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Original Article

MicroRNA-27b inhibits the migration and invasion of oral squamous cell carcinoma cells via direct suppression of vascular endothelial growth factor-C

Jian Wu, Xin Tan, Xiaohong Zhou

Department of Head and Neck Oncology, Chongqing Cancer Hospital & Institute & Cancer Center, Chongqing 400030, China

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Abstract: Studies have indicated that miR-27b was down-regulated in OSCC, however, the exact role of miR-27b in OSCC remains unclear. In this study, we aimed to investigate the role of miR-27b and its target gene in OSCC. We fist confirmed the down-regulation of miR-27b in both OSCC tissues and cells by using qRT-PCR, and the results were consistent with the previous study. TargetScan and Miranda databases were used to predict the target genes of miR-27b, and the prediction was verified by Dual-luciferase Reporter Assays. To investigate the role of miR-27b in OSCC, a stable miR-27b-over-expression cell line was established by using miR-27b mimics. CCK8, Wound healing assay and Transwell invasion assay were preformed to investigate the proliferation, cell migration and invasion of CAL27 cells. Additionally, the mRNA and protein expression level of VEGF-C was measured by qRT-PCR and Western blot analysis respectively. Our results suggested that VEGF-C is a target gene of miR-27b, and it can be inhibited by miR-27b over-expression. miR-27b over-expression could suppress the proliferation, migration and invasion of CAL27 cells. In conclusion, miR-27b functions as a tumor inhibitor in OSCC though suppressing the migration and invasion of OSCC cells by repressing VEGF-C.

Keywords: miR-27b, OSCC, VEGF-C, target, migration, invasion

Introduction

As a significant global disease, oral squamous cell carcinoma (OSCC) is the sixth most common human malignant tumor in the world. The incidence rate of OSCC is now in an increasing trend, particularly among younger people [1, 2]. Due to the lower overall survival, OSCC is considered an important public health threat [3]. In China, more than 11,900 cases of oral cancers are newly diagnosed every year and about 5,000 patients die of the disease [4]. Various factors can cause the increase of risks of oral cancer, including alcohol and tobacco consumption, age, race and human papilloma virus infection [5-7]. The main treatments for OSCC are surgery, chemotherapy and radiotherapy, although many progresses have been made, the treatment effect is limited and the 5-year survival rate of OSCC is still very low [8, 9]. Therefore, it is very urgent to find new treatment therapies for OSCC.

MicroRNAs (miRNAs), a class of endogenous, non-coding RNA molecules, about 22 nucleotides in length, can inhibit protein translation or cleave mRNA by binding to the 3' untranslated region of the target genes [10-13]. Abnormal expression of miRNAs play critical role in cancer development [14-16]. In OSCC, for example, over-expression of miR-99a inhibited cell proliferation [17], and decreased expression of miR-155 in oral cancer cells suppressed tumor progress [18].

miR-27b plays important roles in the development of cancer, including colon cancer, breast cancer and glioma [19-21]. Previous study suggested that miR-27b may be a valuable biomarker of OSCC [22], and miR-27b was down-regulated in both the plasma and the tumor tissues of OSCC patients [23]. However, the precise role of miR-27b in OSCC remains unclear. Thus, in the present study, we focused on the role of miR-27b in OSCC. We hope that

