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MicroRNA-200c suppresses tumor metastasis in oral squamous carcinoma by inhibiting epithelial-mesenchymal transition

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Abstract. – OBJECTIVE: To examine the potential mechanisms implicating miR-200c and epithelial-mesenchymal transition (EMT) in oral squamous carcinoma (OSC).

MATERIALS AND METHODS: 32 pairs of OSC tissue samples and matched para-carcinoma normal tissue from patients undergoing routine surgery in the Xuzhou Stomatological Hospital from 2014-2016. HOC313 cells were cultured and transfected with miR-200c mimics and scrambled mimics. Cell migration, invasion assays, Luciferase reporter assay, and Western blot assay were conducted.

RESULTS: miR-200c was downregulated in OSC tissues compared with adjacent normal tissues (n=32). miR-200c knockdown in the human oral cancer cell line HOC313 significantly suppressed cell invasion and migration, indicating the ability to inhibit tumor progression. Luciferase reporter assay indicated that miR-200c directly bound to the 3'-untranslated regions (3'-UTR) of Zinc finger E-box-binding homeobox (ZEB1) directly. Moreover, miR-200c significantly inhibited HOC313 cell EMT via negatively regulating ZEB1 protein expression.

CONCLUSIONS: MiR-200c plays a pivotal role in controlling OSC metastasis via inhibiting EMT, which provides potential therapeutic targets for OSC.

Key Words:
miR-200c, OSC, EMT, ZEB1.

Introduction

Oral squamous carcinoma (OSC) is the most frequent type of malignant tumor of the oral and maxillofacial region. It has a high degree of malignancy, high likelihood of lymphatic metastasis, and poor prognosis[1]. OSC originate primarily *in situ*, but can be transferred from a distant origin, or caused by infringement of adjacent tissue lesions[2]. Despite recent advances in diagnosis and treatment of OSC, the mortality remains high, with a 5-year survival rate less than 50%[3]. Therefore, further understanding of the molecular mechanisms of OSC progression and development will support the identification of new therapeutic targets for OSC.

MicroRNA (miRs) are small non-coding RNA transcripts of 18 to 25 nucleotides in length[4]. MiRs suppress gene expression through binding and promoting degradation of specific mRNA and play a fundamental role in most biological processes[5]. Among other functions, miRs play an important role in various cellular states, including cancerous transformation[5]. Therefore, the study of miRs may be of great value in explaining the occurrence and development of tumors.

MiR-200c is a member of the microRNA-200 family that is downregulated and plays an anti-oncogene role in several kinds of cancer, such as non-small cell lung cancer (NSCLC)[6], glioblastoma multiforme (GBM)[7], and colorectal cancer (CRC)[8]. Previous evidence has demonstrated that the levels of miR-200c are decreased in OSC[9]. However, the mechanism of miR-200c in progression and development of OSC remains unknown.

Epithelial-mesenchymal transitions (EMT) is the cellular process by which epithelial tumor cells lose polarity and transforms into mesenchymal phenotypes[10]. One characteristic of EMT is the downregulation of epithelial biomarkers like E-cadherin and the upregulation of mesenchymal biomarkers like N-cadherin[11]. Studies have revealed that EMT is involved in cell growth and metastasis in several types of tumors, including

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OSC[12]. Various oncogenes are known to participate in EMT, including Zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2)[9]. The main objective of this work was to examine the potential mechanisms implicating miR-200c and EMT in OSC.

Materials and Methods

Clinical Samples

We collected 32 pairs of OSC tissue samples and matched para-carcinoma normal tissue from patients undergoing routine surgery in the Xuzhou Stomatological Hospital from 2014-2016. All surgical specimens were frozen immediately in liquid nitrogen after collection. The tumors were diagnosed and confirmed by pathological examination. This study was approved by the Ethics Committee of the Xuzhou Stomatological Hospital. All participants read and signed informed consent.

Cell Culture

The human oral cancer cell line HOC313 was purchased from Shanghai Model Cell Bank (Shanghai, China). HOC313 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Cell Transfection

miR-200c mimics and scrambled mimics were obtained from RiboBio (Guangzhou, China) and were transfected into the cells by using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, MA, USA) according to the manufacturer's protocols.

