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MicroRNA-378 promotes the malignant progression of oral squamous cell carcinoma by mediating FOXN3

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Abstract. – OBJECTIVE: This study aimed to detect the expression of microRNA-378 in OSCC, and further studies its effects on clinicopathology and prognosis of OSCC patients.

PATIENTS AND METHODS: Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was used to detect the expression levels of microRNA-378 in 96 pairs of OSCC tissues and paracancerous tissues. The relationship between microRNA-378 expression and pathological parameters and prognosis of OSCC patients was analyzed. The expression level of microRNA-378 in OSCC cells was detected by RT-qPCR as well. Also, microRNA-378 knockdown expression model was constructed using small interfering RNA in OSCC cell lines CAL-27 and Tca8113. Biological functions of OSCC cells were determined using cell counting kit-8 (CCK-8), colony formation, and transwell assay. Western blot was conducted to detect the protein expression of FOXN3 in OSCC cells.

RESULTS: RT-qPCR results showed that the expression level of microRNA-378 in OSCC tissues is remarkably higher than that in paracancerous tissues. Compared with OSCC patients with lower expression of microRNA-378, patients with higher expression of microRNA-378 had higher incidences of lymph node metastasis and distant metastasis, as well as shorter overall survival. MicroRNA-378 knockdown significantly decreased proliferative, invasive, and metastatic abilities of OSCC cells. Western blot results showed that microRNA-378 downregulates FOXN3 expression in OSCC cells. Rescue experiments found that microRNA-378 could regulate FOXN3, thus promoting malignant progression of OSCC.

CONCLUSIONS: MicroRNA-378 is highly expressed in OSCC, which is significantly associated with tumor staging, distant metastasis, and poor prognosis of OSCC. It is shown that microRNA-378 may promote malignant progression of OSCC by regulating FOXN3.

Key Words
MicroRNA-378, FOXN3, OSCC, Malignant progression.

Introduction

Oral squamous cell carcinoma (OSCC) is a malignant tumor originating from epithelial tissue, which accounts for more than 80% of oral and maxillofacial malignancies[1-3]. In the United States, 90% of cases of carcinoma of mouth are OSCC[4]. Although the treatment of OSCC has made significant progress, postoperative recurrence and metastasis are still frequently observed, leading to poor prognosis in OSCC patients. The five-year survival rate of OSCC is only 50%-60%[5,6]. Therefore, researches on OSCC pathogenesis are well concerned[7]. Currently, biological characteristics and functions of oncogenes and tumor-suppressor genes have been widely explored. These specific genes may be served as diagnostic markers and therapeutic targets of OSCC[7-9].

MicroRNAs are a class of stable, non-coding, small-molecule RNAs. They participate in the regulation of target genes at the post-transcriptional level[10,11]. Although they could not be directly served as oncogenes and tumor-suppressor genes, they could regulate these gene expressions by inhibiting or degrading target mRNA[12,13]. Recent studies have found that some microRNAs are differentially expressed in OSCC patients. These microRNAs are crucial for diagnosis, treatment, and prognosis of OSCC, which may be utilized as new tumor hallmarks[14,15]. MicroRNA-378 is found to be differentially expressed in multiple tumors[16,17]. Our previous study has already found the high expression of microRNA-378 in peripheral blood of OSCC patients. This study further explored the biological functions of microRNA-378 in OSCC and its underlying mechanism.

In this investigation, we analyzed the expression of microRNA-378 in 96 pairs of OSCC tissues and paracancerous tissues, and subsequently explored the effects of microRNA-378 on the biological functions of OSCC cells. Previous researches

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have indicated that microRNA-378 can promote the process of tumor cell division and metastasis, thus affecting tumor development. We aim to investigate the role of microRNA-378 in pathological parameters and prognosis of OSCC patients.

Patients and Methods

Patients and OSCC Samples

We collected 96 pairs of tumors and paracancerous tissues from OSCC patients undergoing surgical resection. All patients were pathologically diagnosed as OSCC according to the 8th edition of UICC/AJCC, and they did not receive preoperative anti-tumor treatments. This study has been approved by the Ethics Committee. Patients and their families have been informed consent.

Cell Culture

Four human OSCC cell lines (Fadu, SCC-25, CAL-27, and Tca8113) and one normal human oral cell line (Hs 680.Tg) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured in DMEM containing 10% FBS and maintained in a 5% CO_2 incubator at 37°C.

Transfection

The si-RNA negative control and si-microRNA-378 were obtained from GenePharma, Shanghai, China. Cells were seeded in 6-well plates and grown to a cell density of 70%, followed by cell transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were harvested 48 h later for the following experiments.

