European Review for Medical and Pharmacological Sciences 2020; 24: 12717-12726

Inhibition of miR-133b indicates poor prognosis and promotes progression of OSCC *via* SOX4

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Abstract. – OBJECTIVE: Oral squamous cell carcinoma (OSCC) accounts for the first largest proportion of oral and maxillofacial malignancies worldwide. Increasing studies have indicated that miRNAs are involved in the regulation of various tumors, including OSCC. However, the exact role of miR-133b in OSCC has not been fully elucidated. Here, we aimed to explore the effects of miR-133b on the development and progression of OSCC and its related mechanisms.

PATIENTS AND METHODS: Expression of miR-133b in 44 paired OSCC tissues and adjacent normal tissues were detected using quantitative real-time polymerase chain reaction (qRT-PCR). Clinicopathological characteristics were collected from OSCC patients, and the relationship between miR-133b expression and the prognosis of patients was analyzed. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and colony formation assays were employed to measure the proliferation of OSCC cells transfected with miR-133b inhibitors or mimics. Cell invasion and migration were detected using transwell and Matrigel experiments, respectively. Bioinformatics and Western blot were applied to investigate the possible underlying mechanism of miR-133b in OSCC.

RESULTS: MiR-133b was lowly expressed in OSCC tissues compared to adjacent normal tissues ($p<0.05$). Lower expression miR-133b indicated a significantly worse prognosis of OSCC patients ($p<0.05$). Over-expression of miR-133b reduced the growth and metastasis of SCC9 cells ($p<0.05$). Transfection of miR-133b inhibitors obviously enhanced the proliferation, migration and invasion of TSC-15 cells ($p<0.05$). SRY-Box Transcription Factor 4 (SOX4) was verified as a specific target for miR-133b. Up- or down-regulation of miR-133b decreased or increased the protein expression level of SOX4 in OSCC, respectively ($p<0.05$).

CONCLUSIONS: MiR-133b was lowly expressed in OSCC tissues and cell lines. Down-regulation of miR-133b reduced the proliferation, invasion and migration of OSCC cells via regulating SOX4. All our findings suggested that miR-133b could be used as a potential target for the treatment of OSCC.

Key Words:
MiR-133b, Oral squamous cell carcinoma (OSCC), Prognosis, Proliferation, Metastasis, SOX4.

Introduction

Oral squamous cell carcinoma (OSCC) remains one of the common malignant tumors, accounting for more than 90% of oral and maxillofacial malignancies[1]. OSCC progresses rapidly, with an approximant 60% lymphatic metastasis when diagnosed at the advanced stage. Meanwhile, it can cause nerve damage in a very short period. Due to the special oral anatomy and unclear boundary, it is difficult to perform radical surgery for OSCC patients[2,3]. Therefore, specific biomarkers for early detection and treatment of OSCC are still needed, which is of great significance for improving the survival rate and life quality of affected patients.

MicroRNAs (MiRNAs) are endogenous, single-stranded, non-coding RNA molecules with about 19 to 25 nucleotides in length[4]. MiRNAs have been identified to influence cell proliferation, apoptosis, autophagy, metabolism, invasion, migration and other biological processes. Meanwhile, they can participate in the regulation of tumor development and progression[5]. Of note, miR-155-5p acts as a oncogene in OSCC to accelerate its progression *via* regulating ARID2[6]. On contrast, miR-101 is considered as a tumor suppressor gene to retard cell cycle and metastasis by targeting TGF-βR1[7]. MiR-504 or miR-29b-3p inhibits the invasion and migration of OSCC cells *via* regulating CDK6 or IL32/AKT signaling axis[8,9]. In addition, miR-486-3p, targeting DDR1, suppresses the progression of oral cancer[10].

