



Silencing circular RNA hsa_circ_0004491 promotes metastasis of oral squamous cell carcinoma

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ABSTRACT

Aims: Oral squamous cell carcinoma (OSCC) is an oral cavity malignancy which is the eighth most common cancer. However, the molecular mechanisms underlying the pathogenesis of OSCC remain largely unknown, and effective methods for the prognosis and diagnosis of this disease are lacking. Circular RNAs (circRNAs) are widely expressed among mammals. A growing number of studies have shown that circRNA expression is altered in cancers. In this study, we investigated the role of hsa_circ_0004491 in OSCC cell migration and invasion and examined the clinical characteristics associated with its expression.

Main methods: Hsa_circ_0004491 expression was examined in 40 paired OSCC and normal tissue samples using quantitative real-time PCR (qRT-PCR). Wound healing, transwell and western blotting assays were conducted in OSCC cells up- or down-regulation of hsa_circ_0004491 to evaluate changes in cell migration, invasion and protein expression.

Keyfindings: qRT-PCR analysis revealed that hsa_circ_0004491 was significantly downregulated in OSCC tissues compared with paired normal tissues ($P < 0.001$), and this low expression was associated with lymph node metastasis ($P = 0.0398$; area under ROC curve = 0.7510). Hsa_circ_000449 silencing or overexpression significantly affected the invasion and migration abilities of OSCC cells. Western blotting analysis showed that EMT-related proteins expression was significantly changed after hsa_circ_000449 silencing or overexpression.

Significance: The expression level of hsa_circ_0004491 affects the migration and invasion of OSCC cells. Hsa_circ_0004491 may therefore play a role in the progression of oral squamous cell carcinoma.

1. Introduction

Oral cancer is a malignant disease and the eighth most common cancer type, with 540,000 new cases diagnosed globally each year. It is the 15th most common cause of cancer mortality worldwide [1]. Oral squamous cell carcinoma (OSCC) accounts for more than 90% of malignant tumors of the oral cavity. The 5-year survival rate for early stage OSCC (stages I and II) is approximately 80%, but approaches 20% for late stage disease (stages III and IV) [2,3]. The pathogenesis of OSCC is complex and, despite many years of research, the molecular mechanisms driving this process remain unclear. The cells of OSCC tumors exhibit marked changes in their RNA and protein expression profile and such changes may highlight potential diagnostic markers or treatment targets for this disease.

Circular RNA (circRNA) is a special type of non-coding RNA.

CircRNA was first identified in 1976 in RNA viruses, and was initially considered to be a byproduct of RNA mis-splicing [4,5]. With the development of high-throughput sequencing technologies and bioinformatics, a large number of circRNAs have now been discovered in a variety of tissues. And there is a growing body of evidence supporting roles for circRNAs in multiple physiological and pathological processes, such as cell proliferation, differentiation and survival. Consequently, there has been renewed focus on circRNAs in the field of RNA research in recent years [6–8]. Unlike traditional linear RNA (with exposed 5' and 3' ends), circRNA molecules have a closed circular structure and are not affected by RNA exonucleases. They are more resistant to degradation than many other species of RNA and their expression is, therefore, more stable [9]. CircRNA molecules are typically rich in microRNA (miRNA) binding sites and they are thought to primarily act as miRNA sponge in cells [10], suppressing the inhibitory effects of

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miRNAs on target genes. One such example is the antisense strand of the cerebellar degeneration-related protein 1 transcript (CDR1), which contains more than 70 binding sites for miR-7 and functions as a miR-7 miRNA sponge [11]. This process of miRNA inhibition is called the competitive endogenous RNA mechanism. Thus, circRNAs may play an important regulatory role in disease through their interactions with disease-associated miRNAs. Previous studies have reported that circRNAs are aberrantly expressed in a variety of epithelial tumors, including ovarian cancer, laryngeal cancer, and cancers of the digestive system [12]. However, a potential role for circRNAs in OSCC has yet to be established.

We have previously shown in a high-throughput sequencing study that the circRNA hsa_circ_0004491 exhibits significantly reduced expression in OSCC [13]. In this study, we investigated the expression of hsa_circ_0004491 in 40 paired OSCC and normal tissue samples. The potential relationship between hsa_circ_0004491 expression and lymphatic metastasis was explored. We also examined the role of hsa_circ_0004491 in OSCC cell migration and invasion, and its association with clinical characteristics. Our results suggest that hsa_circ_0004491 may play a role in the metastasis of OSCC and be a potential biomarker for this disease.

