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ORIGINAL ARTICLE

# MicroRNA-182-5p Modulates Oral Squamous Cell Carcinoma Migration and Invasion Via Targeting MTSS1 Gene

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## Abstract

Oral squamous cell carcinoma (OSCC) characterized with invasive growth, local metastasis and later stage diagnosis was a common malignancy in head and neck region. The aim of this study was to explore the relationship between miR-182-5p and OSCC, which will contribute to find potential biomarker for OSCC metastasis. MiR-182-5p expression level was detected by the quantitative real-time PCR (qRT-PCR). Cell migration and invasion ability were examined by scratch and transwell assay. Loss-of function together with luciferase reporter assay were used to verify the miR-182-5p modulated OSCC cells migration and metastasis was mediated by MTSS1. The expression of MTSS1 protein was examined by western blotting. MiR-182-5p up-regulated in OSCC, was involved in the migration and invasion of OSCC and the increased miR-182-5p expression was correlated with lower OSCC differentiation grade, higher T and N stage. Bioinformatics analysis predicted MTSS1 gene was a potential target of miR-182-5p. Following co-transfection, qRT-PCR, luciferase activities assay and western blotting confirmed that MTSS1 gene was a direct target of miR-182-5p and silence of MTSS1 could reverse the effects of miR-182-5p on OSCC migration and invasion. MiR-182-5p was up-regulated in OSCC and the ability of miR-182-5p to promote MTSS1 repression may precipitate in the OSCC through bypassing cell migration and invasion control.

**Keywords** Oral squamous cell carcinoma · miR-182-5p · MTSS1 · Migration · Invasion

## Introduction

Oral cancer is the general name of the malignant tumors occurring in the oral cavity, the majority of which belong to oral squamous cell carcinoma (OSCC). Up till now, oral cancer is prevalent among Asian countries and the incidence rate is still rising. OSCC is characterized with invasive growth and local metastasis, high recurrence, poor prognosis and often diagnosed at a late stage [1, 2]. Although tremendous progress has been made in the diagnosis and clinical treatment such as surgery resection in combination with radiotherapy, neoadjuvant chemotherapy and targeted biological therapy, the general OSCC patients survival rate remained slightly improved, with a 3-year of 50% ~ 75% and a 5-year of around 50% [3, 4]. Therefore, it's imperative to find suitable prognostic or metastatic biomarkers and therapeutic strategies to improve OSCC patients' prognosis and survival rates.

MicroRNAs (miRNAs), a class of small molecule RNAs ranging from 20 to 24 nucleotides with little ability to encode protein, usually play essential part in posttranscriptional manipulation of targeted genes. Evidencing suggested that miRNAs emerge complex physiological functions and participate in complex diseases generation especially in various types of malignancies [5–10]. Some studies have showed that miR-182-5p was up-regulated and acted as an oncogene correlated with prostate cancer, bladder cancer, gastric cancer and breast cancer [11–15]. However, *Xu X* et al reported that miR-182-5p was down-regulated in renal cell carcinoma and functioned as a tumor suppressor [16]. The reasons for the contradictory roles of miR-182-5p might due to the different binding targets and signaling pathway regulation in different varieties

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of tumors. Up till now, no published literatures between miR-182-5p and OSCC has been reported.

In view of the important value of miRNAs in tumor prognosis and treatment prediction, we attempted to explore the regulatory roles and molecular mechanisms of miR-182-5p in OSCC by using bioinformatics analysis together with loss-of function technology in this study, which will contribute to find potential biomarker for OSCC therapy.

## Materials and Methods

### Clinical Specimens

Totally 60 patients pathologically diagnosed with OSCC no matter any kind of therapeutic measures had been selected in this study (Stomatology Hospital affiliated to China Medical University). Samples were obtained from surgery patients and immediately stored at −80 °C. Informed consent for all the patients was obtained and the protocol has been approved by the Ethics Committees of Stomatology Hospital affiliated to China Medical University. Detailed patients' information is shown in Table 1.

### RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA including miRNAs was extracted by using miRNeasy Mini Kit (Qiagen, Ger) and DNA was removed with RNase-free DNase Set (Qiagen, Ger) following manufacturer's instructions. After quantified by NanoDrop 2000C (Life Technologies, USA), the RNA was reversely transcribed into cDNA and amplified on a 7500 real time PCR System (Applied Biosystems, USA) according to the manufacture's instruction (Life Technologies, USA). U6 and GAPDH were used as reference controls respectively and the RNA expression level was determined through the $2^{-\Delta\Delta CT}$ method.

**Table 1** Correlation between miR-182-5p. expression and clinical parameters in OSCC patients

| Factors | | | Mean ± SD | *P* value |
|---|---|---|---|---|
| Gender | Male | 37 | 3.29 ± 0.58 | 0.7593 |
| | Female | 23 | 3.24 ± 0.66 | |
| Age | ≤50 | 16 | 3.36 ± 0.63 | 0.5067 |
| | >50 | 44 | 3.24 ± 0.61 | |
| Position | Tongue | 29 | 3.10 ± 0.57 | 0.2992 |
| | Gingiva | 14 | 3.47 ± 0.67 | |
| | Buccal mucosa | 7 | 3.52 ± 0.59 | |
| | Lip | 4 | 3.22 ± 0.64 | |
| | Palate | 6 | 3.36 ± 0.71 | |
| Differentiation | well/moderately | 43 | 3.14 ± 0.53 | 0.0060 ** |
| | poorly | 17 | 3.61 ± 0.68 | |
| T stage | T1 and T2 | 39 | 3.12 ± 0.57 | 0.0121 * |
| | T3 and T4 | 21 | 3.54 ± 0.65 | |
| N stage | N0 | 40 | 3.09 ± 0.67 | 0.0045 ** |
| | N1-N3 | 20 | 3.62 ± 0.62 | |

* $P < 0.05$, ** $P < 0.01$

### Cell Culture and Transfection

The cell lines including Tca8113, Cal27 OSCC cell lines and normal oral keratinocyte cell line (hNOK) were purchased from Chinese Academy of Sciences (Shanghai, China) and all these cell lines were cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) as previously described [17]. As for transfection, cells were seeded into a 6-well plate one day before, and the Lipofectamine™ 3000 (Life Technologies, USA) were used to finish the process at approximately 60% confluence according to the manufacturer's protocol. Both mimics of miR-182-5p (agomiR-182-5p and antagomiR-182-5p) and their corresponding negative controls (agomiR-NC and antagomiR-NC) were designed and synthesized by Life Technologies Corporation. The pSilencer 4.1-CMV puro-MTSS1 (pS-MTSS1) vector and corresponding negative control (pS-NC) were constructed by GeneScript Corporation (Nanjing, China). The transfection efficiency was verified using the qRT-PCR assay.

### Cell Migration and Cell Invasion Assay

We use scratch assay to detect cell migration. Scratches were made by using a 100 μl pipette tip at 80% cells confluence post-transfection. The wound closure was observed by exhaustively washed with PBS and the migration rate was calculated according to the ratio of migrated cell surface area to total surface area.

Cell invasion assays were performed with Transwell chamber (Costar, USA). To create an invasion assay, the pores in the membrane (0.8 μm) were blocked with fibronectin (10 μg/ml, 50 μl) for 2 h at 37 °C to mimic the typical matrices that tumor cells encounter during the invasion process in vivo [18]. Briefly, $5 \times 10^4$ cells with 10% serum medium were seeded to the top chambers and the bottom chambers were filled with 500 μl supernatant of human NIH3T3 which was collected when exchanging the culture medium in triplicate. After a 12 h incubation in 37 °C, cells occurring transmembrane were stained with hematoxylin and eosin. Finally, five random fields under microscope for each group were selected and counted.

### 3′ UTR Luciferase Reporter Assay

The luciferase reporter vectors, no matter pmiR-MTSS1-mut1 (containing mutant binding site 1), pmiR-MTSS1-mut2 (containing mutant binding site 2), pmiR-MTSS1-mut1&2 (containing mutant binding site 1 and 2) or pmiR-MTSS1-wt (containing wild-type binding site) were designed and synthesized by GenScript Corporation. Then cells were co-transfected with miR-182-5p and MTSS1 reporter vectors using Lipofectamine™ 3000 Reagent (Life Technologies, USA). The luciferase reporter system (Promega, USA) was applied to detect the combination of miR-182-5p and MTSS1 48 h after co-transfection.