Cell Proliferation Assay

After 48 h of transfection, cells were seeded into 96-well plates at 2000 cells per well. After cell culture for 6 h, 24 h, 48 h, and 72 h, respectively, the cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well. 2 hours later, the optical density (OD) value of each well was measured using a microplate reader.

Colony Formation Assay

After 48 h of transfection, 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was replaced after one week, and then replaced twice a week. 2 weeks later until colony formation, cells were washed with phosphate-buffered saline (PBS) twice and fixed with 2 mL of methanol for 20 min. Cells were then washed with PBS and stained with 0.1% crystal violet staining solution for 20 min. Finally, colonies were observed and captured using a microscope.

Transwell Assay

After transfection for 48 hours, the cells were collected and resuspended in serum-free medium. Cell density was adjusted to 2.0×10^5/mL. Transwell chamber containing Matrigel or not was placed in a 24-well plate. 200 μL of the cell suspension was added to the upper chamber of the chamber, and 500 μL of medium containing 10% FBS was added to the lower chamber. 48 hours later, the chamber was removed, and cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 15 min. The inner layer cells were carefully removed. 5 randomly selected fields of each sample were observed for capturing the migratory and invasive cells.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from OSCC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). RT-qPCR was performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real-time PCR System (Applied Bios Systems, Foster City, CA, USA). Primers used in this study was as follows: MicroRNA-378: forward: 5'-ACUGGACUUGGAGUCAGAAGG-3', reverse: 5'-UUCUGACUCCAAGUCCAGUUU-3'; U6: forward: 5'-UUCUCCGAACGUGUCACGUTT-3', reverse: 5'-ACGUGACACGUUCGGAGAATT-3'; FOXN3: forward: 5'-CAAAGGCGTCGTCAATCACC-3', reverse: 5'-TTCCAAACCTTTGAGGGCGA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data analysis was performed using the ABI Step One software and relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The protein was washed three times with precooled PBS at 4°C and extracted by cell lysate. Protein concentration was determined by bicin-

choninic acid (BCA) method (Abcam, Cambridge, MA, USA), and the volume of the sample was adjusted according to the protein quantification result with 30 μg per well. Protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 50 μg/L skim milk for 1 h, incubated with FOXN3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, 1:1 000 dilution, Cambridge, MA, USA) overnight at 4°C. Subsequently, they have incubated with the goat anti-rabbit anti-IgG antibody (1:5 000 dilution) for 1 h. Band exposure was performed by chemiluminescence, and the expression level of each protein was normalized with β-actin as an internal reference.

Statistical Analysis

Data analyses were performed using the Statistical Product and Service Solutions (SPSS) 22.0 procedure (IBM, Armonk, NY, USA). Data were expressed as mean ± standard deviation. The continuous variables were analyzed using the *t*-test, and the categorical variables were analyzed using the χ^2-test or Fisher's exact test. Kaplan-Meier method was used to calculate the survival time of OSCC patients, and the difference between different curves was compared by Log-rank test. $p<0.05$ was considered to be statistically significant.

Results

MicroRNA-378 was Highly Expressed in OSCC Tissues and Cell Lines

We examined the expression of microRNA-378 in 96 pairs of OSCC tissues and paracancerous tissues by RT-qPCR. The data showed higher expression of microRNA-378 in OSCC tissues than that of paracancerous tissues (Figure 1A and 1B). Similarly, microRNA-378 was highly expressed in OSCC cells than normal oral cells (Figure 1C). In particular, CAL-27 and Tca8113 cells expressed a relatively high expression of microRNA-378, which were selected for the following experiments.