2. Materials and methods

2.1. Patient samples

Forty paired samples of OSCC and adjacent normal tissues were obtained from the Department of Oral and Maxillofacial Surgery, Peking University Shenzhen Hospital (Guangdong, China) between December 2016 and May 2017. All samples were verified by histopathology and then stored at -80°C until required for downstream analysis. The clinical study was approved by the Ethics Committee of Peking University Health Science Center (IRB00001053-08043) and written informed consent was obtained from each patient.

2.2. Total RNA extraction

Total RNA was extracted from OSCC and normal tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration and quality of extracted RNA was determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

2.3. Quantitative real-time PCR

cDNA synthesis was performed with Oligo (dT) and random primers using the PrimeScript RT Reagent Kit (Takara Bio, Noshigashi, Kusatsu, Japan) according to the manufacturer's instructions. SYBR-Green Premix Ex Taq (Takara Bio) was used to determine the relative expression levels of circRNAs by qRT-PCR. The reaction conditions were as follows: A preincubation step at 95°C for 5 min followed by 40 cycles of 95°C for 5 s, 55°C for 30 s and 72°C for 30 s. The expression levels of hsa_circ_0004491 were normalized to those of β -actin. All data were analyzed using the ΔCt method [14]. Forward and reverse primers for the amplification of hsa_circ_0004491 were 5'-GGATCAGAAAACTA TGGAAGCCTG -3' and 5'-GCCTTGACAGACAGACAGCA -3', respectively. Forward and reverse primers for the amplification of β -actin were 5'-AAACTG GAACGTTGAGAGTG-3' and 5'-AGTGGTCTGGCTTTT AGGT-3', respectively.

2.4. Cell culture

The human OSCC cell lines, SCC15, SCC25, and CAL27, were obtained from the College of Stomatology, Wuhan University (Wuhan, China). Human oral keratinocytes (HOK) were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). SCC15,

SCC25, CAL27, and HOK cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, GIBAO, Grand Island, NY, USA), at 37°C in a humidified 5% CO_2 atmosphere.

2.5. Cell transfection

The sequence of the hsa_circ_0004491-targeting siRNA used in RNA interference assays was 5'-CTATGGAAGCCTGGAGCCA-3'. Control and hsa_circ_0004491 siRNAs were synthesized by Guangzhou RiboBio Co. (Guangzhou, China). The control and hsa_circ_0004491 overexpression plasmids were constructed by GenePharma (Suzhou, China) using pcDNA3.1 vector. OSCC cells were transfected using Lipofectamine 3000 (Gibco) according to the manufacturer's protocol.

2.6. Wound healing assays

SCC15 and CAL27 cells were trypsinized and reseeded in 6-well plates. Confluent cell cultures were scratched with a 200 μl sterile pipette tip to generate a single scratch-wound. The plate was washed three times with phosphate-buffered saline (PBS) to remove detached cells. The migration rate was monitored using an inverted microscope and data were obtained from three independent experiments.

2.7. Migration and invasion assays

Migration and invasion assays were performed using uncoated and Matrigel pre-coated Transwell chambers, respectively (Corning Life Sciences, Corning, NY). Matrigel matrix (100 μl of a 1 mg/ml solution; Sigma-Aldrich, USA) was added to Transwell chambers in a 24-well plate. Transfected cells in serum-free DMEM (2×10^4 cells in 100 μl medium) were added to the upper chamber and DMEM containing 10% FBS (700 μl medium) was added to the lower chamber. After culture for 36 h, the cells in the upper chamber were removed using a cotton swab, and those in the lower chamber were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet, washed with PBS, and dried. Images were acquired using an inverted epifluorescence microscope and processed using ImageJ. The migration assay was performed as described above, but without pre-coating the Transwell membrane with Matrigel matrix. For each assay type, data were analyzed from three independent experiments.

2.8. Western blotting

Total cellular proteins were extracted using lysis buffer, separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Following hybridization with a primary antibody at 4°C overnight, the membranes were immunoblotted with a secondary antibody at room temperature for 1 h. Finally, enhanced chemiluminescence (ECL kit, Millipore) was used for visualization of immunoreactivity and Image J software (National Institutes of Health, USA) was used for the analysis of band intensity. Primary antibodies and their dilutions were as follows: anti-E-cadherin (1:1000), anti-vimentin (1:1000) and anti-GAPDH (1:1000). All antibodies were from Cell Signaling Technology (Danvers, MA). Protein expression data were analyzed from three independent experiments.