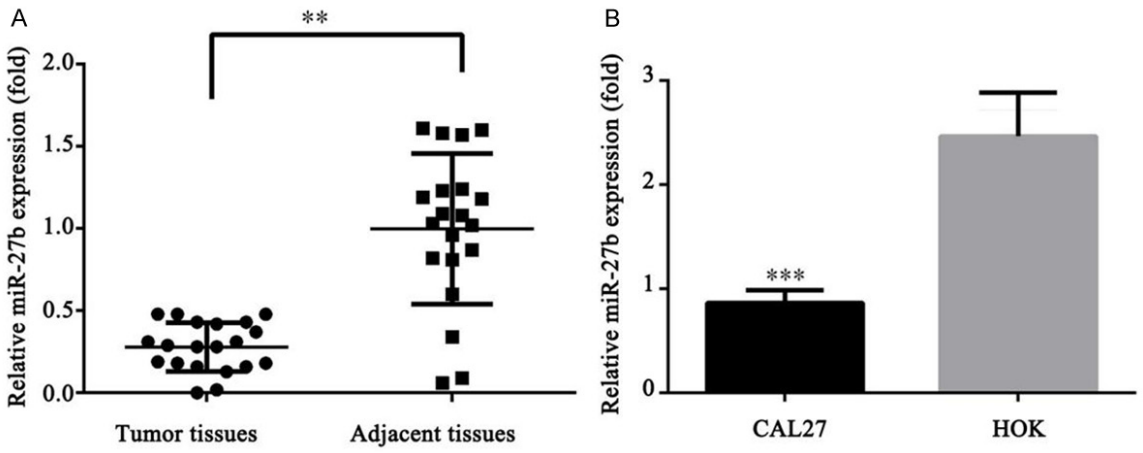

Figure 1. miR-27b expression level in OSCC. The relative expression level of miR-27b in OSCC tissues and cells was measured by qRT-PCR. A: Relative expression of miR-27b in OSCC tissues; B: Relative expression of miR-27b in OSCC CAL27 cells and the primary normal human oral keratinocyte (HOK) cells. **P<0.01, ***P<0.001, tests were performed in triple.

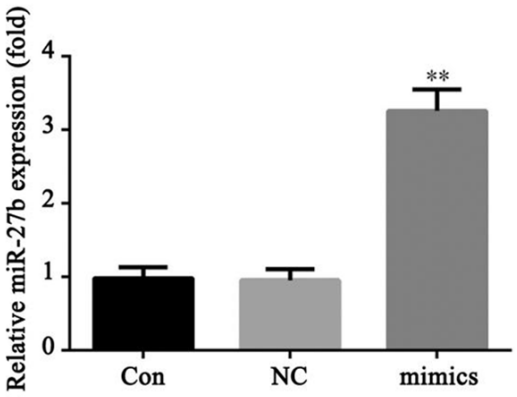

Figure 2. Over-expression of miR-27b. 48 h after CAL27 cells were transfected with miR-27b mimics, relative expression of miR-27b in CAL27 cells was determined. **P<0.01, tests were performed in triple. Con: cells without any treatment; NC: negative control; mimic: CAL27 cells transfected with miR-27b mimics.

the results of this study will provide a potential therapeutic target for OSCC treatment.

Materials and methods

Human tissue specimens

The study was approved by the Human Ethics Committees Review Board at *Chongqing Cancer Hospital* and informed consent was obtained from all patients. Paired primary TSCC samples from anterior portions of the tongue and adjacent histological normal tissues were obtained from 20 patients who were admitted to *Chongqing Cancer Hospital*. None of the patients received treatment before surgery. Tumor tissues and adjacent normal tissues that were snap-frozen in liquid nitrogen and then stored at -80°C until use.

Cell cultures and transfection

Primary normal human oral keratinocyte (HOK) cells were obtained from ScienCell Research Laboratories (San Diego, CA, USA) and were cultured in a keratinocyte growth medium (ScienCell Research Laboratories Inc.) according to the manufacturer's instructions. Human tongue cancer cell lines CAL27 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), grown in fed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. CAL27 cells were seeded into a 6-well plate the day before transfection, then miR-27b mimics or its negative control were transfected into CAL27 cells with Lipofectamine 2000 transfection reagent (Invitrogen, USA) in accordance with the manufacturer's instruction. Cells without treatments act as the blank control. 4 h after incubation, fresh culture medium were replaced. After incubating for another 24 h, the transfected cells were used for following experiments analysis.