MiR-133b has been detected down-regulated in glioma, renal cancer, non-small cell lung cancer, ovarian cancer and breast cancer. It can inhibit epithelial-to-mesenchymal transition, cell

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growth and metastasis *via* several specific target genes[11-14]. So far, the relationship between miR-133b and OSCC tumorigenesis has rarely been elucidated. Therefore, this study aimed to explore the role of miR-133b in the progression of OSCC. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of miR-133b in OSCC tissues and cell lines. The relationship between miR-133b level and clinicopathological features or patients' prognosis was analyzed. SCC9 and TSC-15 cells were cultured *in vitro*, and the influence of miR-133b on cell proliferation and metastasis was analyzed. Furthermore, we explored the downstream targets of miR-133b in OSCC. All our results might help to find a potential target for OSCC patients on further prognosis prediction and treatment.

Patients and Methods

Clinical Samples and Characteristics

The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). From January 2017 to January 2019, 44 OSCC patients who underwent radical mastectomy at the Department of Oral and Maxillofacial Surgery of The First People's Hospital of Lianyungang were enrolled in this study. Monthly follow-up visits and outpatient reviews were performed. All patients received initial surgery, and none of them received other adjuvant treatments such as radio-chemotherapy before surgery. Clinical pathological data were collected from OSCC patients. Adjacent normal tissues were collected from at least 5 cm from the original lesion. This investigation was approved by the Ethics Committee of The First People's Hospital of Lianyungang. Signed written informed consents were obtained from all participants before the study.

Cell Transfection and Culture

Four OSCC-derived cell lines (SCC9, SCC15, TSCCA, TSC-15) and one human normal oral keratinocytes cell line (HOK) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and maintained in an incubator with 5% CO_2 at 37°C. Cell passage was performed at a density of about 95%. According to the relative expression level, SCC9 and TSC-15 cells were used as experimental cells. SCC9 cells in logarithmic growth phase were transfected with miR-133b mimics or negative control (NC), while TSC-15 cells were transfected with miR-133b inhibitors or inhibitor negative control (INC) according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). MiR-133b mimics, NC, miR-133b inhibitors and INC were synthesized by GeneWiz Co. (Suchow, China). Transfection efficiency was confirmed using qRT-PCR.

RNA Extraction and qRT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA in OSCC tissues and cells. Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Master Mix (TaKaRa Bio, Inc., Komatsu, Japan). Three replicate wells were set for each sample. PCR reaction was completed by using SYBR Premix ExTaq kit (TaKaRa Bio, Inc., Komatsu, Japan) with ABI PRISM 7500 Real-Time system (Applied Biosystems, Foster City, CA, USA). The cycle threshold (Ct) of each sample was analyzed. The relative expression of miR-133b was calculated by the $2^{-\Delta\Delta Ct}$ method. U6 was used as an internal reference. This experiment was repeated for three times. Primer sequences used in this study were as follows: miR-133b, F: 5'-GGCCTTGGGTACATCCCGACGGG-3', R: 5'-GAATGGGTCTCGTAGAGACAG-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] Assay

Cell proliferation was determined in strict accordance with Cell Growth Determination Kit MTT Based (Sigma-Aldrich, St. Louis, MO, USA). Transfected SCC9 or TSC-15 cells for 24 h were trypsinized and inoculated into 96-well plates at a density of 2,000 cells per well. Six replicate wells were set in each group. 30 μL of MTT working solution was added to each well at 24 h, 48 h, 72 h and 96 h, respectively, followed by incubation for 2 h at 37°C. Absorbance at 470 nm was finally detected by a micro-plate reader (Bio Tek Instruments, Winooski, VT, USA).

Colony Formation Assay

Transfected SCC9 or TSC-15 cells were seeded into 6-well plates at a density of 1000 cells

per well. Then, the cells were cultured for 2 to 3 weeks until the clone was visible to naked eyes. Next, the cells were fixed in 4% paraformaldehyde (Beyotime, Shanghai, China) for 15 min and stained with crystal violet (Beyotime, Shanghai, China) for 30 min, followed by slowly washing with running water. Formed colonies were observed under a microscope, and the number of colonies with more than 50 cells was counted.

