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MiR-126 inhibits cell migration and invasion by targeting ADAM9 in oral squamous cell carcinoma

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Abstract. – OBJECTIVE: Oral squamous cell carcinoma (OSCC), the most frequent head and neck cancer, has a high potential for metastasis. MiR-126 plays an important role in the tumorigenesis of many tumors; however, there were little studies in OSCC. The purpose of our study was to explore how miR-126 and ADAM9 worked on migration and invasion in OSCC.

PATIENTS AND METHODS: The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was applied to detect the mRNA level of miR-126 and ADAM9. The transwell assay was utilized to calculate the migratory and invasive capacities in the OSCC cells. The luciferase report assay was utilized to verify that ADAM9 was a direct target of miR-126.

RESULTS: MicroR-126 was downregulated in OSCC tissues and cell lines SCC25 and HSC3. ADAM9 was predicted to be a direct target of miR-126 and was upregulated in the OSCC cells. In addition, miR-126 suppressed the migratory and invasive ability via mediating the expression of ADAM9 by directly targeting its mRNA 3'-noncoding region (UTR), whose partial functions was reversed by ADAM9.

CONCLUSIONS: MiR-126 inhibited the migratory and invasive capacities of OSCC by directly targeting the 3'-UTR of ADAM9 mRNA. It is suggested that miR-126/ADAM9 axis may play an essential role in inhibiting the abilities of migration and invasion in oral squamous cell carcinoma cells.

Key Words:
Oral squamous cell carcinoma, Migration, Invasion, MiR-126, ADAM9.

Introduction

Oral squamous cell carcinoma (OSCC), ranking the sixth most common cancer worldwide, is the most frequent head and neck cancer[1]. Unlike other types of cancers, OSCC has a great metastatic potential and is often restricted to the head and neck area. In the past thirty years, the 5-year overall survival has a bare improvement, and it was less than 50%[2,3]. Although patients in the early stage account for more than 80%, approximately 70% of OSCC patients at an advanced-stage cannot be cured through therapy[4]. Whereas, the molecular mechanism of migration and invasion of OSCC is still not clear.

MicroRNAs (miRNA) are 22-28 nucleotides small noncoding RNAs, which could inhibit gene expression through directly binding the 3'-UTR of mRNA or cutting mRNA of the target gene at a post-transcriptional level[5-9]. MicroRNAs have been reported to play great roles in the progression and metastasis of multiple tumors. MiR-126, an endothelial-specific miRNA which located within intron 7 of epidermal growth factor-like domain 7 (EGFL7), has been found to be involved in the process of blood vessel formation[10-13]. MiR-126 has been reported[11,14-16] to be downregulated and play important roles of suppression in serious tumors including non-small-cell lung cancer, bladder cancer, hepatocellular carcinoma, and gastric cancer. MiR-126 could inhibit the proliferation and angiogenesis through the downregulation of EGFL7 in the hepatocellular carcinoma[11]. What's more, miR-126 suppresses the viability and invasion *via* targeting IRS-1 in a diabetic retinopathy model[17]. In addition, miR-126 inhibits the proliferation, colony formation and induces cell apoptosis in oral squamous cell carcinoma cell OSCC-15[18]. There are plenty of target genes of miR-126 including IRS-1, TGFβ, VEGF-A, and ADAM9[15,17,19-21]. It has been reported that miR-126 suppresses cell growth, invasion, and migration *via* the downregulation of ADAM9 in osteosarcoma cells[21].

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A disintegrin and metalloproteinases (ADAMs) family play a great role in the proteolytic process, which could be divided into membrane-anchored group and secreted-type proteins group. ADAM9, a member of ADAMs family, has been reported to be overexpressed and dysregulated in several types of cancers, including bladder cancer, breast cancer, hepatocellular carcinoma, lung cancer, and glioblastoma[15,22-25]. Qin et al[22] had discovered that the upregulation of ADAM9 could inhibit the proliferation, migration, and invasion of breast cancer cells. In lung cancer, Lin et al[24] had reported that ADAM9 promotes cell progression through vascular remodeling. In the oral squamous cell carcinoma, ADAM9 has been reported to be overexpressed; however, little studies illuminate how it works[26]. We began this research to observe the roles of miR-126 and ADAM9 in the oral squamous cell carcinoma. In our study, we aim to detect the functions of miR-126 and ADAM9 on migration and invasion in the oral squamous cell carcinoma cells.