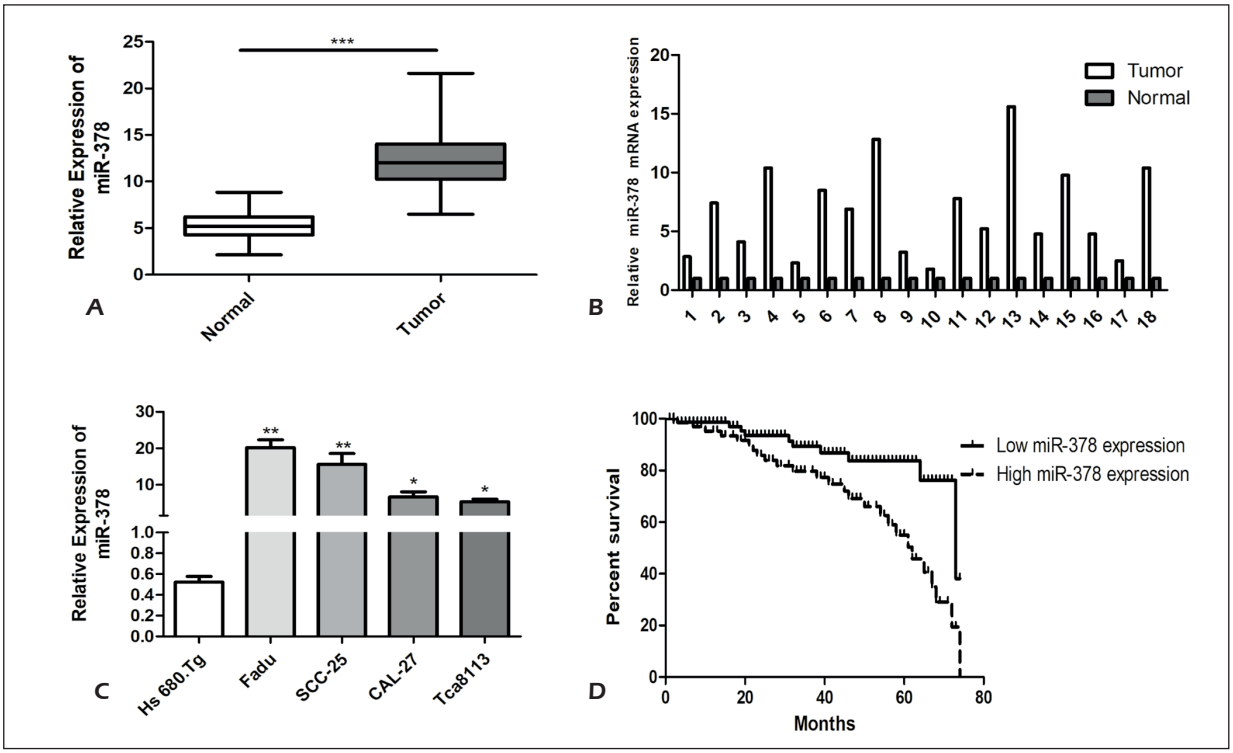

Figure 1. ***A-B***, Expression of microRNA-378 in 96 pairs of OSCC tissue and paracancerous tissues. ***C***, Expression levels of microRNA-378 in 4 OSCC cell lines (Fadu, SCC-25, CAL-27, Tca8113) and normal human oral cell line (Hs 680.Tg). ***D***, Kaplan-Meier survival curves of OSCC patients based on their microRNA-378 expressions. Patients in the high expression group had an unfavorable prognosis than those in the low expression group. A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

Table I. Association of miR-378 expression with clinicopathologic characteristics of oral squamous cell carcinoma.

Parameters	Number of cases	miR-378 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.569
<60	42	25	17	
≥60	54	29	25	
Gender				0.143
Male	47	30	17	
Female	49	24	25	
T stage				0.032
T1-T2	53	35	18	
T3-T4	43	19	24	
Lymph node metastasis				0.035
No	55	36	19	
Yes	41	18	23	
Distance metastasis				0.023
No	70	45	25	
Yes	26	10	16	

MicroRNA-378 Expression Was Correlated With Clinical Stage, Lymph Node Metastasis, Distance Metastasis and Overall Survival of OSCC Patients

According to the expression level of microRNA-378, OSCC patients were divided into high microRNA-378 level group and low microRNA-378 level group, respectively. The relationship between microRNA-378 expression with age, sex, tumor stage, lymph node metastasis, and distant metastasis of OSCC patients was analyzed. It is shown that high expression of microRNA-378 is positively correlated to tumor stage, lymph node metastasis and distant metastasis, whereas not correlated to age and sex of OSCC patients (Table I). Follow-up data of enrolled patients were collected for analyzing the correlation between microRNA-378 expression and prognosis of OSCC patients. Kaplan-Meier survival curves elucidated that high expression of microRNA-378 is correlated to the poor prognosis of OSCC (Figure 1D). These results suggested that microRNA-378 may serve as a new prognostic hallmark for OSCC.

Knockdown of MicroRNA-378 Inhibited Proliferation of OSCC Cells

To explore the regulatory effect of microRNA-378 on OSCC cells, we constructed si-NC and si-microRNA-378. Their transfection efficacies in OSCC cells were first verified (Figure 2A and 2B). The CCK-8 assay was conducted to determine the proliferative ability after the microRNA-378 knockdown in OSCC cells. The data demonstrated that transfection of si-microRNA-378 in OSCC cells remarkably decreases proliferative rate (Figure 2C and 2D). Colony formation assay showed the similar results. MicroRNA-378 knockdown reduced the colony formation ability of OSCC cells (Figure 2E).