Transwell Assay and Matrigel Assay

Transwell and Matrigel assays were completed using 8-μm transwell inserts (Corning, Corning, NY, USA). Briefly, transfected SCC9 and TSC-15 cells in serum-free medium were seeded into the upper layer of the transwell chamber. Meanwhile, DMEM medium containing 10% FBS was added in the lower layer of the chamber. After 48 hours of incubation, inserts were removed and placed in ice-cooled ethanol for fixation. After staining with crystal violet, non-penetrating cells were carefully wiped off using a cotton swab. The number of cells passing through the chamber was observed under a microscope, and 5 fields of view were randomly selected for each sample. For Matrigel assay, the membrane of the insert was covered with 50 μL Matrigel (Corning, Corning, NY, USA), and the remaining steps were consistent with tranwell assay.

Dual-Luciferase Reporter Gene Assay

The targeting relationship between SOX4 and miR-133b was identified using the Dual-Luciferase reporter gene assay. Wild-type (WT) and mutant (Mut) full-length 3'-UTR of SOX4 were amplified and ligated to the psi-CHECK-2 vector. Cells were co-transfected with miR-186 mimics and 100 ng plasmid, followed by incubation for 48 h. Fluorescence intensity of *Renilla* relative to firefly Luciferase was analyzed with a dual Luciferase reporter assay system (Promega, Madison, WI, USA). The experiment was repeated for three times.

Western Blotting

Total protein in SCC9 or TSC-15 cells was extracted using a radioimmunoprecipitation assay (RIPA) reagent (KGI, Nanjing, China) containing protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) (KGI, Nanjing, China). After centrifugation, the supernatant was collected. The concentration of extracted protein was quantified by the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membranes were incubated with primary antibodies of SOX4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA, USA) overnight at 4°C. After washing, the membranes were incubated with goat anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Immuno-reactive bands were exposed using the enhanced chemiluminescence (ECL) Western blotting kit (Millipore, Billerica, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 statistical software (SPSS Inc., Armonk, NY, USA) was used for all statistical analysis. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Kaplan-Meier analysis was used for survival analysis. $p<0.05$ was considered statistically significant.

Results

MiR-133b Was Down-Regulated in OSCC Tissues and Cell Lines

To verify the expression of miR-133b in OSCC tissues, we first collected 44 pairs of OSCC tissues and adjacent normal tissues. MiR-133b expression was detected using qRT-PCR. The results showed that miR-133b was lowly expressed in OSCC tissues compared with adjacent normal tissues ($p<0.05$, Figure 1A). Subsequently, the expression of miR-133b in four OSCC-derived cell lines (SCC9, SCC15, TSCCA, TSC-15) and HOK cells was detected as well. QRT-PCR results demonstrated that miR-133b expression in OSCC cell lines was significantly lower than that in HOK cells ($p<0.05$, Figure 1B). These results indicated that miR-133b might act as a tumor suppressor gene in OSCC.

Down-regulation of MiR-133b Indicated Poor Clinicopathological Characteristics and Prognosis of OSCC

To validate the relationship between OSCC characteristics and miR-133b expression, clinic-pathological features including sex, gender, smoke, alcohol, tumor grade, tumor stage and lymph node