Cell proliferation assay

The effects of miRNA-27b over-expression on cell proliferation were assessed by using the

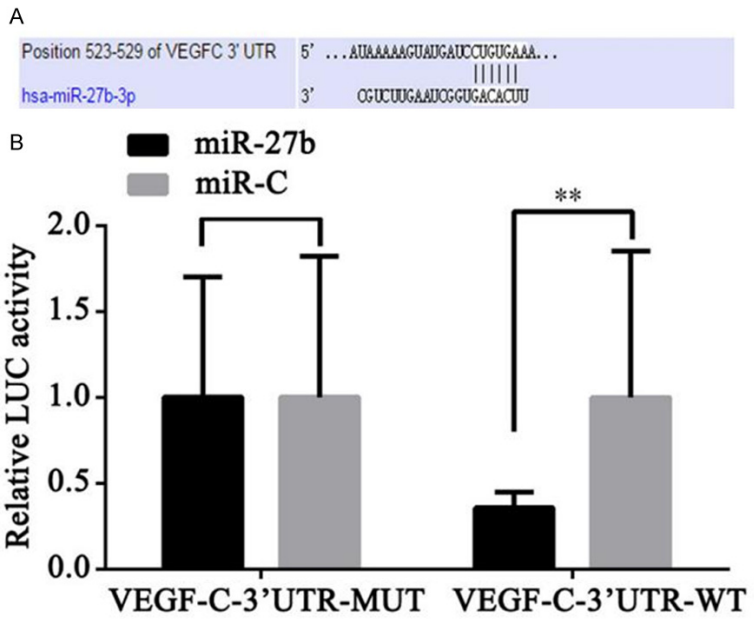

Figure 3. miR-27b directly target VEGFC. A: Interaction between miR-27b and 3'UTR of VEGFC was predicted using TargetScan and Miranda databases; B: Luciferase activity of a reporter containing a wild-type VEGFC 3'UTR or a mutant VEGFC 3'UTR are shown in the bar graph (**P<0.01 *vs* control). Here, "VEGFC 3'UTR-MUT" indicates the VEGFC 3'UTR with a mutation in the miR-27b binding site. UTR, untranslated region.

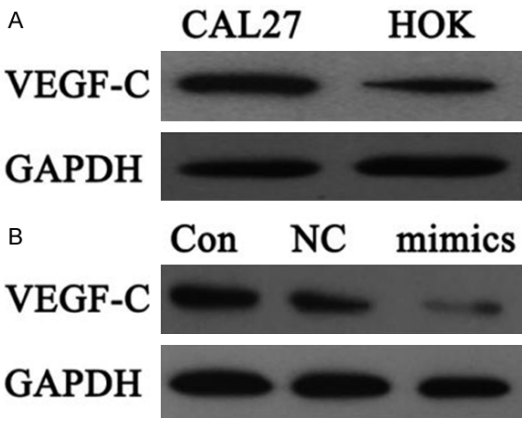

Figure 4. VEGFC protein expression in OSCC cells. Protein expression of VEGFC was detected by using western blotting. A: VEGFC protein expression in OSCC CAL27 cells and the primary normal human oral keratinocyte (HOK) cells; B: VEGFC protein expression in CAL27 cells after transfection with miR-27b mimics. Con: cells without any treatment; NC: negative control; mimic: CAL27 cells transfected with miR-27b mimics.

Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, 24 h after transfection, CAL27 cells were seeded into a 96-well plate (2000 cells per well). CCK-8 (10 ml) was added to each well and incubated at 37°C for 3 h. The absorbance at 450 nm was detected by performing a microplate spectrophotometer (Bio-Tek Instruments Inc, Winosski, VT).

Wound healing assay

CAL27 cells were cultured in 6-well plates and transfected with miR-27b mimics or its negative control using Lipofectamine 2000. Subsequently, a 200 ml pipette tip was used to created wounds in the confluent cells. The debris was discarded by washing with serum-free cell culture medium. 24 h after incubation, cells were photographed under an inverted microscope. Each experiment was independently performed at least three times.