### Western Blotting

Western blotting was finished according to the previously described [17]. Total 20 mg of protein was subjected into SDS-PAGE electrophoresis and transferred to NC membranes. The membranes were probed with indicated antibodies of MTSS1 (#4385) and β-tubulin (#2128) from Cell Signaling Technology at 4 °C overnight and then incubated with secondary antibodies (IRDye-conjugated anti-mouse and anti-rabbit IgG from LI-COR) for 1 h at room temperature. Immunoblots were evaluated using the Odyssey imaging system. Image J Software (NIH, USA) was used to quantify the protein density.

### Statistical Analysis

All statistical analyses were performed with SPSS software (IBM, USA) and GraphPad Prism 5.0 (GraphPad Software, USA). Student's *t* test was used to compare differences between two groups. Measurement data were reported as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance (*ANOVA*) was used to finish the comparisons among multiple groups. All tests were two-tailed and a *p value* < 0.05 was considered statistically significant (* or #, $P < 0.05$; ** or ##, $P < 0.01$).

## Results

### miR-182-5p Was Up-Regulated and Correlated with OSCC Progression

To investigate the significance of miR-182-5p in OSCC, we first detected the miR-182-5p expression level in 60 clinical samples and 2 OSCC cell lines. qRT-PCR results showed that miR-182-5p was highly expressed in OSCC tissues with a significant 3.27 ± 0.64 fold up- regulation than that in its matched controls (Fig. 1a, $P < 0.01$). Meantime, miR-182-5p expression was significantly increased in both OSCC cell lines (Tca8113 and Cal27) while compared with its expression in hNOK cells (Fig. 1b, $P < 0.01$).

To get assessment of the clinical significance of miR-182-5p, we decided 60 patients' tissues into 2 groups based on the median value and evaluate the correction between miR-182-5p level and clinic-pathological parameters. The results showed that the increased miR-182-5p expression was closely correlated with lower differentiation grade, higher T and N stage (Table 1, $P < 0.05$), while was independent of other parameters including gender, age and position in OSCC (Table 1, $P > 0.05$). These results revealed that miR-182-5p participated in the progression of OSCC and could be a potential prognostic biomarker for OSCC patients.

### Silence of miR-182-5p Inhibited Migration and Invasion in OSCC Cells

In order to further investigate the miR-182-5p function, we examined OSCC cells migration and invasion ability using the

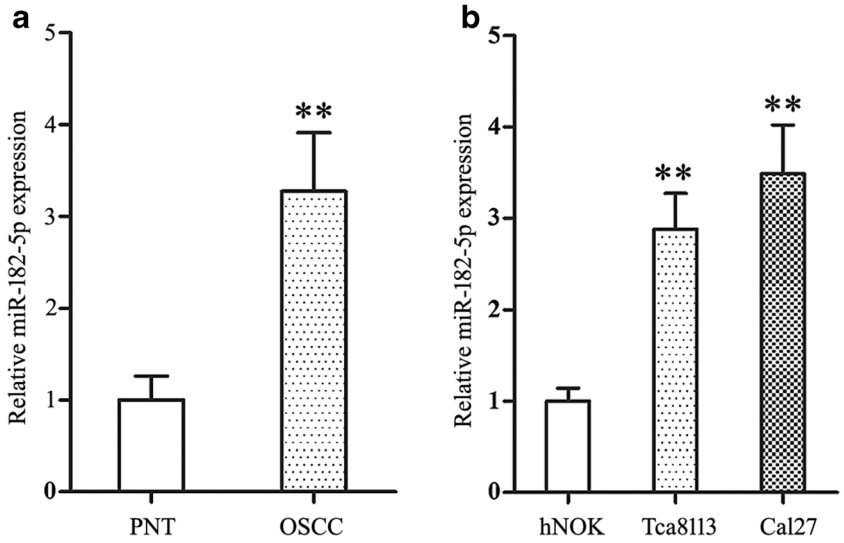


**Fig. 1 miR-182-5p was up-regulated in OSCC. a** The expression of miR-182-5p in PNT and OSCC specimens. PNT = paraneoplastic normal tissues, OSCC = oral squamous cell carcinoma. ** $P < 0.01$ vs PNT group. **b** The expression of miR-182-5p in hNOK and OSCC cells (Tca8113 and Cal27). hNOK = normal oral keratinocyte cell line. ** $P < 0.01$ vs hNOK group