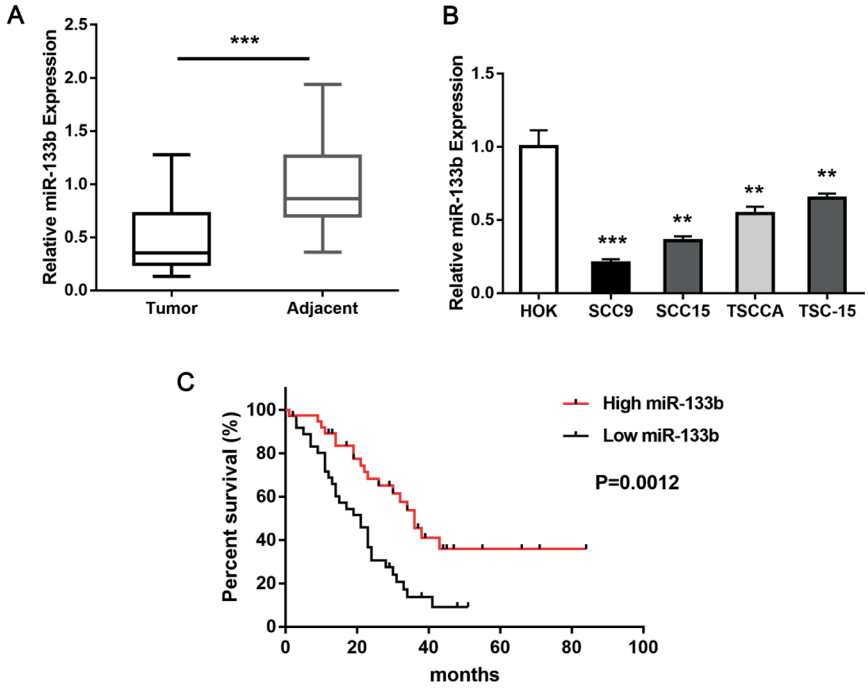

Figure 1. MiR-133b was lowly expressed in OSCC tissues and cell lines. **A,** Analysis of the expression level of miR-133b in 44 OSCC tissues and adjacent normal tissues. **B,** Analysis of miR-133b expression level in OSCC cell lines (TSC-15, SCC15, TSCCA, SCC9) and human normal oral keratinocytes cells (HOK). **C,** Kaplan-Meier survival assay indicated that patients with high miR-133b expression exhibited significantly poor survival compared with patients with low miR-133b expression.

metastasis were collected. Based on the median expression level of miR-133b, OSCC patients were divided into two groups, including high miR-133b expression group and low miR-133b expression group. As shown in Table I, there was no statistically significant difference in sex, gender, smoke and alcohol between the two groups ($p>0.05$). However, significant differences were observed in tumor grade, tumor stage and lymph node metastasis between the two groups ($p<0.05$). Down-regulation of miR-133b suggested poor tumor grade, worse tumor stage and positive lymph node metastasis. In addition, we performed a Kaplan-Meier plot based on the expression of miR-133b. The results found that lower miR-133b expression indicated significantly worse survival of OSCC patients ($p<0.05$, Figure 1C). These results indicated that miR-133b might provide as an optimistic factor in the progression of OSCC.

MiR-133b Effected OSCC Cell Proliferation

To further demonstrate the influence of miR-133b on OSCC progression, we established SCC9 cells with miR-133b over-expression and TSC-15 cells with miR-133b down-expression. Comparing with relative control group, SCC9 cells showed markedly increased miR-133b level after miR-133b mimics treatment, while TSC-15 cells displayed reduced miR-133b expression after miR-133b inhibitors treatment ($p<0.05$, Figure 2A, 2B). Subsequently, cell proliferation ability was detected using MTT assay. The results demonstrated that over-expression of miR-133b significantly inhibited the proliferation of SCC9 cells, while down-regulation of miR-133b remarkably promoted the proliferation of TSC-15 cells ($p<0.05$, Figure 2C, 2D). Similarly, SCC9 cells treated with miR-133b mimics formed fewer colonies than NC group, whereas TSC-15 cells transfected with miR-133b inhibitors formed more colonies than INC group ($p<0.05$, Figure 2E, 2F). These data elucidated that miR-133b inhibited the proliferation of OSCC cells.

Ectopic MiR-133b Influenced OSCC Cell Invasion and Migration

We detected the invasion and migration of OSCC cells to demonstrate the effect of miR-133b on cell metastasis. Transwell assay found that cell invasion was obviously reduced in miR-133b mimics treated SCC9 cells compared with NC group ($p<0.05$). However, TSC-15 cells trans-

fected with miR-133b inhibitors displayed significantly promoted invasion ability compared with INC group ($p<0.05$, Figure 3A, 3B). Similarly, Matrigel analysis showed that the number of migrated cells decreased remarkably in miR-133b over-expressed SCC9 cells, whereas increased significantly in miR-133b down-expressed TSC-15 cells compared with negative control cells ($p<0.05$, Figure 3C, 3D). These results indicated that miR-133b could inhibit the invasion and migration of OSCC cells.