RNA Extraction

Total RNA was extracted from the frozen tissue samples and cultured cells using the TRIzol reagent (Invitrogen, Carlsbad, MA, USA) following the manufacturer's protocols. The PrimeScript RT reagent kit (TaKaRa, Dalian, China) was used to synthesize cDNAs. The stem-loop primer for miR-200c was 5'-GTC GTA TTC GCG TGT CGT GAA GGC ACT TTC ACT GGA TAC AGT CCA TC-3'. U6 was used for normalization. Then, we performed Polymerase Chain Reaction (PCR) reactions using the following primers: miR-200c, Fw, 5'-GGC CTA ATA CCG GGT AAT-3' and Rv, 5'-CAG TAC TGG TGG GAT-3'; U6, Fw, 5'-CCT GCT TAG GCA GAA CA-3' and Rv, 5'-AGC GCT TTA CGA CTT TGC GT-3'. ZEB1 mRNA level was quantified by SYBR Green Real-Time PCR and normalized to GAPDH using the following primers: ZEB1, Fw, 5'-CTC GAG CAT TTA GAC ACA AGC G-3' and Rv, 5'-TGG CCC TTC CTT TCC TGT GT-3'; *GAPDH*, Fw, 5'-CGG AGT TGT TCG TAT TCG G-3' and Rv, 5'-TAC TGG ACG ATG ATG GCA TT-3'. QRT-PCR was performed with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, MA, USA).

Cell Migration and Invasion Assays

The wound-healing assay was used to measure cell migration. Cells were cultured in six-well plates marked by a horizontal line on the back and transfected with the *miR-200c* mimics or scrambled mimics. The cells were scratched by a pipette tip across the confluent cell layer, washed gently, and continued to culture in serum-free medium for 24-48 h. Wound closure was documented with a light microscope (DFC500, Wetzlar, Germany). The transwell assay was performed to measure cell invasion. Cells were cultured in the upper invasion chamber (BD Biosciences, Franklin Lakes, NJ, USA) coated with Matrigel. Serum-free medium was added into the upper chamber, and 10% FBS medium supplemented was added to the lower chamber. After 48 h, the cells cultured above the filter, which did not invade through the chamber, were removed. Then, the lower chamber was fixed in 100% precooled methanol, stained with 0.05% crystal violet, and inspected under the microscope (Olympus, Tokyo, Japan). The values for the invasion cells were measured by counting five fields per membrane.

Analysis of miR-200c Targets

TargetScan (http://www.targetscan.org/vert_71/) was utilized to predict the target genes. As shown in the database, ZEB1 was the candidate gene that we chose. The result of bioinformatics software indicated that miR-200c binds the 3'-UTR of ZEB1.

Luciferase Reporter Assay

We purchased the ZEB1 3′-UTR vector for luciferase reporter from Genechem (Shanghai, China). We co-transfected miR-200c mimics or scrambled mimics and pmiR-ZEB1 or pmiR-control into HOC313 cells for 48 h. We used Renilla luciferase (pRL-TK Vector) as control. The Duo-Glo luciferase assay kit (Promega, Madison, WI,

USA) was used to detect the luciferase signal following the manufacturer's instructions.

Western Blot Assay

To investigate the expression level of ZEB1, E-cadherin, and N-cadherin, we homogenized HOC313 cells using lysis buffer. Then, we measured protein concentration using the protein assay kit from Beyotime (Nanjing, China). 20 μg of protein extract was denatured and chilled on ice. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins and transferred to PVDF membranes (Millipore, Billerica, MA, USA). 5% fat-free milk was used to block non-specific protein interactions in TBST buffer containing Tris-HCl (50 mM), NaCl (150 mM) and Tween 20 (0.05%) at 4°C for 1 h. The membranes were incubated at 4°C in fat-free milk overnight with the following primary antibodies: polyclonal rabbit anti-human E-cadherin (1:1,000), polyclonal rabbit anti-human N-cadherin (1:1,000), and polyclonal rabbit anti-human ZEB1 (1:2,000). TBST buffer was used to wash the unbound antibody (10 min each, three times). The membranes were then incubated at room temperature with secondary antibody conjugated with horseradish peroxide for 1 h. After washing the membranes three times in TBST, we developed them using ECL (Millipore, Billerica, MA, USA) following the manufacturer's instructions.