Patients and Methods

Patients and Clinical Samples

We obtained paired oral squamous cell carcinoma tissues and the corresponding paracancerous tissues from 50 OSCC patients who underwent surgery in the Zhongshan Hospital, affiliated to Xiamen University from 2015 to 2017. Fresh tissues were promptly frozen in liquid nitrogen and stored in -80°C freezer after the operation. All patients had no radiotherapy nor neoadjuvant chemotherapy before the surgery. All the specimens of this study got the informed consent from patients. The investigation was approved by the Ethical Committee of Zhongshan Hospital Affiliated Xiamen University.

Cell Lines and Culture Condition

A normal oral epithelial cell line CGHNK2 and two OSCC cell lines (SCC25 and HSC3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco, Rockville, MD, USA) cultured at 37°C in a humidification atmosphere containing 5% of CO_2.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total mRNAs or miRNAs were extracted by the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) or the MIRcute Extraction and Separation of miRNAs Kit (Tiangen, Beijing, China). The PrimeScript™ II 1st Strand complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (TaKaRa, Dalian, China) was applied to synthesize the first cDNA chain through reverse transcription. And then, the SYBR Premix Kit or the SYBR Prime Script miRNA qRT-PCR Kit (both purchased from TaKaRa, Dalian, China) were employed to perform the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). We calculated the levels of mRNA and miRNA by the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 as internal references. The primers were synthesized as follows: ADAM9 forward 5'-GGAAGAGTGTGACTGTGGTAC-3' and reverse 5'-CCTCGGCATAAAGTACCTCC-3'; GAPDH forward reverse 5'-ACAACTTTGGTATCGTGGAAGG-3' and 5'-GCCATCACGCCACAGTTTC-3'; miR-126 forward 5'-UCGUACCGUGAGUAAUAAUGCG-3' and reverse, 5'-CAUUAUUACUCACGGUACGAUU-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. Amplification conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and at 60°C for 60 s.

Transfection

The miR-126 mimic or the inhibitor were transfected to upregulate/downregulate the expression of miR-126 in OSCC cells SCC25 and HSC3. Meanwhile, ADAM9 was overexpressed using pcDNA3.1-ADAM9 plasmid in OSCC cell lines SCC25 and HSC3.

We inoculated suitable cells with a density of 1×10^5 into a 6-well plate and we cultured them in 37°C incubator overnight. The Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) was employed to transfect the special vectors in OSCC cells SCC25 and HSC3.

Protein Extraction and Western Blotting

Total proteins were lysed from the OSCC tissues and cell lines by the Radioimmunoprecipitation Assay (RIPA) lysis buffer supplemented with proteinase inhibitor (both from Beyotime, Shanghai, China). Proteins with the same quality, which was quantified using the Bradford

Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), were separated by 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked in Tris-Buffered Saline and Tween (TBST) at room temperature for 1 h, incubating with mouse anti-ADAM9 polyclonal antibody (1:1000, ab186833, Abcam, London, UK) overnight at 4°C, which normalized with anti-GAPDH mouse monoclonal antibody (1:2000, G8795, Sigma-Aldrich, St. Louis, MO, USA). Washed the extra antibody with TBST and blocked in 5% of skim milk, the membranes were incubated with mouse horseradish peroxidase-conjugated antibody (1:5000; sc-362280, Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing horseradish peroxidase-conjugated at room temperature for about 2 h. Odyssey CLx Western Blot Detection System (LI-COR Biosciences, Lincoln, NE, USA) was applying to perform the interest proteins (ADAM9 and GAPDH).

Transwell Assay

The transwell chamber (Corning Incorporated, Corning, NY, USA) which covered with 20 μL Matrigel (Clontech, Mountain View, CA, USA) on the surface of the membrane was placed into a 24-well plate. For invasion assays, 200 μL cell suspensions which were suspended by a serum-free medium were seeded into the top chamber, while the bottom chamber was filled with 500 μL medium containing 15% of FBS served as chemoattractant. Once incubated for 48 h at 37°C, we removed the cells still at the upper surface of the membrane using a cotton swab; we fixed the invading cells using 4% of formaldehyde, and then, stained the cells by 0.1% of crystal violet solution at room temperature. An inverted microscope was employed to photograph the invading cells.