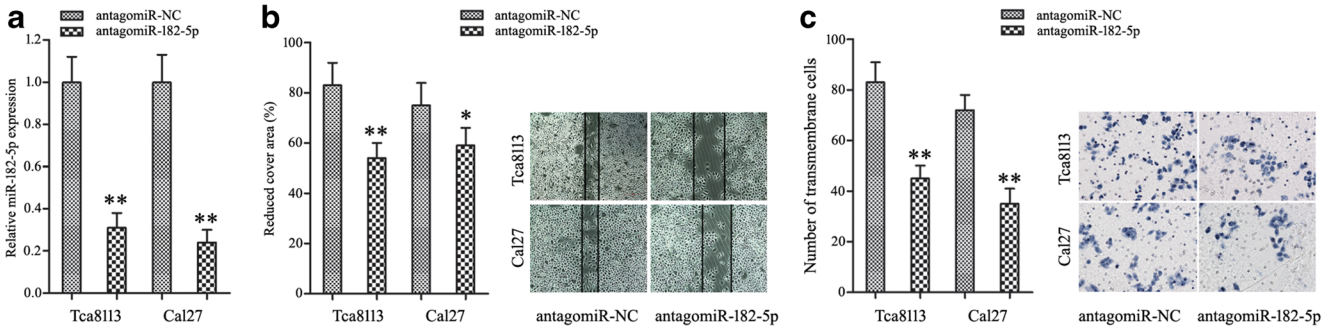


**Fig. 2 The effect of miR-182-5p on OSCC cells migration and invasion. a** qRT-PCR detected the transfection efficiency of miR-182-5p in Tca8113 and Cal27 cells. **b** The migration ability of Tca8113 and Cal27 cells with miR-182-5p silence. **c** The invasion ability of Tca8113 and Cal27 cells with miR-182-5p silence. * $P < 0.05$ vs matched antagomiR-NC group, ** $P < 0.01$ vs matched antagomiR-NC group

loss-of function technology to silence miR-182-5p expression. We used qRT-PCR to verify the miR-182-5p knock-down efficiency after antagomiR-182-5p was transfected into both Tca8113 and Cal27 cells. The results showed that cells transfected with antagomiR-182-5p had a significantly lower expression level compared with that in the antagomiR-NC group (Fig. 2a, $P < 0.01$).

And then, we used wound healing assay and Transwell chamber to detect the miR-182-5p effect on OSCC cells migration and invasion abilities. Would healing assay showed that antagomiR-182-5p transfection cells with decreased miR-182-5p expression showed obvious suppression of wound closure in comparison to control cells (Fig. 2b, $P < 0.01$). Consistently, transwell chamber assay indicated that antagomiR-182-5p transfection cells had a significant inhibition of cell invasion in both OSCC cell lines compared with control cells (Fig. 2c, $P < 0.01$). Our study revealed that manipulating miR-182-5p expression could influence OSCC cells invasion and migration, which further indicated that miR-182-5p was involved in the migration and invasion of OSCC.