2.9. Online prediction

CircRNA/miRNA interaction was predicted with miRNA target prediction software CircInteractome (<https://circinteractome.nia.nih.gov>).

2.10. Statistical analysis

Statistical data were analyzed using GraphPad Prism 6.0 Software

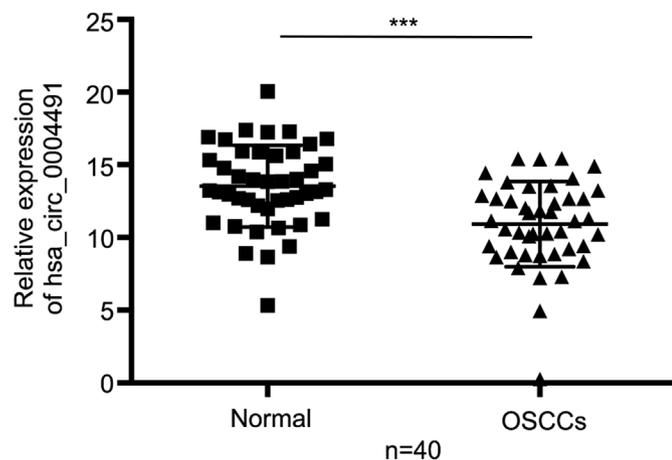


Fig. 1. Expression of hsa_circ_0004491 in OSCC tissues. Relative expression level of circular RNA hsa_circ_0004491 was detected in 40 paired oral squamous cell carcinoma tissues by qRT-PCR, which was down-regulated evidently compared to adjacent normal tissues.

(GraphPad Software, La Jolla, CA, USA). Results were expressed as the mean \pm SD. Data were analyzed using a two-tailed paired *t*-test. A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Hsa_circ_0004491 expression in OSCC tissues and cell lines

We have previously shown that a large number of circRNAs demonstrate altered expression in OSCC, when compared with matched normal tissues [13]. Among these, we found that hsa_circ_0004491 was significantly reduced in OSCC. Specific divergent primers were designed to verify hsa_circ_0004491 expression in 40 paired OSCC and adjacent normal tissues. The experimental results confirmed that hsa_circ_0004491 was expressed in OSCC tissues and that this expression was significantly downregulation, when compared with adjacent normal tissues (Fig. 1). Furthermore, an analysis of hsa_circ_0004491 expression levels in HOK cells and three human OSCC cell lines (SCC15, SCC25, and CAL27) revealed that hsa_circ_0004491 expression was significantly lower in OSCC cells, when compared with normal keratinocytes (Fig. 2).

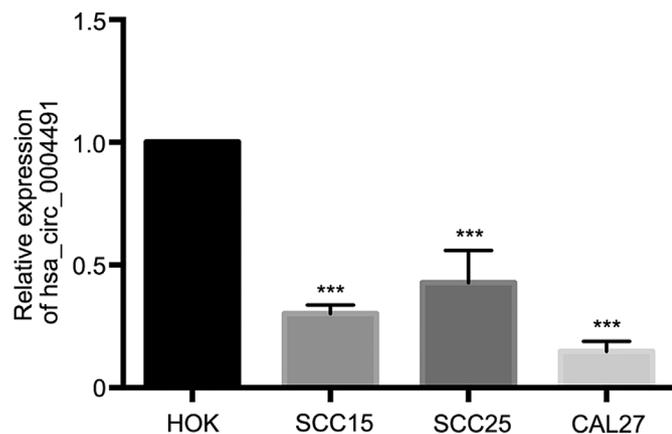


Fig. 2. The expression of hsa_circ_0004491 in different OSCC cell lines. The relative expression level of circular RNA hsa_circ_0004491 was detected in 3 OSCC cell lines (SCC15, SCC25, CAL27) and the human oral keratinocytes by qRT-PCR (***, *P* < 0.001).

3.2. Characterization of hsa_circ_0004491

Hsa_circ_0004491 is encoded by the *ORC4* gene and is located on chromosome 2 at chr2:148730307-148739650. Hsa_circ_0004491 is composed of four exons and has a spliced sequence length of 396 bp (Fig. 3).