Statistical Analysis

SPSS 20.0 software (IBM SPSS, Statistics for Windows, Armonk, NY, USA) was used for statistical analysis. Quantitative data were expressed as mean ± SD; statistical data were presented with Graph PAD prism software (GraphPad Software, La Jolla, CA, USA). In the current study, the independent sample *t*-test was used to compare samples. Regression and correlation analyses were done using the Spearman chi-squared test. The relative mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method. Results were considered statistically significant at $p < 0.05$.

Results

miR-200c Expression is Downregulated in OSC Tissue and HOC313 Cells

To determine the role of miR-200c in oral cancer, we first measured the expression level of miR-200c in the 32 pairs of OSC tissues and adjacent tissues by qRT-PCR. We found that the expression level of miR-200c was significantly lower in samples than paraneoplastic tissue (Figure 1A). This is consistent with the role of aberrant miR-200c expression in OSC progression and development. Then, we examined the expression of miR-200c of in HOC313 transfected with miR-200c mimics and scrambled mimics.

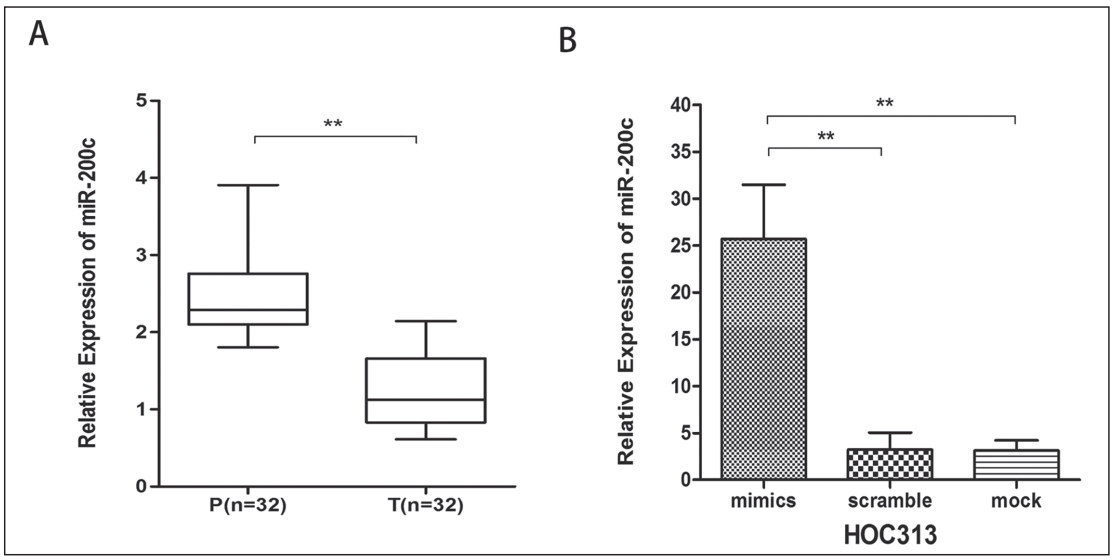

Figure 1. Expression of miR-200c in OSC tissue and cell lines. **A,** Analysis of *miR-200c* expression level in para-carcinoma tissues (P) and tumor tissues (T). *MiR-200c* is significantly decreased in OSC tissue compared with the para-carcinoma tissue. **B,** Transfection of miR-200c mimics and scrambled mimics in HOC313. MiR-200c expression was elevated by mimics compared with scrambled and mock control. GAPDH was used as internal control. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$

The expression of miR-200c was elevated in HOC313 transfected with miR-200c mimics compared with scrambled mimics and empty controls (Figure 1B).

miR-200c Inhibits OSC Cell Migration and Invasion

Since miR-200c was downregulated in OSC, we next explored the role of miR-200c in the tumorous properties of HOC313 cells. As shown in the wound-healing assay, overexpression of miR-200c inhibited HOC313 cell migration when compared with the scrambled group (Figure 2A). Then, we examinined the effect of miR-200c overexpression on cell invasion by using the transwell assay. MiR-200c overexpression inhibited HOC313 cell invasion (Figure 2B). These results suggest that miR-200c is critical for the migration and invasion properties of HOC313 cells.