## MTSS1 Was a Direct Target of miR-182-5p in OSCC Cells

Metastasis suppressor 1 (MTSS1), initially identified in bladder cancer, was recognized as an essential tumor suppressor through inhibition of cancer metastasis. It has been predicted as a potential target for miR-182-5p predicted by TargetScan and microRNA.org. In accordance with the published studies [13, 19], we choose position 262–268 and 1928–1935 bp in MTSS1 3'UTR as the binding sites of miR-182-5p for the dual-luciferase reporter assay (Fig. 3a). As shown in Fig. 3b, agomiR-182-5p could significantly decrease the luciferase activity of pmiR-MTSS1-wt, pmiR-MTSS1-mut1 and pmiR-MTSS1-mut2 ($P < 0.01$), while could not affect the luciferase activity of pmiR-MTSS1-mut1&2 activity when compared with agomiR-NC which had no effect on the luciferase activity of pmiR-MTSS1, no matter wide type or mutant vectors. Since MTSS1 protein is relatively lower expressed in OSCC cells compared with hNOK cells [17], we performed western assay to monitor the alteration of MTSS1 expression after antagomiR-182-5p transfection. As shown in Fig. 3c,

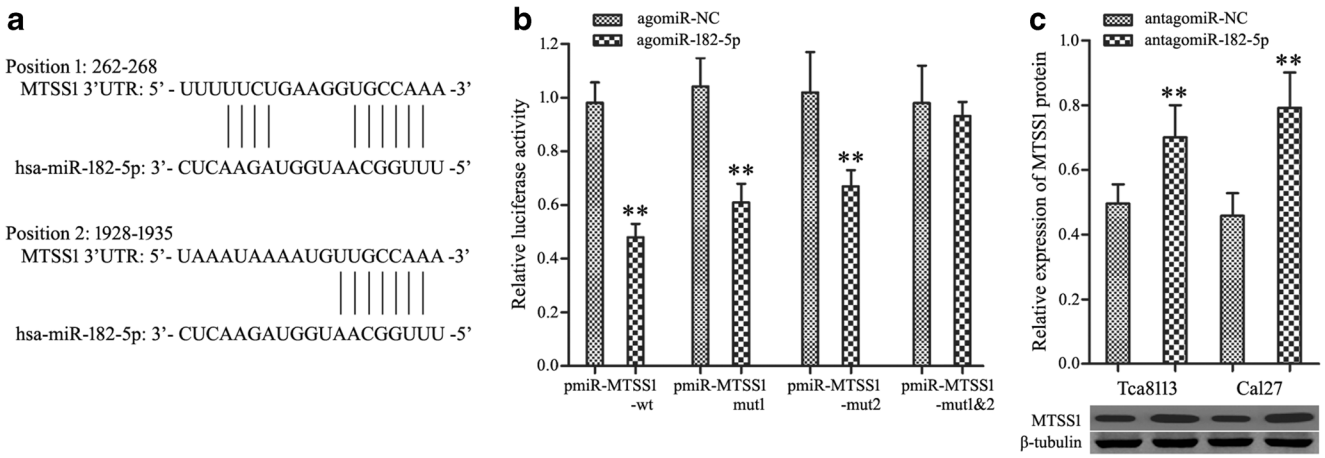


**Fig. 3 MTSS1 was a direct target of miR-182-5p in OSCC cells. A**: The binding sites of miR-182-5p in the 3' UTR of MTSS1. **B**: The effect of miR-182-5p on the luciferase activity of pmiR-MTSS1-mut and pmiR-MTSS1-wt. ** $P < 0.01$ vs agomiR-NC + pmiR-MTSS1-mut1&2 groups. **C**: The relative expression of MTSS1 protein in Tca8113 and Cal27 cell lines. ** $P < 0.01$ vs matched antagomiR-NC group