3.3. Correlations between hsa_circ_0004491 expression and clinical parameters

We next investigated the potential relationship between hsa_circ_0004491 expression and the clinical characteristics of OSCC patients. As shown in Table 1, the level of hsa_circ_0004491 expression was negatively associated with lymph node metastasis (*P* = 0.0398). Other clinical factors were not significantly associated with the level of hsa_circ_0004491 expression. To test whether hsa_circ_0004491 had diagnostic value as an indicator of OSCC, a ROC curve was constructed using expression data from the paired OSCC and adjacent normal tissues. The area under the ROC curve (AUC) was 0.7510 (Fig. 4), suggesting that hsa_circ_0004491 expression had diagnostic value as an indicator of OSCC.

3.4. Up- or down-regulation of hsa_circ_0004491 affects the migration and invasion of OSCC cells

To examine the potential role of hsa_circ_0004491 in OSCC cell migration, we performed wound healing assays in SCC15 and CAL27 cells transfected with control or hsa_circ_0004491-specific siRNAs (hsa_circ_0004491 RNAi). The scratch-wound area was measured at 0, 24 and 36 h after wounding. The results showed that siRNA-mediated suppression of hsa_circ_0004491 expression enhanced the rate of wound closure, when compared with the control group (Fig. 5A). To further confirm the role of hsa_circ_0004491 in OSCC cell migration, a transwell migration assay was also performed using SCC15 and CAL27 cells transfected with control or hsa_circ_0004491 siRNAs (hsa_circ_0004491 RNAi). In both cell lines, siRNA-mediated suppression of hsa_circ_0004491 expression significantly inhibited transwell migration after 36 h of culture, when compared with controls (Fig. 5B). In consist, overexpression of hsa_circ_0004491 suppressed the migration of OSCC cells (Fig. 5C). Findings from transwell invasion assays showed that the invasive rates of SCC15 and CAL27 cells were also enhanced following hsa_circ_0004491 suppression with siRNA (Fig. 5D) while overexpression of hsa_circ_0004491 suppressed the invasion abilities of SCC15 and CAL27 cells (Fig. 5E).

3.5. Up- or down-regulation of hsa_circ_0004491 alters epithelial-mesenchymal transition process in OSCC

Epithelial-mesenchymal transition (EMT) is a cellular process in which cells lose their epithelial characteristics and acquire mesenchymal features. EMT is often defined by the loss of the epithelial marker E-cadherin and a gain of expression of the mesenchymal marker vimentin, changes which are closely associated with invasion and metastasis in oral cancer. To examine the role of hsa_circ_0004491 expression in EMT, cell lysates from siRNA-transfected SCC15 and CAL27 cells were examined by western blotting. The results of the protein expression analysis revealed that siRNA-mediated suppression of hsa_circ_0004491 expression was associated with reduced E-cadherin expression and increased vimentin expression (Fig. 6A). When overexpressed hsa_circ_0004491, the vimentin expression was significantly decreased, meanwhile the E-cadherin expression was increased (Fig. 6B). Thus, these results indicates that hsa_circ_0004491 regulates EMT process in OSCC cells.

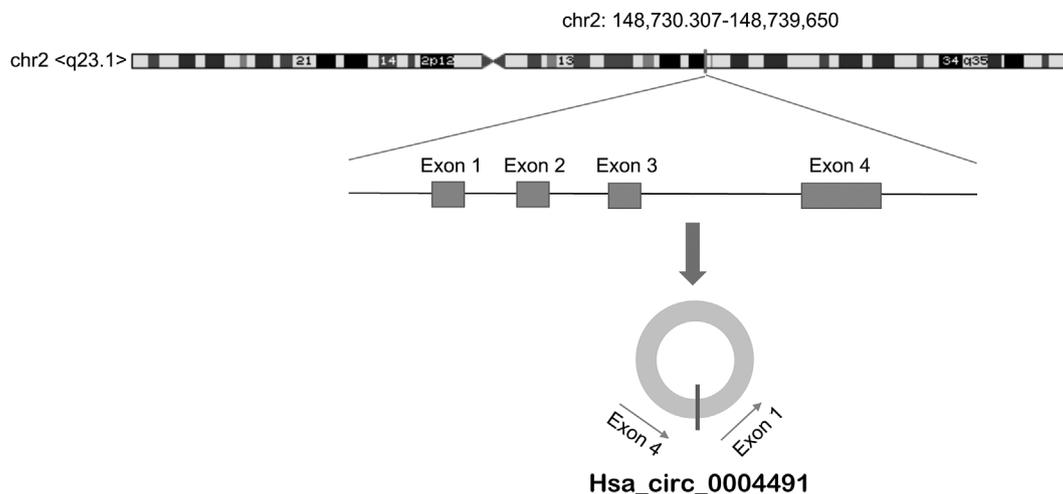


Fig. 3. Relationship of hsa_circ_0004491 genomic DNA, mRNA and circRNA. Hsa_circ_0004491 is encoded by chromosomal region 2. Its gene is located at chr2:148,730,307–148,739,650. Four exons form it by back splicing.