miR-200c Directly Targets ZEB1 in HOC313 Cells

To understand the mechanism mediating the activity of miR-200c in HOC313 cells, we identified ZEB1 as a putative downstream target of miR-200c using the TargetScan database (Figure 3A). According to the database predictions, miR-200c was co-transfected with a ZEB1 3'UTR luciferase reporter gene into HOC313 cells. We found that the luciferase activity from the ZEB1 reporter was lowered in HOC313 cells transfected with miR-200c mimics compared with cells transfected with miR-200c scrambled mimics (Figure 3B).

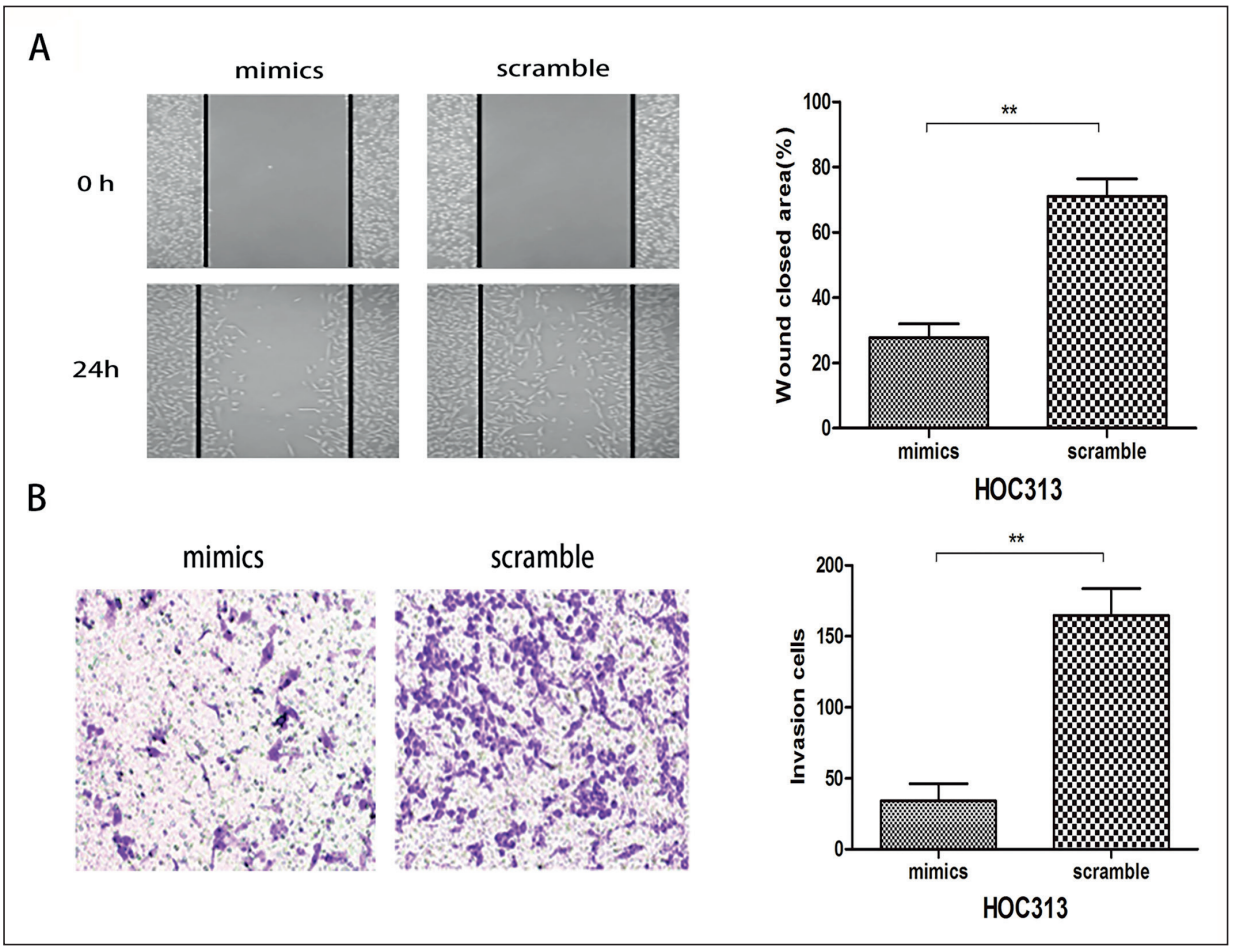

Figure 2. Effects of miR-200c on OSC cell migration and invasion. **A,** Wound healing assay was performed to determine the migration of HOC313 cells transfected with miR-200c mimics and scrambled mimics. **B,** Transwell assay was employed to determine the invasion of HOC313 cells transfected with miR-200c mimics and scrambled mimics. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$.