Luciferase Reporter Assay

The online software TargetScan (http://www.targetscan.org/vert_71/) was applied to predict potential target genes of miR-126, and ADAM9 was predicted to be one. To verify this conjecture, the nucleotide fragments of the 3'-untranslated region (3'-UTR) of ADAM9 mRNA which contained the predicted binding site of miR-126 were cloned and inserted into the pmirGlo vector (pmirGlo-ADAM9-WT; WT). Then, once the target sequences had been changed with the QuickChange Multi Site-Directed Mutagenesis Kit (Santa Clara, CA, USA) from 5'-GGUACG-3' to 5'-UUGGAA-3', we inserted the mutated sequences into the pmirGlo vector (pmirGlo-ADAM9-MUT; MUT). Next, the miR-133a mimic and WT or MUT were co-transfected into oral squamous cell carcinoma cell lines SCC25 and HSC3. After 48 h, the Luciferase reporter activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis used the Student's *t*-test or ANOVA and Scheffe test for the comparison between two groups or multiple groups. The correlation between miR-126, ADAM9 and clinicopathologic characteristics of OSCC was performed by the Pearson χ^2-test. *p*-value less than 0.05 showed a statistical significance for all the results.

Results

Expression of MiR-126 and ADAM9 in OSCC Tissues and Cell Lines

The level of miR-126 in 51 paired of OSCC tissues and corresponding paracancerous tissue samples were measured using qRT-PCR. We discovered that miR-126 was significantly low expressed in all 51 OSCC tissue samples compared with the paracancerous tissues ($p<0.001$) (Figure 1A). Furthermore, we also detected the mRNA level of miR-126 in OSCC cell lines SCC25 and HSC3 and a normal oral epithelial cell line CGHNK2. Compared with the normal oral epithelial cell CGHNK2, the mRNA levels of miR-126 were still lower in the OSCC cell lines SCC25 ($p=0.0014$) and HSC3 ($p=0.0068$) (Figure 1B).

Meanwhile, the expression of ADAM9 in OSCC cell lines and normal oral epithelial cell CGHNK2 was also assessed using qRT-PCR. Contrary to the results of miR-126, the mRNA levels of ADAM9 were significantly upregulated in SCC25 ($p=0.0003$) and HSC3 ($p=0.0015$) *versus* CGHNK2 (Figure 1C). In addition, the expression of miR-126 had a negative correlation with the expression of ADAM9 in the OSCC tissues ($p<0.0001$, r=-0.6914), as shown in Figure 1D.

MiR-126 Inhibits Migration and Invasion of OSCC Cells

We employed the transwell assay to detect whether miR-126 was able to suppress the migration and invasion of OSCC. MiR-126 mimic or inhibitor were applied to up/downregulate the expression of miR-126 in SCC25 ($p=0.0016$ and