Table 1
Correlation between the expression of hsa_circ_0004491 and clinicopathological characteristics in 40 patients with OSCC.

Characteristics	No. Patients	Mean ± SD	P value
Gender			
Male	28	13.60 ± 0.4909	0.6863
Female	12	13.25 ± 0.6573	
Age			
< 60	26	12.48 ± 0.5913	0.4191
≥ 60	14	11.52 ± 0.6960	
T stage			
T1, T2, T3	21	12.14 ± 0.6516	0.5484
T4	19	12.15 ± 0.6522	
Lymph node metastasis			
N0	25	12.86 ± 0.5645	0.0398 ^a
N1, N2, N3	15	10.95 ± 0.6842	
Differentiation			
Well & Moderate	34	13.41 ± 0.4241	0.6039
Poor	6	13.99 ± 1.106	

^a Indicated statistical significance.

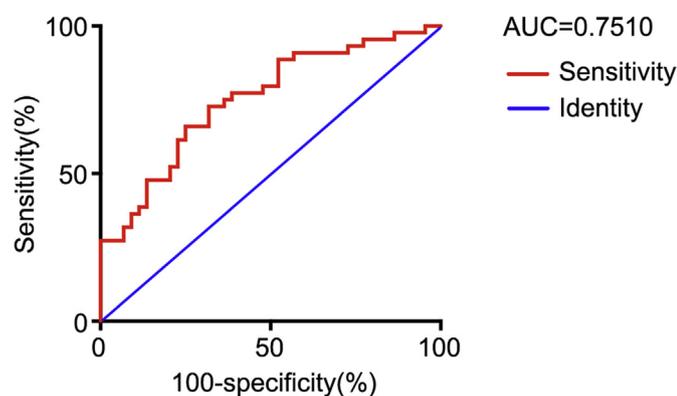


Fig. 4. ROC curve was used to evaluate the diagnostic value of hsa_circ_0004491 in OSCC. The area under the ROC curve was 0.7510.

3.6. Hsa_circ_0004491 may interact with several miRNAs

In order to explore the molecular mechanism and function of circRNA hsa_circ_0004491, potential interaction between circRNAs and miRNAs were investigated. By the online CircInteractome tool, we found that hsa_circ_0004491 may interact with hsa-miR-149-5p, hsa-miR-136-5p, and hsa-miR-155-5p (Fig. 7A). Among them, hsa-miR-

155-5p has been reported to be an oncogene in many cancers, including oral cancer. We examined the expression level of hsa-miR-155-5p and found it was upregulated when knock down hsa_circ_0004491 (Fig. 7B).

4. Discussion

Oral cancer is the third most common cancer in developing countries and the eighth most common cancer in the world [15,16]. OSCC is the most common malignant tumor among all oral cancers. The five-year survival rate for this disease has not improved significantly over the past few decades, despite continuous improvements in treatment strategies, such as surgery, radiation therapy and chemotherapy. Patients with advanced OSCC often present with regional lymph node metastasis at the time of diagnosis. Lymph node metastasis is considered to be an important factor affecting prognosis and recurrence in OSCC patients. Therefore, in recent years, a large number of studies have been conducted to unpick the biological mechanisms underlying OSCC tumorigenesis and progression, in hopes of finding effective therapeutic targets that can improve patient survival.