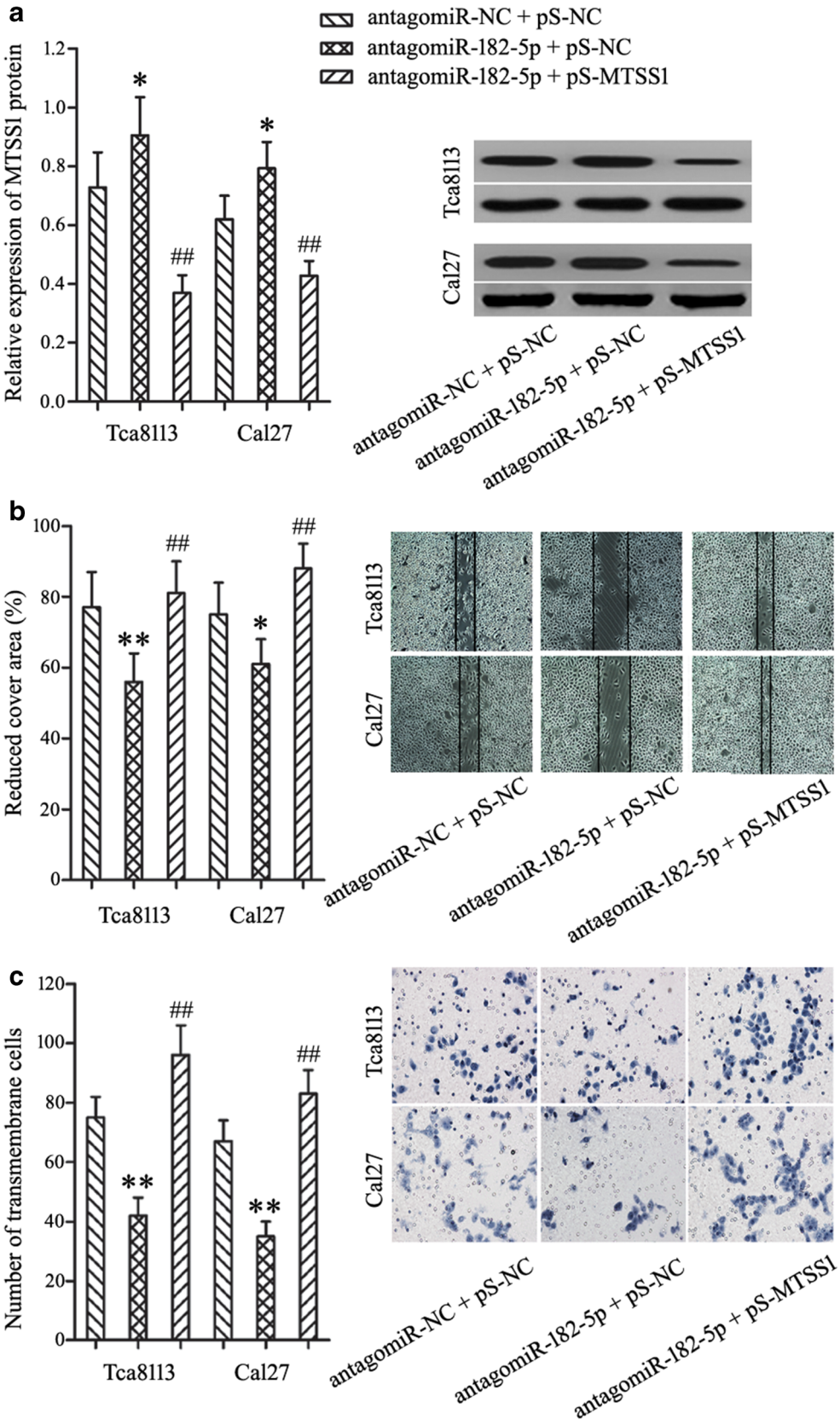


**Fig. 4 The modulation of miR-182-5p and MTSS1 on OSCC cells migration and invasion. a** The relative expression of MTSS1 protein in Tca8113 and Cal27 cells with miR-182-5p and MTSS1 transfection. **b** The migration ability of Tca8113 and Cal27 cells with miR-182-5p and MTSS1 transfection. **c** The invasion ability of Tca8113 and Cal27 cells with miR-182-5p and MTSS1 transfection. * $P < 0.05$ vs antagomir-NC + pS-NC, ** $P < 0.01$ vs matched antagomir-NC + pS-NC, ## $P < 0.01$ vs matched antagomir-182-5p + pS-NC

transfection of antagomiR-182-5p could significantly increase MTSS1 expression in both cell lines compared with cells transfected with antagomiR-NC ($P < 0.01$). Collectively, these results suggest that miR-182-5p directly manipulated MTSS1 expression and MTSS1 gene is a direct target of miR-182-5p.