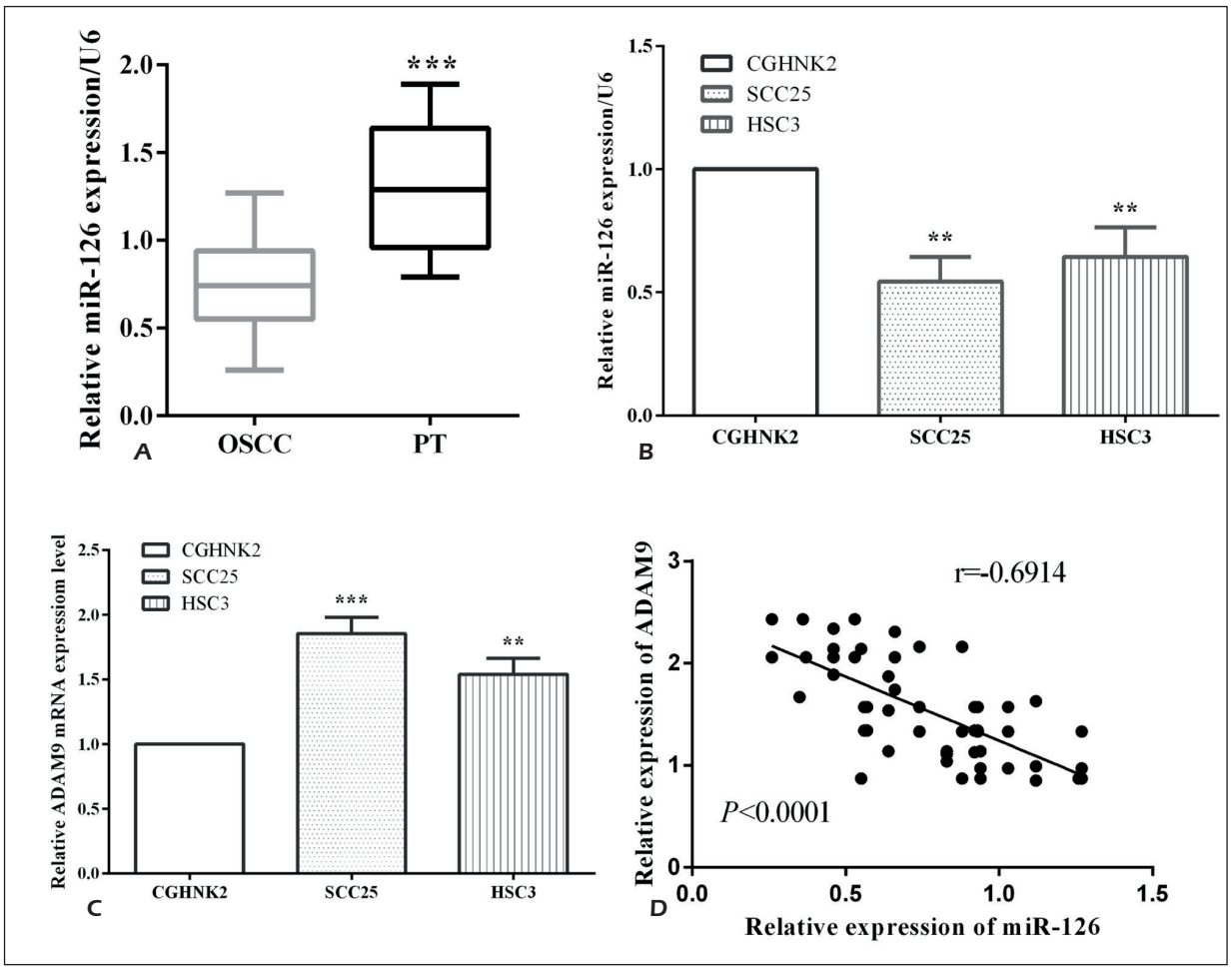

Figure 1. MiR-126 was low expressed in oral squamous cell carcinoma tissues and cell lines. **A**, MiR-126 was downregulated in the OSCC tissues versus paracancerous tissues. **B**, MiR-126 was low expressed in OSCC cell lines SCC25 and HSC3 compared to the normal oral epithelial cell CGHNK2. **C**, The upregulation of ADAM9 was detected in the OSCC cell lines SCC25 and HSC3. **D**, The mRNA level of miR-126 has a negative correlation with ADAM9 in OSCC tissues. **, $p<0.01$; ***, $p<0.001$; PT, paracancerous tissues; OSCC, oral squamous cell carcinoma tissues.

0.0027) and HSC3 (p=0.0010 and 0.0038) cell lines (Figure 2A). As expected, the results revealed that the overexpression of miR-126 could suppress the migratory and invasive capacities of SCC25 (p=0.0009 and 0.0006) and HSC3 (p=0.0089 and 0.0031) cells. On the contrary, the downregulation of miR-126 improves the migratory and invasive abilities in SCC25 (p=0.0067 and 0.0058) and HSC3 (p=0.0089 and 0.0027) cells (Figure 2B). Our results suggest that miR-126 could inhibit the expression of ADAM9 to inhibit the migration and invasion in OSCC cell.

ADAM9 was a Direct Target of MiR-126 in OSCC Cells

To investigate the mechanism through which miR-126 regulated the OSCC cell migration and invasion, we predicted target genes of miR-126 using the TargetScan software, which identified ADAM9 to be a direct target of miR-126. The potential binding site of ADAM9 for miR-126 was located at 780-786 of its mRNA 3'-UTR. To determine miR-126 direct targeted to the 3'-UTR of ADAM9 mRNA, we mutate the target sequences from 5'-GGUACG-3' to 5'-UUGGAA-3' (Figure 3A). The two kinds of vectors together with the miR-126 mimic, or negative control were co-transfected into the SCC25 and HSC3 cells. The luciferase reporter assay showed that miR-126 suppressed the luciferase activity of the WT reporter compared with the control (p=0.0045 and 0.0032 in SCC25 and HSC3 respectively), while the luciferase activity has no difference after the co-transfected MUT and miR-126 mimic or control in both SCC25 (p=0.6868) and HSC3 (p=0.3585) cells (Figure 3B).