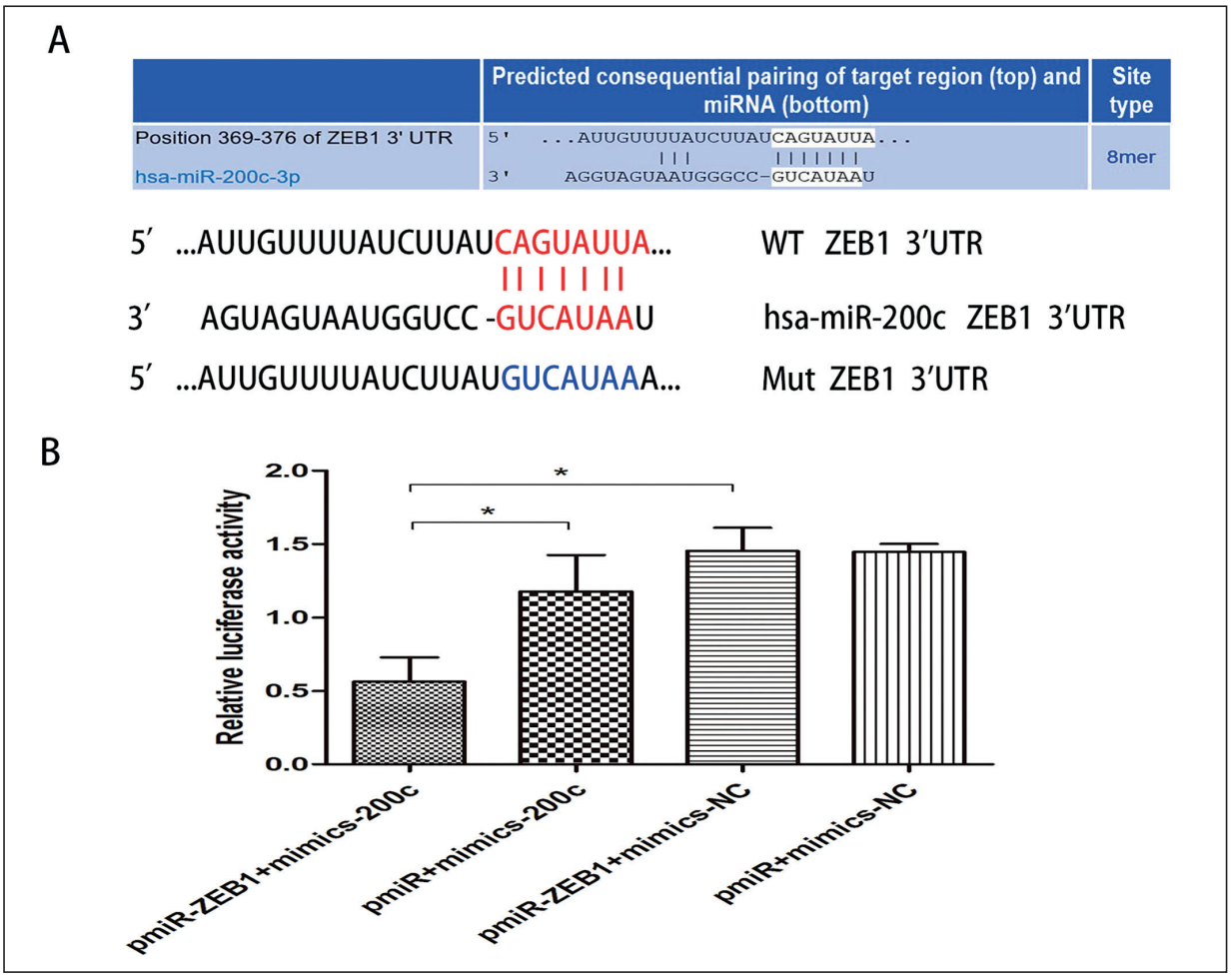

Figure 3. ZEB1 was a direct target gene of miR-200c. **A**, ZEB1 has a miR-200c binding site (7-nt) in its 3'UTR. **B**, Relative luciferase activity of ZEB1 3'-UTR in HOC313 cells normalized to pRL-TK vector luciferase activity. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$

miR-200c Downregulates ZEB1 Expression in OSC Tissues and Cell Lines

To further identify the functional interaction between miR-200c and ZEB1, we measured the expression of ZEB1 in OSC tissues and HOC313 cell (Figure 4A). The expression level of ZEB1 was negatively correlated with miR-200c expression level in OSC tissue (Figure 4B). Additionally, qRT-PCR data also showed that miR-200c significantly induced ZEB1 mRNA degradation (Figure 4C). These results supported the negative regulation of ZEB1 by miR-200c in OSC.

miR-200c Suppresses OSC Cell EMT

To demonstrate whether miR-200c affected EMT in OSC, we employed Western blot to verify the levels of molecular markers for EMT. Results confirmed that the levels of E-cadherin were elevated and the levels of N-cadherin were decreased in HOC313 cells transfected with miR-200c mimics (Figure 5A). As ZEB1 contributes to EMT, we next investigated ZEB1 protein expression. We found that ZEB1 level was decreased in HOC313 cells transfected with miR-200c mimics compared with the scrambled mimic (Figure 5B). The results above indicated that miR-200c attenuated EMT progress in part by targeting ZEB.

Discussion

Recent reports suggest that miRs play a crucial role in carcinogenesis and cancer progression of various types of tumors[13]. MiR-200c is involved in various biological processes of