### MiR-182-5p Modulated OSCC Cells Migration and Invasion by Silencing MTSS1

To determine whether the miR-182-5p mediated OSCC migration and invasion was regulated by MTSS1, OSCC cells were co-transfected with antagomiR-182-5p and pS-MTSS1 or matched controls. Co-transfection of antagomiR-182-5p and pS-MTSS1 led to an obvious decrease of MTSS1 protein expressions in both Tca8113 and Cal27 cell lines (Fig. 4a, $P < 0.01$). The wound healing and transwell chamber assays results revealed that co-transfection of antagomiR-182-5p and pS-MTSS1 significantly increased the migration and invasion of OSCC cells. Exactly, the migration rate of pS-MTSS1 and antagomiR-182-5p co-transfected cells was accelerated obviously compared with the control groups (Fig. 4b, $P < 0.01$). Moreover, the number of cells in pS-MTSS1 and antagomiR-182-5p co-transfected group that invaded into the polycarbonate was increased in comparison with controls (Fig. 4c, $P < 0.01$). Together, our findings indicated that MTSS1 knockdown restored mostly the effecting of miR-182-5p knockdown on migration and metastasis of OSCC cells, MTSS1 mediated the regulation of miR-182-5p on OSCC cells migration and metastasis.

## Discussion

It is well known that abnormal expression of miRNAs can regulate various pivotal biological behaviors, including proliferation, apoptosis, migration and invasion, and many studies have demonstrated that the significant role of miRNAs in cancer were functioned through regulating target mRNA [19–23]. In this study, we revealed that miR-182-5p, up-regulated in OSCC, was involved in the migration and invasion of OSCC. Moreover, increased miR-182-5p expression was closely correlated with lower OSCC differentiation grade, higher T and N stage, which highly indicated that miR-182-5p might be a potential microRNA biological marker for OSCC.

In previous studies, miR-182-5p was detected in various types of cancer and its possible function has been identified. For instance, *Tsuchiyama K* et al. reported that miR-182-5p was statistically up-regulated in higher prostate cancer grade [24] and *Xue* et al. found that miR-182-5p could promote cancer cells migration and invasion in glioma and gastric cancer [25]. Based on loss-of function technology and migration/invasion assays, our study initiatively revealed that, consistent with emerging evidence in other types of cancer, miR-182-5p participate in the migration and invasion in both OSCC cell lines.

In consideration of the prominent roles of miRNAs in tumorigenesis, we speculated that miR-182-5p might regulate invasion of OSCC cells through modifying its target gene. According to bioinformatics analysis combined with previous published studies, MTSS1 gene was predicted to be a potential target of miR-182-5p. As we all known, MTSS1 has been confirmed to be a metastasis suppressor gene in diverse malignant tumors [17, 26, 27]. Moreover, *Shi W* et al. reported that depressed expression of MTSS1 gene was significantly associated with nodal metastases and higher clinical stage, which resulted in tumor recurrence and poor prognosis in intrahepatic cholangiocarcinoma patients [28]. Through a series of experiments, our findings confirmed that miR-182-5p modulated migration and invasion of OSCC cells by silencing MTSS1 expression. Firstly, luciferase reporter assay confirmed that miR-182-5p inhibited luciferase activity of MTSS1 gene through direct combination with its 3'UTR region. Secondly, MTSS1 protein expression could be increased by silence of miR-182-5p and co-transfection of antagomiR-182-5p and pS-MTSS1 led to an obvious decrease of MTSS1 protein expression in Tca8113 and Cal27 cells. Thirdly, antagomiR-182-5p and pS-MTSS1 co-transfection could significantly promote the migration and invasion abilities of Tca8113 and Cal27 cell lines. Collectively, these data strongly suggest that miRNA-182-5p modulates OSCC migration and invasion via targeting MTSS1 gene.

In summary, we identified that miR-182-5p was up-regulated in both OSCC tissues and cell lines, and the ability of miR-182-5p to suppress MTSS1 expression might be involved in the OSCC occurrence and development through manipulating cell migration and invasion.

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**Author Contributions** YG, XQ, LZ and RS carried out the in vitro studies and performed the statistical analysis. YG and XQ conceived of the study and draft the manuscript. All authors read and approved the final manuscript.

### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Approval** This study has been carried out with the approval of the Ethics Committees of School of Stomatology. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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