Transwell assay

24 h after transfection, CAL27 cells were resuspended in serum-free cell culture medium and added to the upper chamber with matrigel-coated membrane matrix. 600 μl DMEM containing 10% fetal bovine serum was added to the lower chamber. Subsequently, cells were incubated at 37°C with 5% CO_2 for 24 h. Cells on the upside of the filter were then struck off by using a cotton swab. Cells on the under-surface of the filter were fixed with 4% paraformaldehyde and then stained with hematine. 20 min after incubation, the filters were washed and then imaged and counted (5 random 200× fields per well).

Dual luciferase reporter assay

To investigate whether miR-27b directly targets the 3'-UTRs of VEGF-C, the vectors named VEGF-C-3'UTR-WT and VEGF-C-3'UTR-MUT with wild-type and mutated 3'UTR of VEGF-C mRNA were established. 293T cells were seeded in a 24-well plate and then co-transfected with VEGF-C-3'UTR-WT or VEGF-C-3'UTR-MUT and miR-27b or its negative control (hsamiR-NC) vector using Lipofectamine 2000 transfection reagent following the manufacturer's instruc-

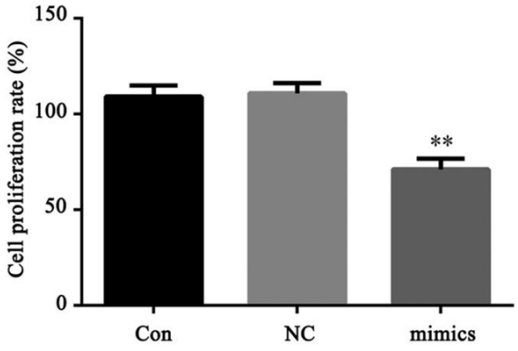

Figure 5. miR-27b inhibits CAL27 cell proliferation. 48 h after CAL27 cells were transfected with miR-27b mimics or its negative control, CCK-8 assay was used to detect cell proliferation. Con: cells without any treatment; NC: negative control; mimic: CAL27 cells transfected with miR-27b mimics. **P<0.01, tests were performed in triple.

tions. 48 h after transfection, we performed the Dual-Luciferase Reporter Assay Kit (Promega, USA) to detect the luciferase activity according to the manufacturer's instructions.

Western blot analysis

CAL27 cells lysed in RIPA lysis buffer (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% NP_40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecylsulfate). Lysates were sonicated and then centrifuged. Protein extracts were resolved by 12% SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were incubated with primary antibodies at 4°C overnight and then incubated with second antibodies (CST, USA; dilution ratio, 1:5000) at room temperature for 1 h. Protein bands were detected by using an ECL kit (Applygen, Beijing, China). The primary antibodies, VEGF-C and β-actin (Cell Signaling Technology, Beverly, MA) were diluted 1:1000 respectively.

RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

Total RNA from tissue samples and cells was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The expression of miR-27b-3p was determined by qRT-PCR. Quantitative PCR was conducted in an ABI 7500 real-time PCR system at following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The relative gene expression level of miR-27b was calculated using the Comparative Ct method.

Statistical analysis

SPSS16.0 was performed for all statistical analyses. Student's t-test and one-way ANOVA were used to analyze the relationship between groups. Data were expressed as mean ± standard deviation (SD). A two-tailed value of *P*, 0.05 was considered to be statistically significant.

Results

miR-27b expression in oral squamous cell carcinoma

We fist determined the expression of miR-27b in both OSCC tissues and cells by using qRT-PCR. As shown in **Figure 1A**, the expression level of miR-27b in tumor tissues was lower than that in the adjacent normal tissues. At the same time, miR-27b expression in OSCC cells was also detected, and the results showed that compared with the normal human oral keratinocyte cells (HOK), the expression level of miR-27b in human tongue cancer cell lines CAL27 was significantly decreased (**Figure 1B**).