SOX4 Was a Direct Target for MiR-133b in OSCC

According to several studies on the mechanism of miRNAs regulating target gene expression *via* binding to its 3'-UTR, we further searched several databases including TargetScan, PiTar, miRWalk and DIANA. Finally, SOX4 was screened out as a potential target for miR-133b in OSCC. Dual-Luciferase reporter gene assay was conducted to certify the assumption using sequences including wild-type or mutant binding region of miR-133b in the 3'-UTR of SOX4 (Figure 4A). Clearly, the activity of Luciferase was remarkably reduced in wild type group ($p<0.05$). There was no significant difference in mutant group ($p>0.05$). This indicated that miR-133b could directly bind to the 3'-UTR region of SOX4 (Figure 4B). Furthermore, the protein expression of SOX4 in SCC9 and TSC-15 cells was measured using Western blot. The results showed that the protein expression level of SOX4 was significantly reduced in SCC9 cells after miR-133b over-expression, whereas it was markedly elevated in TSC-15 cells after miR-133b down-expression ($p<0.05$, Figure 4C, 4D, 4E). These results suggested that miR-133b could directly bind with SOX4 to regulate its expression, thereby influencing OCSS cell proliferation and metastasis.

Table I. Correlation between miR-133b level and clinicopathological features in OSCC.

Characteristics	Total	MiR-133b expression		*p*-value
		Low	High	
Age				
<60 years	28	15	13	0.2636
>60 years	16	7	9	
Gender				
Male	24	11	13	0.8415
Female	20	11	9	
Smoke				
Yes	23	13	10	
No	18	8	10	0.6229
Alcohol				
Yes	26	13	13	0.4668
No	18	11	7	
Tumor grade				
G1-G2	14	2	12	0.0012*
G3-G4	30	20	10	
Tumor stage				
I-II	33	21	12	0.0017*
III-IV	11	1	10	
lymph node metastasis				
Yes	12	9	3	
No	32	13	19	0.0423*

The expression level of miR-133b was cut off by median expression level and * indicated $p<0.05$.