Knockdown of MicroRNA-378 Inhibited Migration and Invasion of OSCC Cell

Migratory and invasive abilities of OSCC cells were detected by transwell assay. We found less penetrating cells after the microRNA-378 knockdown in CAL-27 cells, indicating the inhibited migratory and invasive ability (Figure 3A, 3B). Knockdown of microRNA-378 in Tca8113 cells attained the similar results (Figure 3C, 3D).

Knockdown of MicroRNA-378 Changed FOXN3 Expression in OSCC Cells

To further explore how microRNA-378 promotes the malignant progression of OSCC, we found a possible relationship between FOXN3 and microRNA-378 through bioinformatics analysis. Subsequently, we examined FOXN3 expression after knockdown of microRNA-378 by Western blot. The results showed upregulated FOXN3 after microRNA-378 knockdown

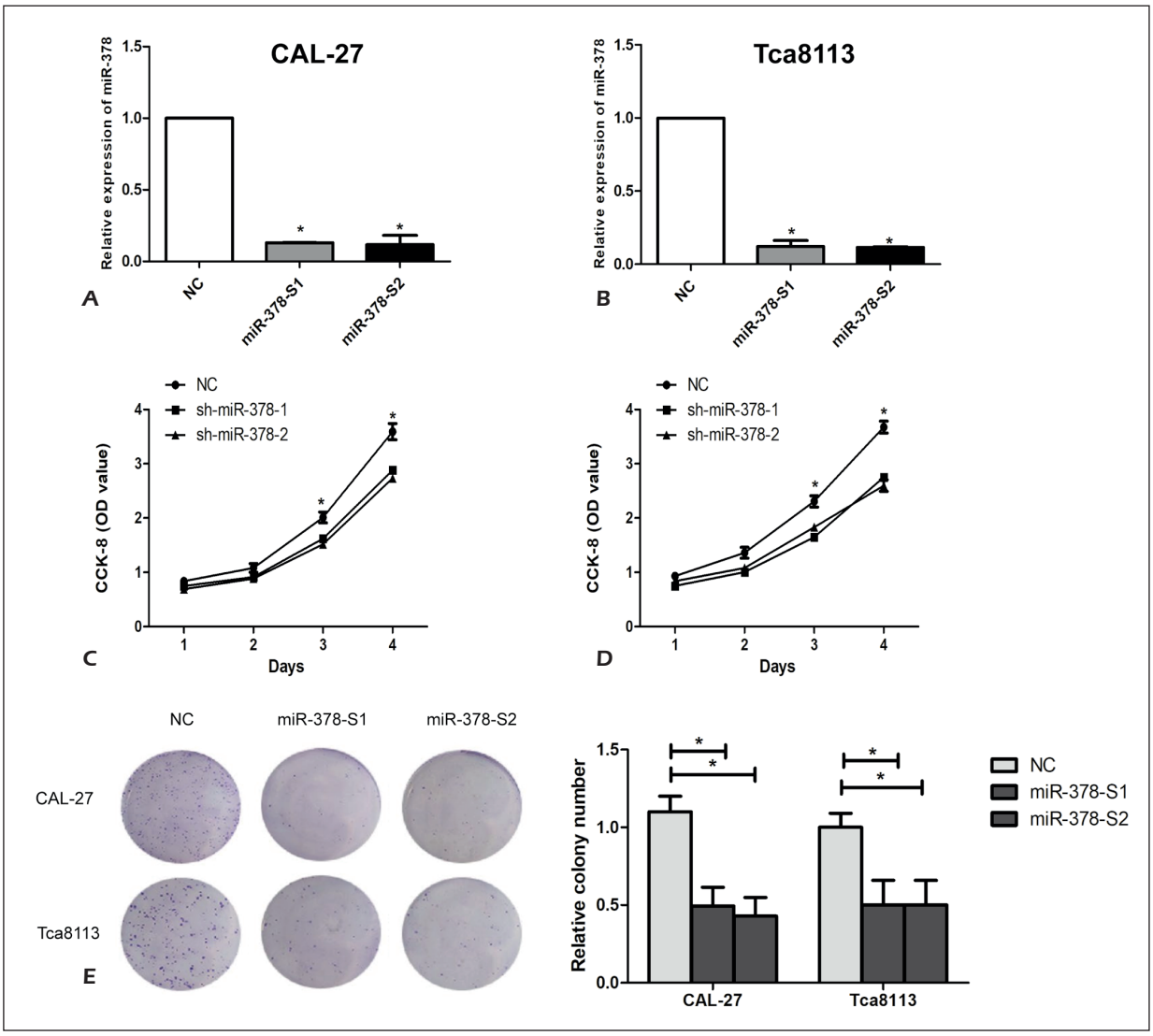

Figure 2. A-B, RT-qPCR was used to verify the transfection efficiency of si-microRNA-378 in CAL-27 and Tca8113 cell lines. **C-D**, Growth curve analysis showed the proliferation of CAL-27 and Tca8113 cells with the microRNA-378 knockdown. E, The efficiencies of cell colony formation in CAL-27 and Tca8113 cells with the microRNA-378 knockdown. A representative data set was displayed as mean ± SD values. *p<0.05, **p<0.01.

(Figure 4A). We also detected FOXN3 expression in OSCC tissues and cell lines. We found that FOXN3 is lowly expressed in OSCC tissues compared with paracancerous tissues (Figure 4B). Similarly, lower expression of FOXN3 was observed in OSCC cells compared with that of Hs 680.Tg cells (Figure 4C). Furthermore, we selected 16 pairs of OSCC tissues and paracancerous tissues from the enrolled 96 OSCC patients for analyzing the relationship between microRNA-378 and FOXN3. It is suggested that both mRNA and protein levels of microRNA-378 are negatively correlated to FOXN3 expression (Figure 4D).

FOXN3 Modulated MicroRNA-378 Expression in Human OSCC Cells

Overexpression plasmid of FOXN3 was conducted to verify its role in malignant progression of OSCC. Firstly, the transfection efficacy of overexpression plasmid of FOXN3 in OSCC cells was determined (Figure 5A and 5B). Subsequently, OSCC cells were co-transfected with si-microRNA-378 and overexpression plasmid of FOXN3. Rescue experiments indicated that the inhibited migration and invasion by microRNA-378 knockdown are reversed after FOXN3 overexpression (Figure 5C and 5D). As a result, we proved that microRNA-378 promotes malignant progression of OSCC by inhibiting FOXN3.