To investigate the role of miR-27b in OSCC, miR-27b was over-expressed by using miR-27b mimics. 24 h after transfection, the higher expression of miR-27b in miR-27b mimics transfected CAL27 cells was revealed by qRT-PCR (**Figure 2**).

miR-27b directly targets VEGF-C

To explore the mechanism of miR-27b function in OSCC, we first predict the target gene of miR-27b by using TargetScan and Miranda databases, and luciferase reporter gene assay was used to confirm our prediction (**Figure 3**). Our results suggested that the luciferase activity was significantly decreased in the HEK293 cells co-transfection of miR-27b with miR-27b-VEGF-C-WT, but co-transfection of miR-27b with miR-27b-VEGF-C-MUT did not (**Figure 4**). This data suggested that VEGF-C is a target of miR-27b.

VEGF-C expression in OSCC cells

VEGF-C expression level in OSCC cells was detected by western blot analysis. As shown in

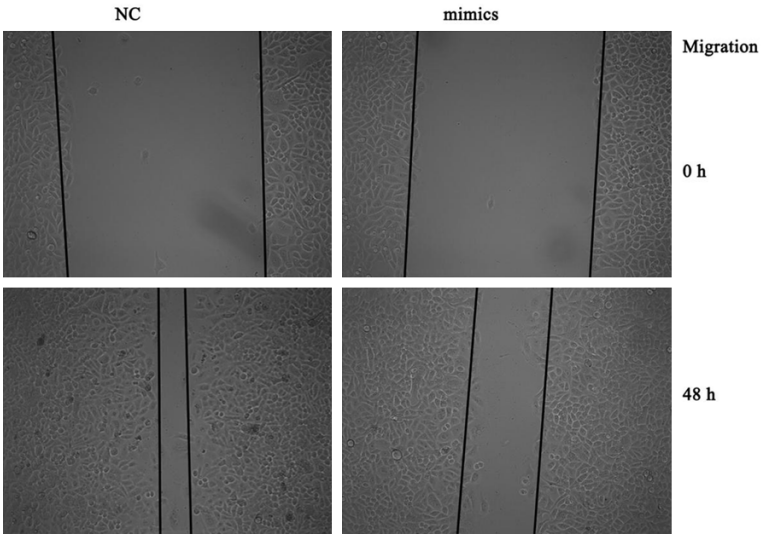

Figure 6. miR-27b inhibits CAL27 cell migration. 48 h after CAL27 cells were transfected with miR-27b mimics, migration ability of the CAL27 cells was determined. NC: negative control; mimic: CAL27 cells transfected with miR-27b mimics.

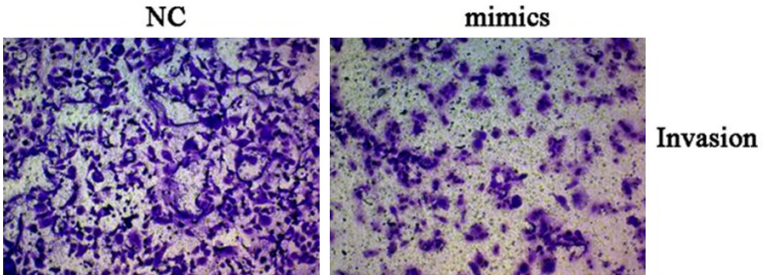

Figure 7. miR-27b inhibits CAL27 cell invasion. 48 h after CAL27 cells were transfected with miR-27b mimics, invasion ability of the CAL27 cells was determined. NC: negative control; mimic: CAL27 cells transfected with miR-27b mimics.