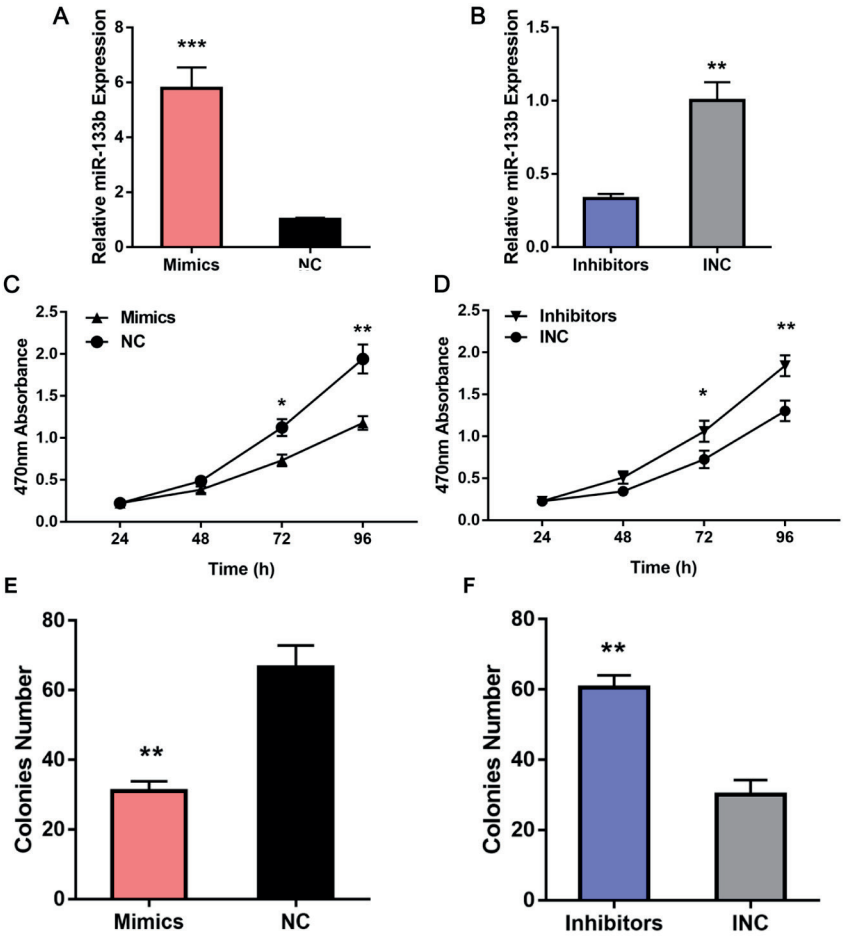

Figure 2. MiR-133b effected the proliferation and invasion of OSCC cells. **A,** Expression of miR-133b in miR-133b mimics treated SCC9 cells. **B,** Expression of miR-133b in miR-133b inhibitors treated TSC-15 cells. * p<0.05, ** p<0.01, ***p<0.001. **C, D,** MTT assay was performed to determine the proliferation of SCC9 (**C**) or TSC-15 (**D**) cells treated with miR-133b mimics or inhibitors compared with negative control cells. **E, F,** Colony formation assay was performed to determine the growth of SCC9 (**E**) or TSC-15 (**F**) cells transfected with mimics or inhibitors, respectively. (magnification: 10×) * $p< 0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

OSCC, one of the common malignant tumors in the department of stomatology, mostly occurs in middle-aged and elderly people. Due to the non-specific initial clinical manifestations, it is generally diagnosed in the middle and late stage. With the development of modern medical technology, the treatment strategy of OSCC has been greatly improved. However, the 5-year survival rate of OSCC still remains poor[1,3,15]. To date, there are no specific early predictors for OSCC diagnosis and prognosis. Therefore, it is of great importance to clarify meaningful molecular markers related to the development of OSCC. With the rapid development of molecular biology and cancer research, a variety of molecules closely related to tumor development have been discovered, including miRNAs[16].

Each miRNA can regulate multiple genes, and several miRNAs can have the same target gene. This complex regulatory network controls the function of multiple genes through a single miRNA, or finely affect the expression of specific genes *via* the combination of several miRNAs[17,18]. It is speculated that one-third of human genes are regulated by miRNAs. Among them, miR-133b is a novel miRNA discovered in recent years. Several studies have shown that miR-133b is abnormally expressed in various systemic tumors and is closely involved in the occurrence and development of malignancies. It suppresses cell stemness and chemoresistance in colorectal cancer through regulating methyltransferase DOT1L[19].