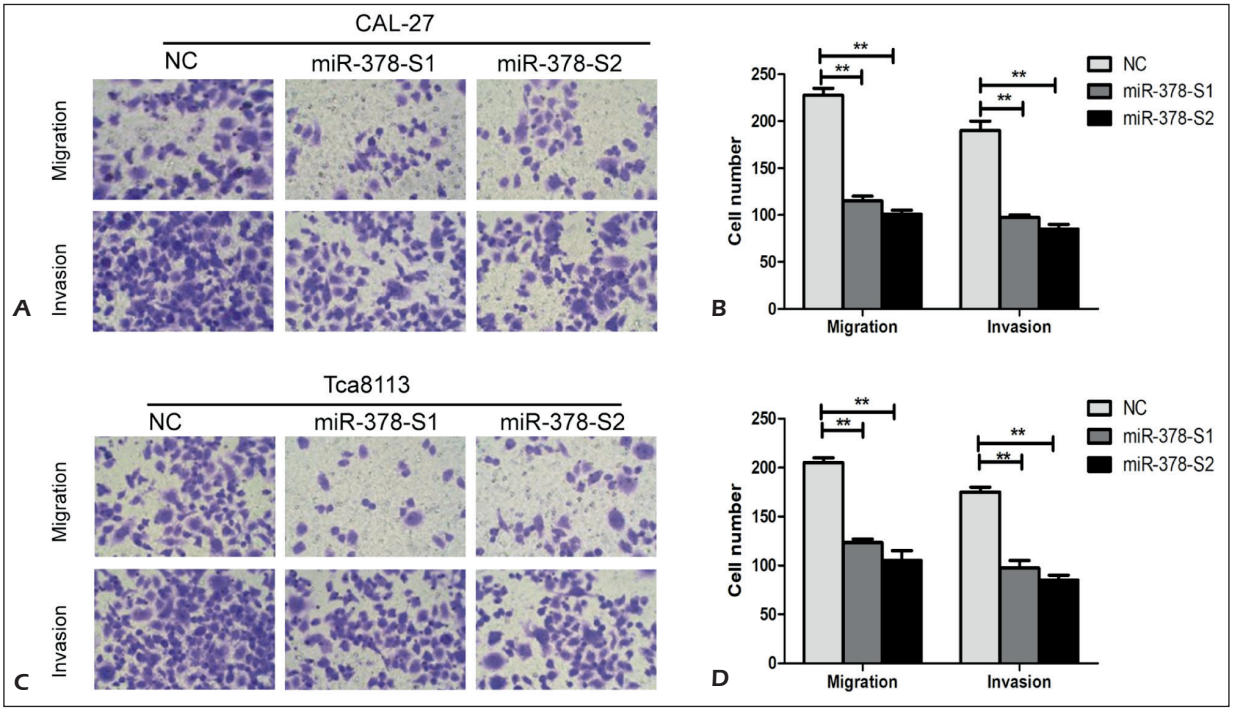

Figure 3. ***A-B***, CAL-27 and Tca8113 cells transfected with si-microRNA-378 displayed significantly lower migratory capacity. ***C-D***, CAL-27 and Tca8113 cells transfected with si-microRNA-378 displayed significantly lower invasive capacity. A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

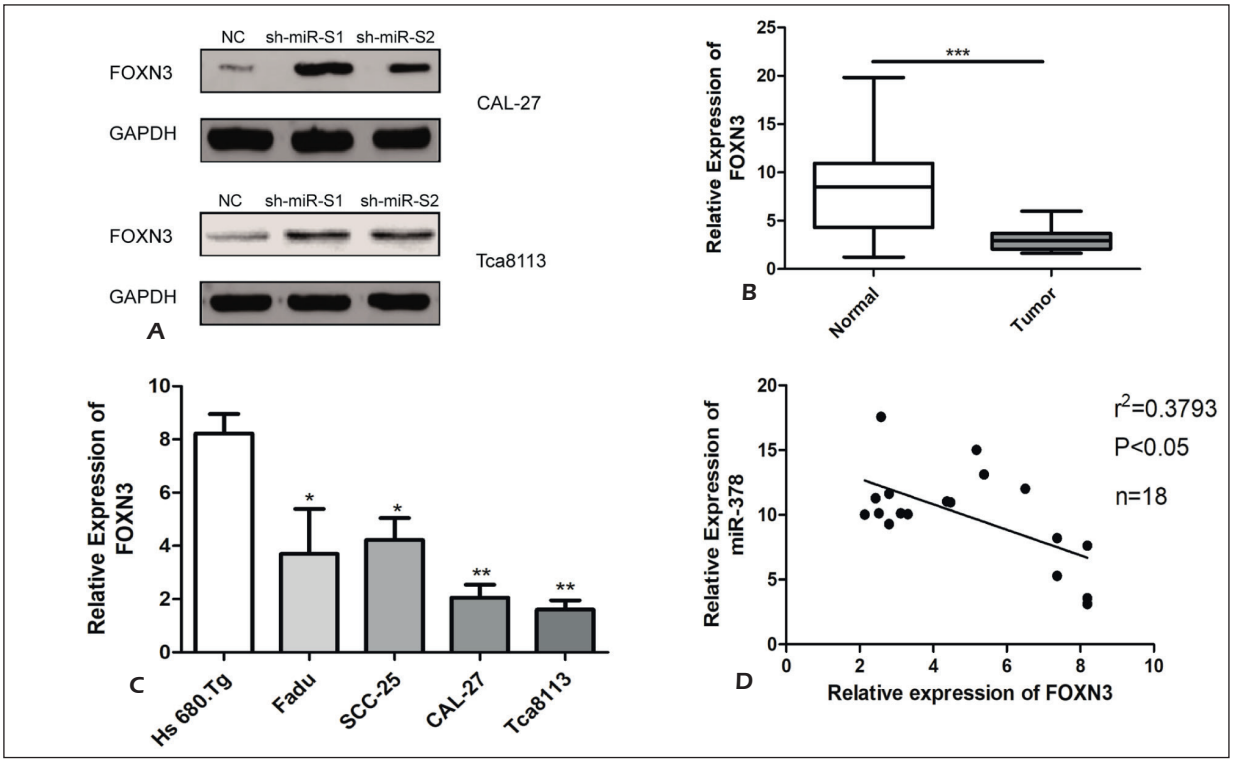

Figure 4. ***A***, Knockdown of microRNA-378 expression significantly increased the expression of FOXN3. ***B-C***, The mRNA expression level of FOXN3 relative to GAPDH in human OSCC tissues and paracancerous tissues, as well as OSCC cell lines, were detected using RT-qPCR. ***D***, A negative correlation was found between microRNA-378 and FOXN3 in tumor samples. A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