Although the existence of circRNAs has been known for decades, these molecules were initially considered to represent transcriptional noise. Owing to rapid advances in high-throughput RNA sequencing that permit a global analysis of circRNA expression, a large number of circRNAs have now been discovered. Because of their stability, circRNAs are promising clinical diagnostic and prognostic markers [17]. Wang et al. have previously reported that hsa_circ_001988 exhibits decreased expression in colorectal cancer where it may have utility as a potential biomarker [18]. Qin et al. have shown that hsa_circ_0001649 is significantly downregulated in hepatocellular carcinoma tissues and may therefore represent a potential novel biomarker for this disease [19]. Li et al. explored the regulatory role of the hsa_circ_0008309-miR-136-5P/hsa-miR-382-5P-ATXN1 network in OSCC and their data suggest that hsa_circ_0008309 may have utility as a potential biomarker for this disease [20]. However, the underlying molecular mechanisms by which circRNA expression contributes to OSCC remain to be elucidated. Hsa_circ_0004491 was first identified by Jeck et al., in 2013. However, to the best of our knowledge, our study is the first to report hsa_circ_0004491 expression in oral cancer.

In this study, hsa_circ_0004491 expression was significantly decreased in 40 OSCC tissues, when compared with adjacent normal tissues, and our ROC analyses revealed that hsa_circ_0004491 had diagnostic value as an indicator of OSCC. Furthermore, hsa_circ_0004491 expression was closely associated with lymph node metastasis (Table 1), suggesting that hsa_circ_0004491 plays a role in disease