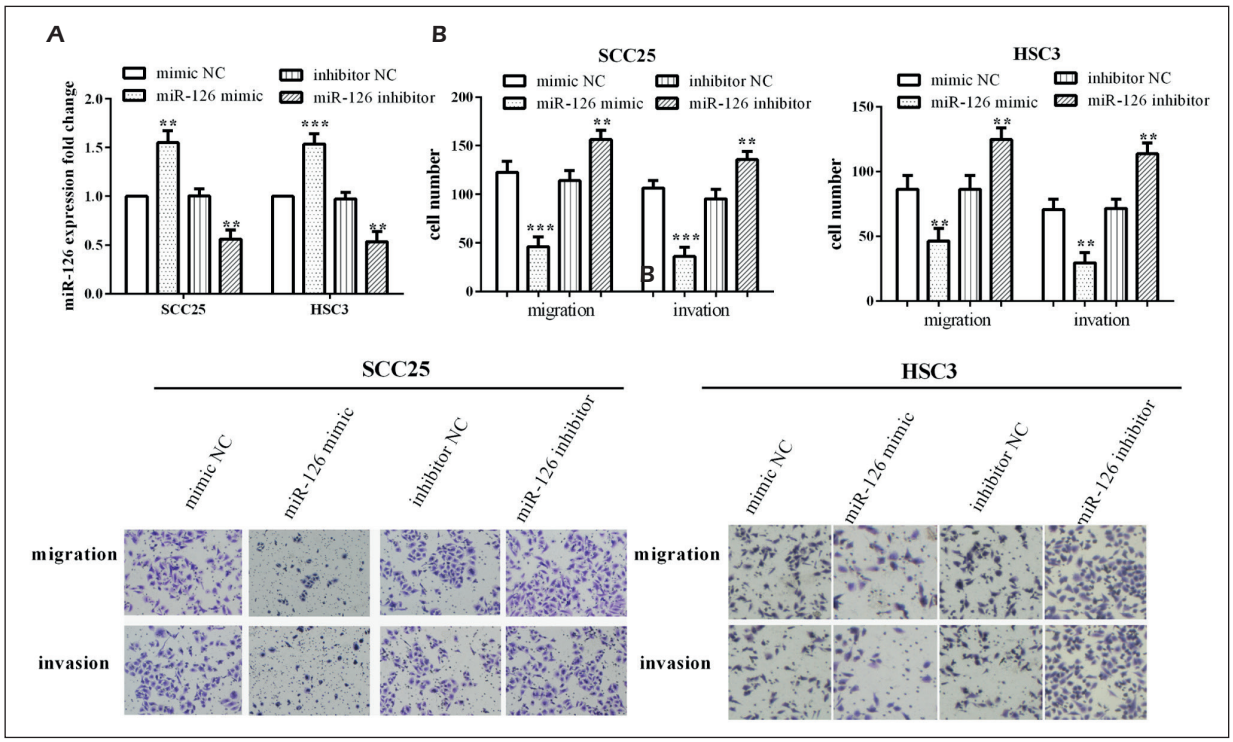

Figure 2. MiR-126 suppresses the migration and invasion in the OSCC cells. **A**, MiR-126 was up/downregulated by transfected with the miR-126 mimic or inhibitor in SCC25 and HSC3 cells. **B**, The migratory and invasive abilities were changed after transfected with the miR-126 mimic or inhibitor in SCC25 and HSC3 cells (magnification: 40×). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