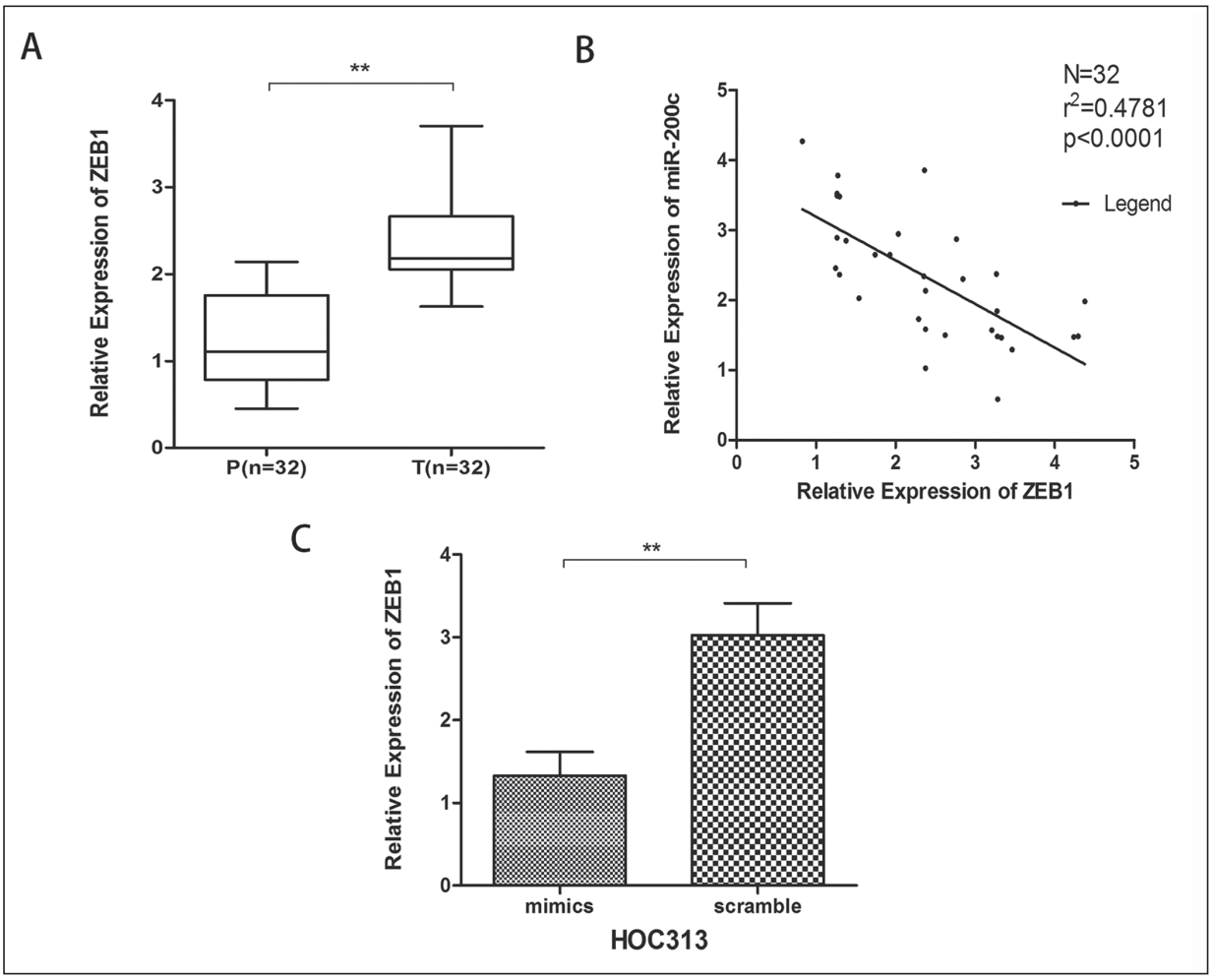

Figure 4. Expression of miR-200c and ZEB1 in OSC tissues and cell lines. **A**, Analysis of ZEB1 expression level in paracarcinoma tissues (P) and tumor tissues (T). ZEB1 was significantly elevated in OSC tumor tissues compared with the paracarcinoma tissues. **B**, ZEB1 expression level was negatively correlated with miR-200c expression level in OSC tissues. **C**, Analysis of ZEB1 expression level in HOC313 cells transfected with miR-200c mimics and scrambled mimics. GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$.

different tumors, including proliferation, invasion, metastasis, drug resistance[14]. MiR-200c is expressed at low levels in some tumors, and can regulate EMT and metastasis[15]. For example, resveratrol inhibits apoptosis, invasion, and switch EMT to MET phenotype through the upregulation of miR-200c in colorectal cancer[16]. Additionally, the increased serum levels of miR-200c have diagnostic value for distinguishing healthy controls and benign tumors from epithelial ovarian cancer[17]. Moreover, miR-200c functions as an oncogene in bladder cancer and may provide a promising therapeutic strategy for the treatment of bladder cancer[18]. Finally, Peng et al[6] uncovered that miR-200c could act as a new therapeutic option for human lung cancer resistance. However, to date, there has been no systematic study of relationship between miR-200c and oral squamous carcinoma.

Here, we demonstrated that the expression level of miR-200c was significantly downregulated in OSC tissue and cell lines, implying that miR-200c may play a role in the progression and development of OSC. Additionally, miR-200c overexpression attenuated OSC cell invasion and migration. These activities of miR-200c could be directly mediated by ZEB1. ZEB1 is an E box-binding transcription factor involved in tumorigenesis of certain kinds of malignancies, including lung cancer, glioma, and OSC[19]. ZEB1 is an oncogene that regulates tumorigenesis primarily via promoting invasiveness, metastasis,

