et al., 2018) were significantly lower in patients with OSCC, and the levels of these circRNAs were related to the tumor differentiation. Similarly, the declines of hsa_circ_001242 (Sun et al., 2018) and hsa_circ_0072387 (Dou et al., 2019) expression in OSCC were associated with the tumor size and stages, as well as the prognosis of patients. Interestingly, a lot of studies explored the possibility of using circRNA levels to predict the diagnosis and treatment of OSCC, and the sensitivity and specificity were satisfactory (Li et al., 2020; Li et al., 2019). Therefore, the circRNAs are essential elements underlying the mechanisms of OSCC. A recent study confirmed, for the first time, that circ_0014359 played a role in the progression of glioma and may be used to predict the prognosis of patients (Shi et al., 2019). In this study, we found that circ_0014359 was highly expressed in OSCC tissues and related to the patient’s prognosis, suggesting a possible role of circ_0014359 in the pathogenesis of OSCC. The results of our study provided a new direction, which was targeting the circ_0014359, for the development of strategies for the diagnosis and treatment of OSCC.

The circRNA-miRNA pathways have been reported to play roles in the pathophysiology of OSCC. For example, circRNA_100553 (Mohamed et al., 2019), circRNA_100290 (Liu et al., 2019), and circ_0001439 (Li et al., 2020) were shown to sponge miRNA-933, miRNA-378a, and miRNA-31, respectively, and interfering with these pathways can inhibit the proliferation and invasion of OSCC cells thereby blocking the progression and metastasis of tumors. In order to further clarify the downstream target of circ_0014359, we used bioinformatics to predict the binding sites of circ_0014359. Consistent with the classic working model, the circ_0014359 was found to interact with miR-149. Previous studies revealed that miRNA-149 can inhibit the expression of transient receptor potential melastatin 7 and affect the activity of mitogen-activated protein kinases pathway, which was involved in the proliferation of airway smooth muscle cells (Zhu et al., 2020). Moreover, miR-149 was essential to maintain the neuronal viability and may be a promising target for the treatment of Alzheimer’s disease (Du et al., 2021). We found that inhibition of miR-149 can partly reverse the inhibitory effects of circ_0014359 knock down on the proliferation, migration, invasion, and EMT of OSCC cells, suggesting the involvement of circ_0014359-miRNA-149 axis
CONCLUSION

This study investigated the role of abnormal circ_0014359 expression in OSCC and further clarified the spliced miRNA of circ_0014359. The results showed that circ_0014359 was highly expressed in OSCC and was related to the poor prognosis of patients. Knock down of circ_0014359 expression in OSCC cells can induce cell apoptosis and inhibit migration, invasion, and EMT. Similarly, the results of the xenograft mouse model showed that silencing the circ_0014359 significantly inhibited tumor growth. MiR-149 was found to be a target of circ_0014359. Knockdown of circ_0014359 may inhibit the growth of OSCC cells by up-regulating miR-149. This study provided novel promising molecular targets for the diagnosis and treatment of OSCC.

REFERENCES

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