Figure 5A, the expression level of VEGF-C in CAL27 cells was significantly higher than that in the HOK cells. The effect of miR-27b on VEGF-C expression in CAL27 cells was also determined by western blotting, and we found that miR-27b mimics significantly decreased the expression of VEGF-C (**Figure 5B**). These data once again proved that VEGF-C is a direct target of miR-27b.

miR-27b inhibits OSCC cell proliferation

To investigate the effect of miR-27b on OSCC cell proliferation, CCK-8 kit was performed. Our results showed that compared with the controls, the cell proliferation ability was significantly decreased when CAL27 cells transfected with miR-27b mimics (**Figure 5**). The data indicated that miR-27b could inhibit OSCC cell proliferation.

miR-27b inhibits OSCC cell migration

To investigate the effect of miR-27b on OSCC cell migration, we performed wound healing assay. CAL27 cells were transfected with miR-27b mimics or its negative control respectively, 48 h after transfection, cell migration ability was measured. As shown in **Figure 6**, miR-27b mimics markedly inhibited CAL27 cell migration.

miR-27b inhibits OSCC cell invasion

CAL27 cells were transfected with miR-27b mimics or its negative control respectively. To investigate the effect of miR-27b on OSCC cell invasion, transwell assay was performed 48 h after cell transfection. We found that the cell invasion ability was notably suppressed when CAL27 cells were transfected with miR-27b mimics (**Figure 7**).

Discussion

Our present results show that miR-27b expression is notably lower in OSCC tissues than in the adjacent normal tissues, and lower miR-27b expression was also determined in human tongue cancer cell lines CAL27. MiR-27b overexpression inhibited OSCC cell lines proliferation, migration and invasion. 3'UTR of VEGFC was targeted by miR-27b. Therefore, we conclude that the low miR-27b expression level in OSCC promoted OSCC cell growth and metastasis.

Our data emphasizes the role of miR-27b in the development of OSCC cells. This is the first study that revealed the role of miR-27b in OSCC. miRNAs have been found to involve in human cancer progress via regulating various

biological processes [24]. The roles of various miRNAs in OSCC have been widely investigated, including miR-21, miR-31, miR-155, miR-145 and miR-429 [25-29]. Previously, miR-27b was reported to play different roles in cancer. In most cancers, miR-27b level is down-regulated [30, 31], which consistent with our findings. However, in breast cancer, miR-27b was significantly up-regulated, and that knockdown of miR-27b substantially suppressed breast cancer growth [32]. In glima, miR-27b acts as an oncogene in glioma, is upregulated in glioma tissues and cells, and can be acted as a therapeutic target for glioma treatment [33]. Furthermore, miR-27b functions as a tumor suppressor by controlling ARFGEF1 and the paxillin/c-Src circuit at focal adhesions [34]. The different roles of miR-27b are possibly due to the fact that miRNAs can negatively regulate various targets, including oncogenes and tumor suppressor genes. A previous study proved that miR-27b could not only repress CRC cell proliferation, colony formation, but also angiogenesis. MiR-27b plays its role in CRC by binding the 3'UTR of VEGFC mRNA [31]. Our present study suggested that miR-27b can inhibit OSCC cell proliferation, migration and invasion, and also revealed the miR-27b-VEGFC pathway in OSCC.

Our data show that VEGFC was negatively regulated by miR-27b, and that miR-27b directly targeted VEGFC. VEGFC has critical roles in angiogenesis in human cancers, which is critical in cancer development.

In summary, our data proves the low expression level of miR-27b in OSCC tissues and cells, and over-expression of miR-27b inhibits OSCC cell growth, migration and invasion. MiR-27b exerts its role by directly targeting VEGFC. These results of our study will provide more theoretical basis for the treatment of OSCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiaohong Zhou, Department of Head and Neck Oncology, Chongqing Cancer Hospital & Institute & Cancer Center, 181 Hanyu Road, Shapingba District, Chongqing 400030, China. Tel: +86-02365075632; Fax: +86-23-65310296; E-mail: zhouxh1234@sina.com

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