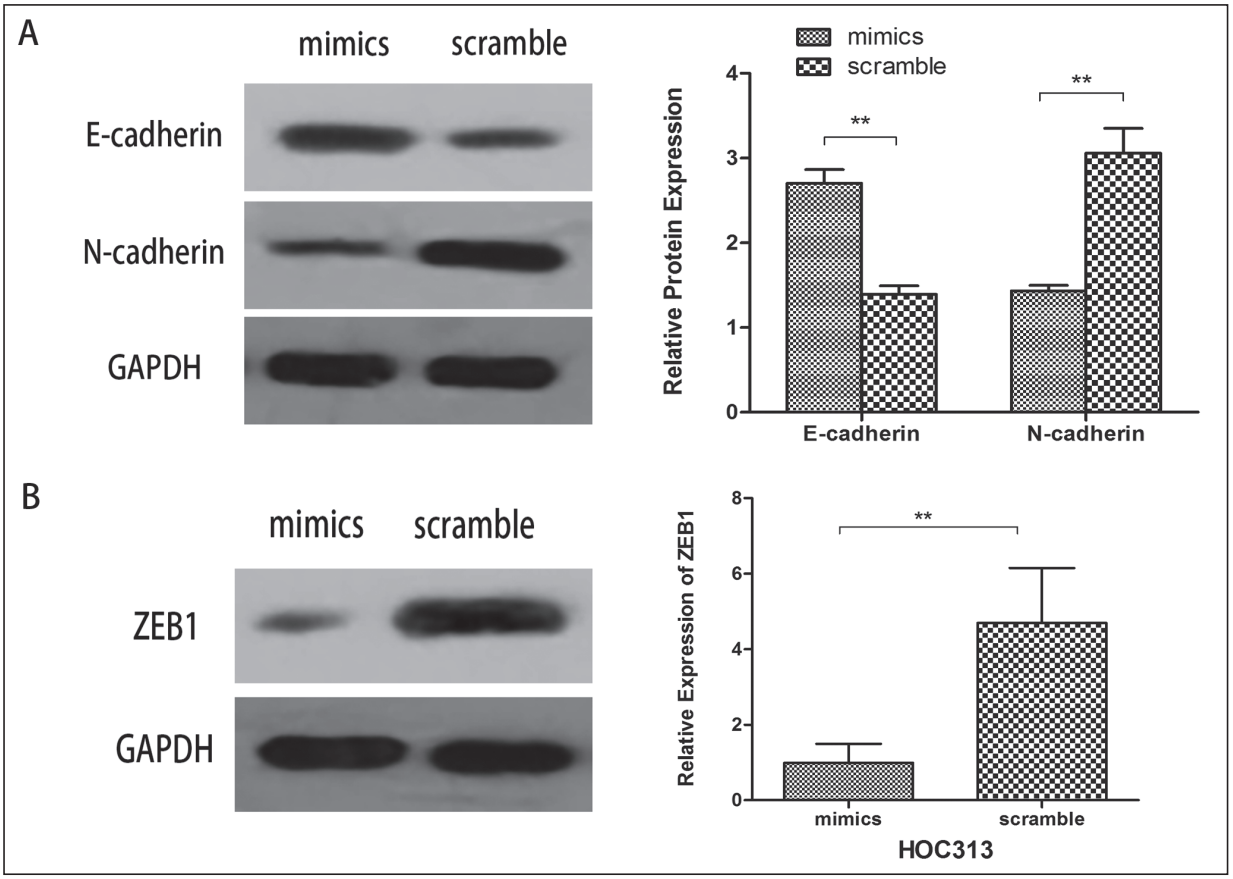

Figure 5. MiR-200c expression inhibited EMT and ZEB1. **A,** The molecular marker protein levels of EMT were detected by Western blot assay. **B,** The protein levels of ZEB1 were detected by Western blot assay. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$.

and EMT of malignancies[20]. We found that ZEB1 expression level was negatively correlated with miR-200c in OSC tissues and that miR-200c overexpression suppressed ZEB1 expression at the mRNA and protein levels. These results suggested that miR-200c exerted a tumor suppressive role by directly targeting ZEB1 in OSC.

Conclusions

We showed that EMT is involved in cell motility in various types of cancer progression, including OSC, and control or reversion of EMT could significantly suppress tumor growth and metastasis of OSC[21]. It is well documented that ZEB1 regulates EMT in several tumors and ZEB1 overexpression can enhance the invasiveness of EMT[22]. Our investigation revealed that miR-200c overexpression upregulated E-cadherin and downregulated N-cadherin and ZEB1.

Overall, the present study demonstrated that miR-200c exhibited tumor-suppression activity on the progression and metastasis oral cancer cells. Additionally, miR-200c suppressed EMT by negatively regulating ZEB1. Our findings imply that miR-200c can serve as a potential and promising therapeutic target for OSC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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