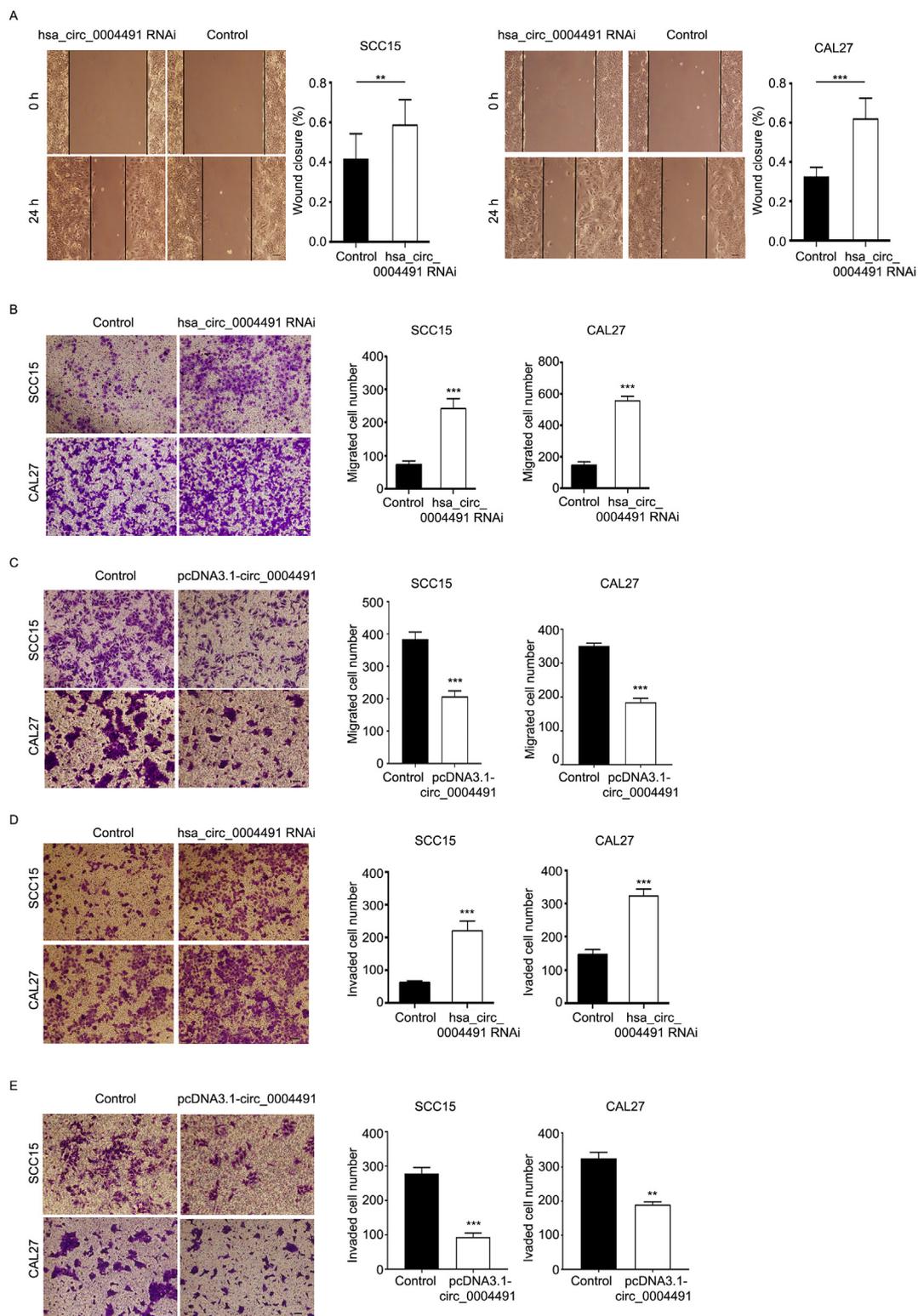


Fig. 5. Up- or down-regulation of hsa_circ_0004491 affects the migration and invasion of OSCC cells. (A) Wound healing assays were performed on SCC15 and CAL27 cells transfected with control or hsa_circ_0004491 siRNA. The scratch area was measured at 0 and 24 h or 36 h, and the percentage was calculated. (B and C) The cell migration capacity and migrative cell number was detected by transwell assays after transfection of control or hsa_circ_0004491 siRNA (B) and control vector or pcDNA3.1-circ_0004491 (C) into SCC15 and CAL27 cells, respectively. (D and E) The cell invasion capacity and invasive cell number was detected by transwell assays after transfection of control or hsa_circ_0004491 siRNA (D) and control vector or pcDNA3.1-circ_0004491 into SCC15 and CAL27 (E) into SCC15 and CAL27 cells, respectively. Scale bars, 10 μ m.

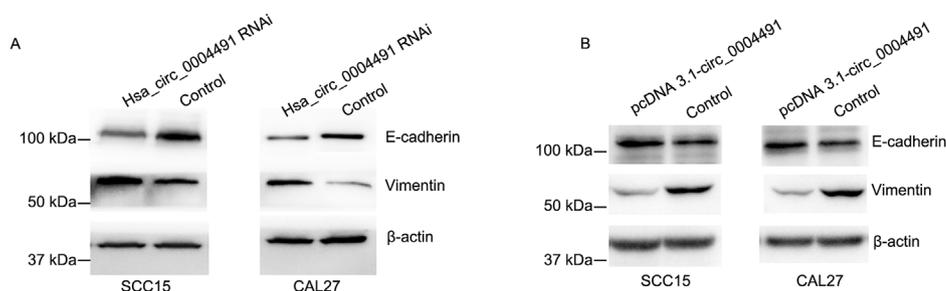


Fig. 6. The expression change of hsa_circ_0004491 regulates EMT process in OSCC. Protein expression levels of E-cadherin and Vimentin were detected by Western blot in OSCC cells (SCC15 and CAL27), transfected with control or hsa_circ_0004491 siRNA (A) and control or hsa_circ_0004491 overexpression plasmid (B), respectively.

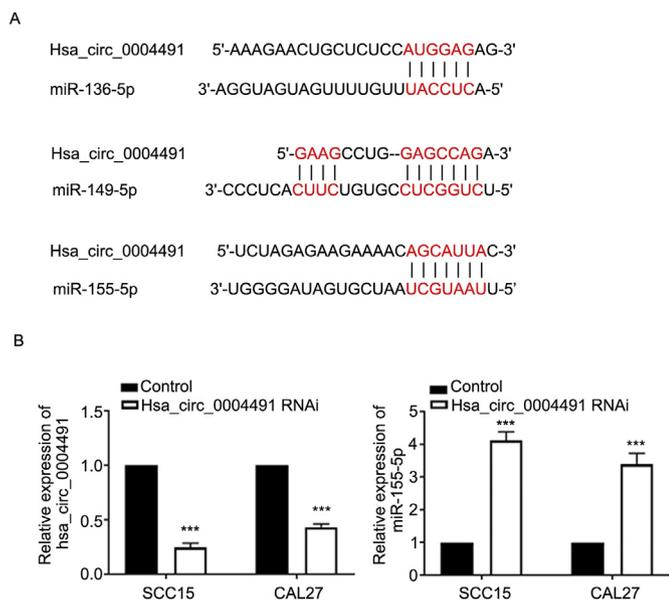


Fig. 7. Hsa-miR-155-5p was regulated by hsa_circRNA_0004491. (A) The predicted binding sites of hsa_circ_0004491 with hsa-miR-136-5p, hsa-miR-149-5p and hsa-miR-155-5p. (B) SCC15 and CAL27 cell lines were transfected with control or hsa_circ_0004491 siRNA, the relative expression levels of hsa_circ_0004491 and hsa-miR-155-5p were analyzed by qRT-PCR.

progression. Lymph node metastasis has been reported to be a predictor of prognosis in OSCC and to be negatively associated with survival and local recurrence. The 5-year survival rate of patients with lymph node metastasis at diagnosis is only 25–40%. This increases to approximately 90% for patients without lymph node metastasis [21]. Lymph node metastasis is also an indicator for the postoperative administration of radiotherapy. Hsa_circ_0004491 expression may therefore have potential diagnostic utility in predicting lymph node metastasis in OSCC.