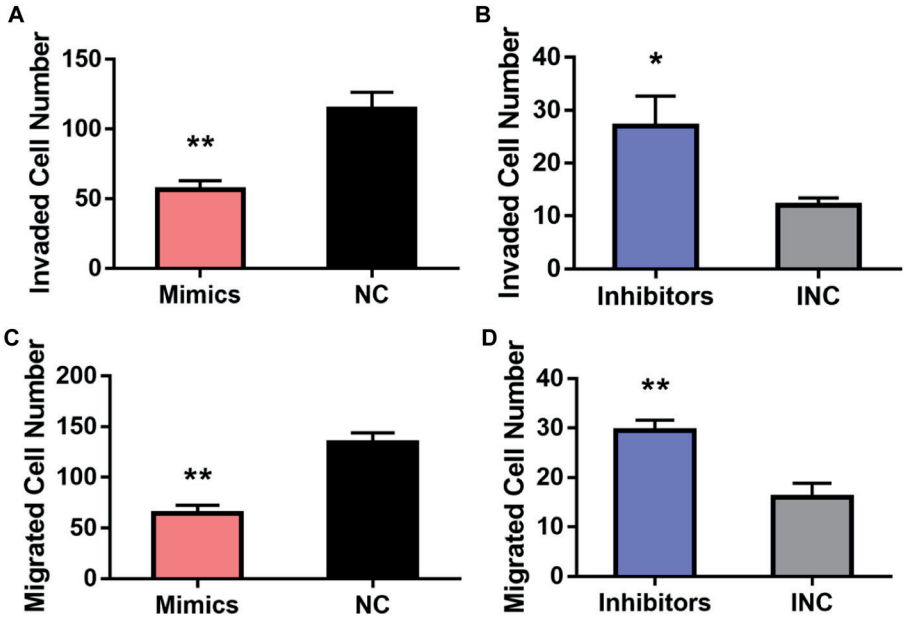

Figure 3. MiR-133b effected the invasion and migration of OSCC cells. **A, B,** Matrigel assay was used to detect the invasion ability of miR-133b mimics treated SCC9 cells (**A**) or miR-133b inhibitors treated TSC-15 cells (**B**). **C, D,** Transwell assay was used to detect the migration ability of miR-133b mimics treated SCC9 cells (**C**) or miR-133b inhibitors treated TSC-15 cells (**D**). Data are presented as mean ± SD of three independent experiments (magnification: 40×). * $p<0.05$, ** $p<0.01$.

MiR-133b inhibits the proliferation and invasion of esophageal squamous cell carcinoma by targeting EGFR[20]. Meanwhile, it displays complex activities of tumor-suppressing *via* FGFR1 in osteosarcoma[21]. MiR-133b inhibits cell cycle of bladder cancer by mediating TAGLN2. In addition, miR-133b suppresses the metastasis of breast cancer by regulating SOX9[14,22].

MiR-133b participates in the occurrence and development of various types of tumors. However, the exact role of miR-133b in OSCC has not been fully elucidated. This study displayed that the expression level of miR-133b was significantly reduced in OSCC tissues and cells. *In vitro* experiments confirmed that miR-133b could inhibit the invasion, migration and proliferation of OSCC-derived cells. However, down-regulation of miR-133b led to significantly increased cell growth and metastasis. These results indicate that miR-133b acts as a tumor-suppressor gene in the development of OSCC.

SOX4, located on 6p22.3, belongs to the group C member of the SOX family. It has been found involved in a number of biological processes, such as the development of the heart, the development and differentiation of T/B lymphocytes, and the growth of pancreas and bone in the period of embryo[23-25]. In recent years, a growing number of studies have shown that SOX4 gene is over-expressed in several cancers. Meanwhile, it is closely correlated with the initiation and progression of malignancies. It promotes or suppresses tumor progression through affecting cell apoptosis, proliferation, EMT, and tumor stem cells[26-29]. SOX4 has been found to promote the metastasis of breast cancer, bladder cancer, HCC, and prostate cancer by regulating EMT. Knocking out SOX4 in invasive HCC cells obviously inhibits tumor growth *in vivo*[30-34]. In OSCC, SOX4 can regulate proliferation, metastasis and cancer stemness, and is associated with treatment failure, chemoradioresistance, differentiation and lymph node metastasis. Multiple researches have shown that it acts as a specific target gene for lncRNA HCP5/miR-140-5p axis, miR-199a-5p and miR-204[35-39]. This study predicted SOX4 as a possible target of miR-133b through bioinformatics prediction. Dual-Luciferase reporter gene assay confirmed that SOX4 was a downstream target gene of miR-133b. Western blotting analysis showed that up-regulation of miR-133b decreased the protein expression of SOX4, while down-regulation of miR-133b increased the protein expression of SOX4, fur-