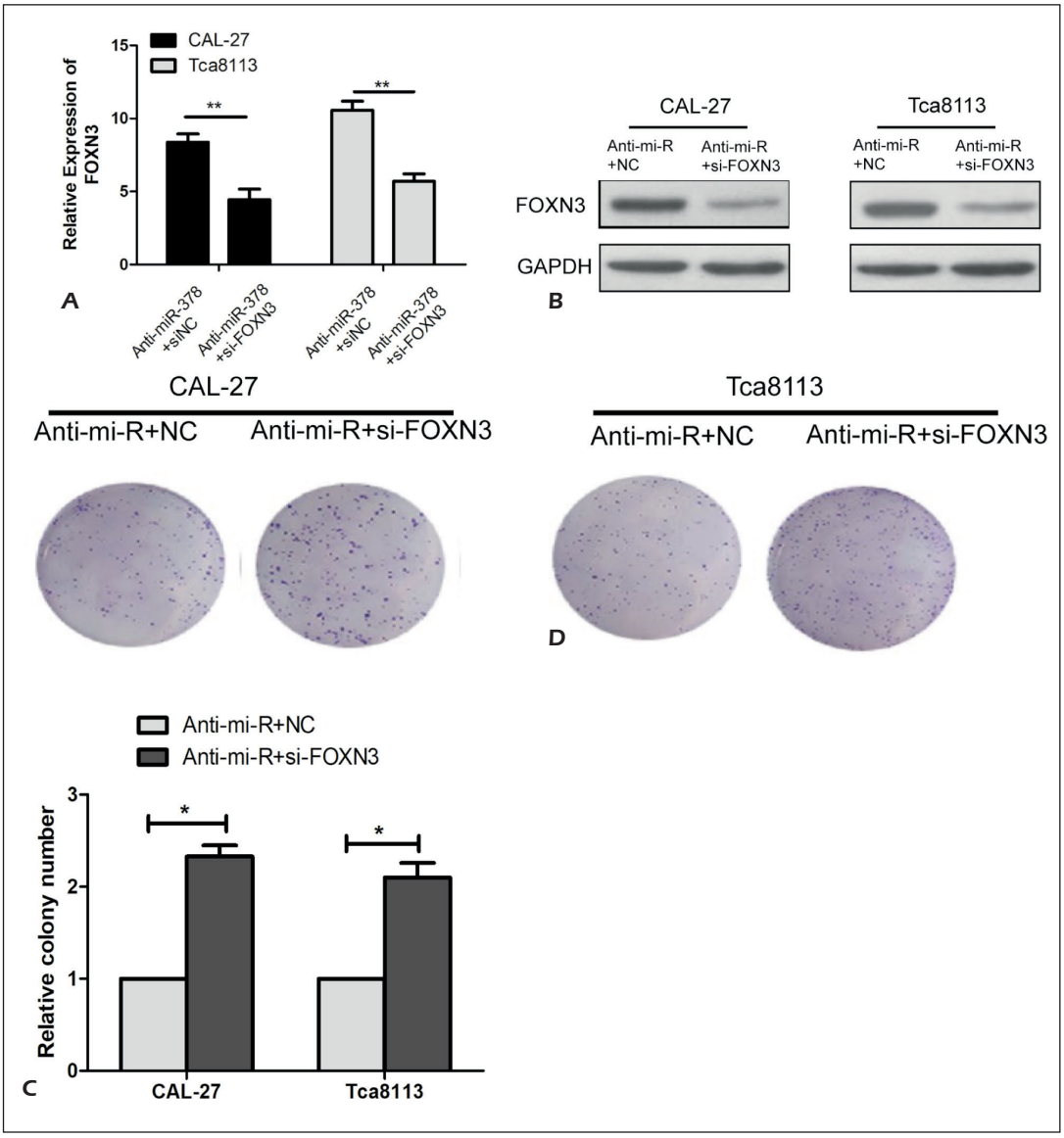

Figure 5. ***A***, The expression of FOXN3 was verified by RT-qPCR in co-transfected cell lines. ***B***, Western blot was used to verify the expression of FOXN3. ***C-D***, The roles of microRNA-378 and FOXN3 in the regulation of OSCC cell proliferation were examined by cell colony formation assay. A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