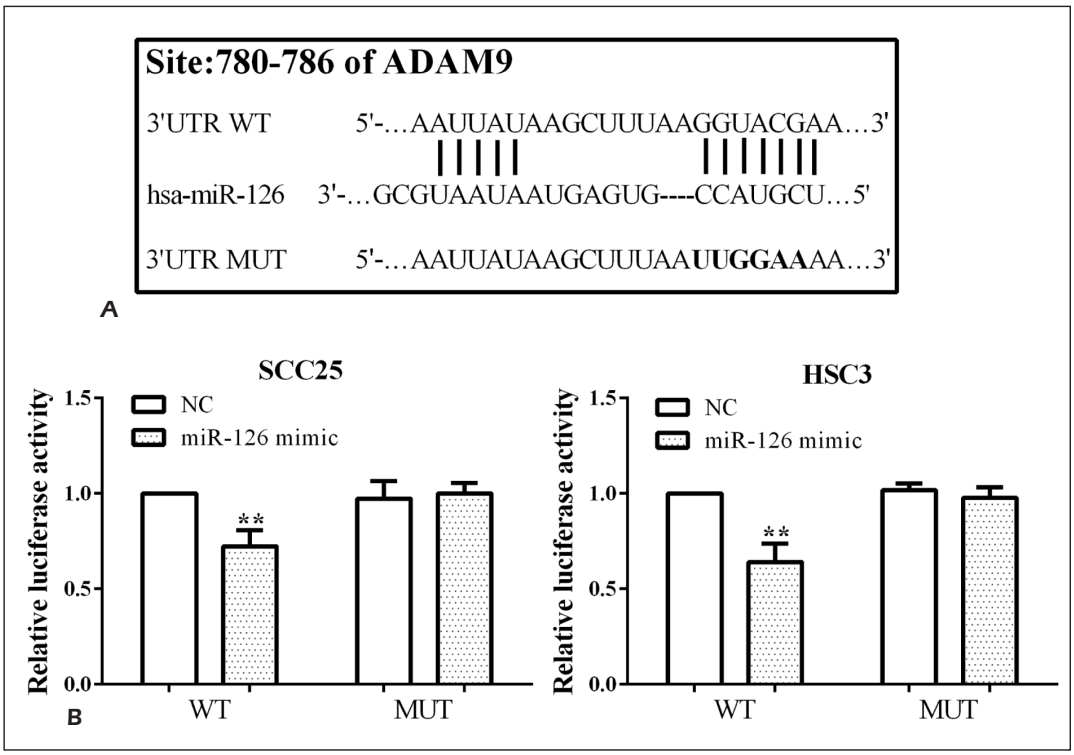

Figure 3. ADAM9 was a direct target of miR-126. **A**, The complementary sequences of ADAM9 3'-UTR and miR-126 were sought from TargetScan. **B**, MiR-126 targeted the WT of ADAM9 but not the MUT. **, $p<0.01$; WT: wild-type of ADAM9 3'-UTR; MUT: a mutant of ADAM9 3'-UTR.