In view of the clinical characteristics associated with hsa_circ_0004491 expression, we sought to further explore the role of this circRNA in OSCC metastasis using *in vitro* assays. We found that alteration of hsa_circ_0004491 expression affects migration and invasion abilities of OSCC cells. EMT plays an important role in the invasion and metastasis of malignant tumors. During EMT, the strong physical connections between neighboring epithelial cells are lost and epithelial polarity is disrupted. Loss of the epithelial marker E-cadherin and a gain of the mesenchymal marker vimentin accompany this transition, and changes in the expression of these proteins are commonly used to evaluate EMT biochemically [22]. In this study, we found that suppression of hsa_circ_0004491 expression in SCC15 and CAL27 cells resulted in a concomitant reduction and increase in the expression of E-cadherin and vimentin, respectively, while overexpression of hsa_circ_0004491 reversed the expression levels of EMT-related proteins. The results suggest that hsa_circ_0004491 regulates EMT progress in OSCC cells.

In many studies, it has been reported that circRNAs can function as

a sponge, sequestering downstream miRNA or protein [23]. We therefore searched for potential miRNA and RNA-binding protein (RBPs) target sites in hsa_circ_0004491 using the CircInteractome database (<https://circinteractome.nia.nih.gov>) and found three potential targets (hsa-miR-136-5p, hsa-miR-149-5p and hsa-miR-155-5p; Fig. 7A). Among these, hsa-miR-155-5p, encoded by the human B-cell integration cluster gene, is regarded as a proto-oncogene [24,25]. It has been reported that elevated levels of hsa-miR-155-5p can promote decreased E-cadherin expression and increased vimentin expression in hepatocellular carcinoma cells, accelerating EMT and cellular invasion and migration, thereby promoting disease progression [26]. Kim et al. have reported that miR-155-5p expression may contribute to EMT-associated OSCC progression and may serve as a biomarker for predicting relapse, especially for patients with early-stage OSCC [27]. In our study, we suppressed the expression of hsa_circRNA_0004491 and detected the increase of hsa-miR-155-5p level (Fig. 7B). In 2017, Yue Wang et al. demonstrated that upregulation of hsa-miR-155-5p inhibits SIRT1, activates P53 and forms a positive feedback loop in renal tubular injury [28]. A previous report has confirmed the lower expression of SIRT1 in OSCC cells and suggests that SIRT1 may prevent oral cancer metastasis by blocking the EMT process [29]. Moreover, Kang et al. revealed that overexpression of SIRT1 suppressed OSCC cell proliferation and invasion, suggesting that SIRT1 can act as a tumor suppressor in OSCC [30]. We speculated that hsa_circ_0004491/miR-155-5p/SIRT1 axis play a significant role in the invasion, migration and EMT process of OSCC. The specific regulation mechanism of hsa_circ_0004491/miR-155-5p/SIRT1 needs further investigation.

5. Conclusions

In conclusion, our study identified the downregulated expression of hsa_circ_0004491 in OSCC. Furthermore, we show that reduced hsa_circ_0004491 expression is associated with lymph node metastasis and may facilitate OSCC cell invasion and migration. These results suggest that hsa_circ_0004491 may be a potential biomarker for OSCC.

Authors' contributions

Xiang Li: Conceptualization, Formal analysis, Investigation, Writing – original draft. Hanyu Zhang: Investigation, Writing – original draft. Yufan Wang: Funding acquisition, Investigation, Writing – original draft. Shuai Sun: Investigation. Yuehong Shen: Conceptualization, Project administration, Supervision, Writing - original draft, Writing - review & editing. Hongyu Yang: Funding acquisition, Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

Ethics approval

The experiments using clinical samples were approved by Ethics Committee of Peking University Health Science Center (IRB00001053-08043).

Declaration of competing interests

The authors declare that there are no conflicts of interest.

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