Discussion

Squamous cell carcinoma is the most common malignancy in the oral cavity, and its incidence increases year by year[1-3]. At present, surgical resection is the preferred option for OSCC, accompanied by radiotherapy, chemotherapy, and other adjuvant treatments. However, OSCC presents high recurrent and metastatic rates even after comprehensive treatments[4,5]. The complex pathogenesis of OSCC involves multiple factors and pathways, such as microRNAs[11-14]. MicroRNAs are a class of endogenous non-coding small RNAs. They could regulate target gene expressions at the post-transcriptional level, thus participating in proliferation, differentiation, drug resistance, and other pathological functions[10,13,15]. Also, differentially expressed microRNAs are closely related to the occurrence and progression of tumors[10,11]. Hence, microRNAs exert important functions in tumor screening, early diagnosis, early treatment, and prognostic evaluation. They provide molecular support for individualized treatment of cancer patients[13]. Scholars[11,16,17] have

found multiple abnormally expressed microRNAs in OSCC. Therefore, finding abnormally expressed microRNAs in OSCC and analyzing their correlation with prognosis of OSCC contribute to improve clinical outcomes of OSCC patients.

Ideal tumor markers should be non-invasive with high specificity, sensitivity, and stability. As a result, serum RNAs are considered to be satisfactory hallmarks[18,19]. More than 1000 microRNAs have been found in the human genome by gene cloning or bioinformatics[10-12]. MicroRNA is a single-stranded small-molecule RNA with a length of about 18-25 nt. It has a hairpin structure and contains 70-90 bases in size. MicroRNA is processed from a double-stranded RNA precursor containing a stem-loop structure by Dicer enzyme[10,13]. Multiple cellular activities are regulated by microRNAs, including proliferation, apoptosis, metastasis, and differentiation[11]. Dysfunctional microRNAs may lead to the incidence of various diseases[15,16].

MicroRNAs are highly conservative, timing and tissue-specific. Serum microRNA is hard to be degraded even under severe physical conditions[20,21]. MicroRNAs are capable of regulating multiple pathological and physiological processes[10,12]. Researches have pointed out that microRNAs are characteristically expressed in different types of tumor tissues and peripheral blood[11]. Many microRNAs are associated with tumor etiology, pathology, clinical staging, tumor hormone secretion, tumor resistance, and prognosis, which are needed to be further confirmed[11]. This study investigated the clinical features of microRNA-378 in OSCC and its role in the malignant progression of OSCC. We first verified the expression of microRNA-378 in 96 pairs of OSCC tissues and paracancerous tissues. The results showed that microRNA-378 is highly expressed in OSCC. MicroRNA-378 expression was positively correlated with tumor staging, lymph node metastasis, distant metastasis, and poor prognosis of OSCC patients. Hence, we believed that microRNA-378 may serve as an oncogene in OSCC. Subsequently, microRNA-378 knockdown in OSCC cells inhibited migratory and invasive abilities. The specific molecular mechanism, however, is still unclear.

FOXN3 is the only protein in the FOXN family that does not have a transcriptional binding domain, and its carboxy terminus acts as a transcriptional repressor in various human cells. FOXN3 possesses an indispensable role in tissue and cell development[22,23]. Previous studies have found that FOXN3 lacks a transcriptional activation domain, indicating that it mainly plays a role in transcriptional regulation by inhibiting the expressions of related genes. Researches have shown that the carboxy terminus of FOXN3 can be fused to a heterologous DNA binding domain and continuously inhibit the transcription of reporter genes. FOXN3 can bind to Sin protein in Sin3/Rpd3 histone deacetylase (HDAC) complex, inhibit histone deacetylation, repair G2/M blockade induced by DNA damage, and compensate for cell cycle checkpoint defects[24,25]. In a study on type I multiple endocrine neoplasia (MEN1), it was found that FOXN3 shows a remarkable effect in patients with invasive pancreatic neuroendocrine tumors (PNENs)[25-27]. The abnormal expression of FOXN3 can affect the metastasis of tumor cells, which may promote migration by regulating the expressions of adhesion molecules. On the other hand, FOXN3 accelerates the metastasis of tumor cells by initiating multiple metastatic pathways[22-24]. In the present work, we found a mutual regulation between microRNA-378 and FOXN3 through rescue experiments. We indicated that microRNA-378 promotes malignant progression of OSCC by regulating FOXN3. In-depth explorations on FOXN3 functions will contribute to better diagnosis, treatment, and prognosis of OSCC.

Conclusions

MicroRNA-378 is highly expressed in OSCC, which is significantly associated with tumor staging, distant metastasis, and poor prognosis of OSCC. It is shown that microRNA-378 may promote malignant progression of OSCC by regulating FOXN3.

Conflict of Interests

The authors declare that they have no conflict of interest.

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