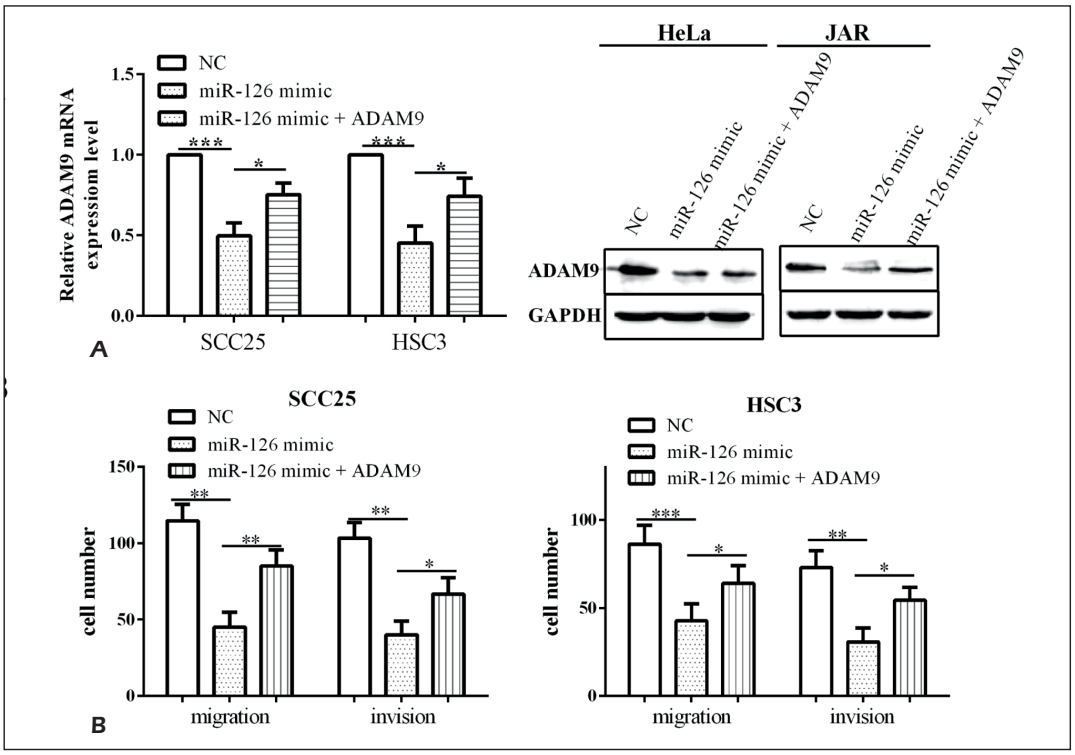

Figure 4. ADAM9 could reverse partial functions of miR-126. **A**, The expression of ADAM9 was measured by the qRT-PCR and Western Blot. **B**, The ADAM9 expression was increased when transfected with the miR-126 mimic, which was re-expressed via overexpressed ADAM9.

ADAM9 could Reverse Partial Function of MiR-126

To determine whether the miR-126 mimic reduced cell migration and invasion through the downregulation of ADAM9, we co-transfected the miR-126 mimic and ADAM9 into SCC25 (p=0.0142) and HSC3 (p=0.0301) cells and next measured the mRNA and protein levels of ADAM9 (Figure 4A). In comparison with only transfected miR-126 mimic, the migratory and invasive abilities were increased in cells co-transfected with the miR-126 mimic and pcDNA3.1-ADAM9 in SCC25 (p=0.0086 and 0.0293) and HSC3 (p= 0.0474 and 0.0197) cells (Figure 4B). Considering these results, we concluded that miR-126 affect the migration and invasion by regulating the expression of ADAM9 in the OSCC cell lines.

Discussion

The oral squamous cell carcinoma (OSCC) is a frequent cancer with high metastasis, which caused extremely poor prognosis[27]. Thus, it is particularly important to discover molecular markers for the early diagnosis and prediction of OSCC. Despite the increasing evidence demonstrated the regulatory roles of microRNAs in the growth and metastasis of the tumor, the adjustment function in OSCC migration and invasion is still poorly understood. MiR-126 is an endothelial-specific miRNA, which was found to be downregulated and plays important roles in serious tumors[11-16]. MiR-126 acted as a predictor and could inhibit the proliferation and invasion in hepatocellular carcinoma, bladder cancer, and gastric cancer, respectively[11,15,16]. Nevertheless, the roles of miR-126 on migration and invasion in OSCC had little research. Our purpose is to identify the functions of miR-126 and its target gene for cell migration and invasion of OSCC. We certified that miR-126 was low expressed, while ADAM9 was upregulated in the OSCC tissues and cell lines compared with paracancerous tissues and normal cell. What's more, the downregulation of miR-126 could improve the abilities of migration and invasion of OSCC cells. In addition, we verified that ADAM9 was a direct target gene of miR-126. Considering these findings, we strongly believe that the roles of miRNA-126 on migration and invasion may be through inhibiting ADAM9.

ADAM9 plays a great role in the proteolytic process and has been reported[15,22-25] to be the overexpression in various cancers that including bladder cancer, breast cancer, hepatocellular carcinoma, lung cancer, and glioblastoma. ADAM9 has been discovered[22] to be upregulated and inhibited the proliferation, migration, and invasion of breast cancer cell. Additionally, ADAM9 has been reported to be a direct target in some tumors. Jia et al[15] have put forward that miR-126 inhibits cell invasion through the target ADAM9 in bladder cancer and breast cancer respectively. Furthermore, Jiang et al[21] discovered that miR-126 suppressed the growth, migration, and invasion in osteosarcoma cells *via* regulation ADAM9. Although miR-126 acts as a tumor suppressor in pancreatic cancer by Hamada et al[28], we first proposed that miR-126 directly targeted the 3'-UTR of ADAM9 mRNA in OSCC. Our findings were consistent with all the results, ADAM9 was overexpressed in the OSCC tissues and cell lines compared to the paracancerous tissues and normal oral epithelial cell line, which were consistent with the present research.

Conclusions

The above results provided evidence that miR-126 was a cancer suppressor in OSCC, which improved the ability of migration and invasion in the OSCC cells. Furthermore, we demonstrated that the expression of miR-126 has a negative association with ADAM9 expression in the OSCC tissues. MiR-126 suppresses the migratory and invasive abilities through targets the 3'-UTR of ADAM9 mRNA in the OSCC cells. This novelty of miR-126 may represent a potential target for the treatment of OSCC.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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