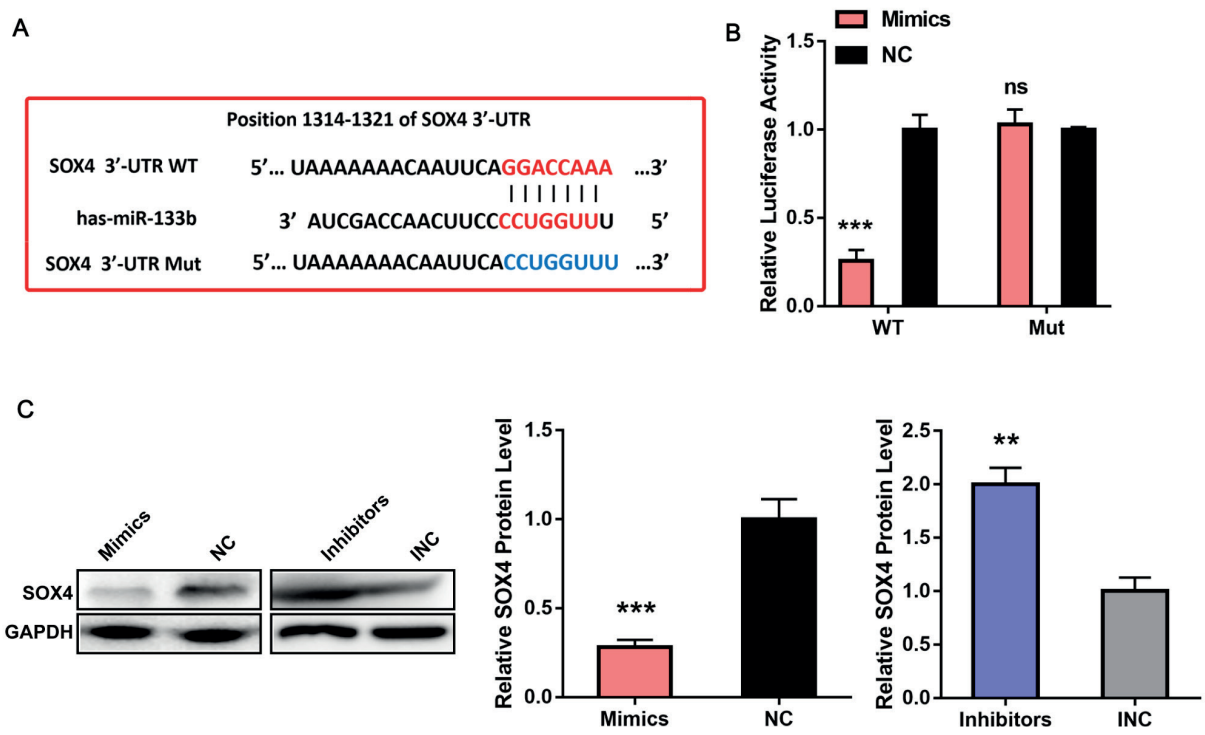

Figure 4. SOX4 was a direct target of miR-133b. **A,** The predicted binding sites of miR-133b in the 3'-UTR of SOX4. **B,** Dual-Luciferase reporter assay was used to determine the binding site. **C,** Protein expression levels of SOX4 and GAPDH measured by Western-blot in miR-133b over-expression SCC9 cells and miR-133b knock-down TSC-15 cells. **D, E,** The relative protein level of SOX4 and GAPDH. Data are presented as mean ± SD of three independent experiments. ** $p<0.01$, *** $p<0.001$, ns: non-sense.

ther confirming miR-133b targeted regulation of SOX4. In view of the important role of SOX4 in malignant tumors, we speculate that miR-133b may regulate the invasion, migration and proliferation of OSCC cells by targeting SOX4. All our findings may lay a theoretical foundation for early diagnosis and targeted therapy for OSCC.

Conclusions

In summary, miR-133b was lowly expressed in OSCC tissues and cells. Meanwhile, it inhibited the proliferation, invasion and migration of OSCC cells by targeting SOX4. The novelty of this study was that miR-133b might become a novel target for OSCC diagnosis and treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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