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Regulatory effect of MiR103 on proliferation, EMT and invasion of oral squamous carcinoma cell through SALL4

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Abstract. – OBJECTIVE: Oral squamous cell carcinoma (OSCC) is a common tumor of head and neck cancer. MiR-103 is involved in several tumors. However, the role and mechanism of miR103 in OSCC remain unclear.

MATERIALS AND MATERIALS: Oral cancer Tca8113 cells were cultured *in vitro* and randomly divided into control group, miR-103 mimics group, and miR-103 inhibitor group, followed by analysis of miR-103 expression by Real Time-PCR, SALL4 expression by Real Time-PCR and Western blot, cell survival by MTT assa[illegible] cell invasion by transwell chamber assay[illegible] mor. Real Time-PCR was performed to me[illegible]e MMP-9 and MMP-2 expression. Western blot[illegible] conducted to detect E-cadherin and Vimen[illegible] expression. The Dual-Luciferas[illegible]porter sy[illegible]tem validated the relationshi[illegible] miR10[illegible] and SALL4.

RESULTS: Transfectio[illegible] miR-1[illegible] mimics into Tca8113 cells signific[illegible]upre[illegible] 103 expression, dec[illegible]sed [illegible]mRNA [illegible]d protein expressio[illegible]nhibited [illegible]feration and invasion of Tca8[illegible]ell, downre[illegible]ed MMP-9 and MMP-2 mR[illegible]ssion, incr[illegible]d E-cadherin, and d[illegible]ased [illegible]entin protein expression ($p<0.0$[illegible]. However, [illegible]103 inhibitor transfection [illegible]n-regulated [illegible]103 expression, promot[illegible]proliferation and i[illegible]sion of Tca8113 cells, [illegible]crease[illegible]MP-9 and MMP-2 mRNA expr[illegible]n, d[illegible]ased E-cadherin expression, and [illegible]t[illegible]imenti[illegible]xpression. Compared with th[illegible]trol g[illegible], the differences were [illegible]tical[illegible]nifi[illegible]t ($p<0.05$). The Dual-Luciferase reporter assay confirmed a targeted relationship bet[illegible]en miR10[illegible]nd SALL4.

CONCLUSIONS: The overexpression of miR-[illegible] MMP-9 and MMP-2 expression by negatively regulating SALL4, inhibiting proliferation and invasion of oral squamous cell carcinoma Tca8113 cells.

Key Words:
MiR103, Oral squamous cell carcinoma, Proliferation, Invasion, EMT, MMP.

Introduction

Oral squamous [illegible]arcinoma (OSCC) is one[illegible]n tumor of [illegible]and neck cancer[1,2]. In[illegible]ent years, its incidence has increased and [illegible]ge has becom[illegible] younger[3]. OSCC can occur [illegible]e mouth, o[illegible]arynx, buccal mucosa, lips, t[illegible], etc., and[illegible]n directly infiltrate or spread to [illegible]ndi[illegible]sues, accompanied by lymph node [illegible], and even with distant metastasis, which seriously threatens human health and [illegible]heavy mental and economic burden to pa[illegible]. There are multiple factors in the pathogenesis of oral cancer, which are related to human papillomavirus infection, smoking and drinking habits. Other factors include poor diet, poor oral hygiene, genetic and environmental factors, etc. It is a multi-step and multi-gene participation process[6,7]. Although the treatment of oral cancer is diversified, which is mainly based on surgical treatment and concurrent adjuvant chemotherapy, such as radiotherapy and chemotherapy, due to individualized differences in patients, the differences in disease and TNM staging and the current treatment effect are still unsatisfactory with poor prognosis. The 5-year survival rate and quality of life of patients did not increase significantly[8,9]. OSCC tumor cell proliferation and invasion causes increased lymph node metastasis rate, which delays the diagnosis and up to 50% of patients with OSCC are in advanced stage. To date, the molecular mechanisms of oral cancer pathogenesis remain unknown, which limit the effective therapeutic strategies to curb oral cancer[10,11]. Understanding the genetic changes in OSCC recurrence and metastasis might be helpful in improving the prognosis.

MicroRNAs (miRNAs, miRs) can bind to the 3' untranslated regions (UTRs) of the target

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genes and negatively regulate their expression[12]. The regulation of epigenetics, transcription, and post-transcription, as well as gene expression regulation, have been thought to participate in regulating tumorigenesis and development. It can be used as a tumor biomarker with tissue sensitivity, and can promote oncogene growth or inhibit the growth of latent malignant cells[12,13]. MiR-103 participates in the development of various tumors, and presents abnormal expression in liver cancer, glioma, colon cancer, etc.[14-16]. However, the role and mechanism of miR103 in OSCC remains unclear.

Materials and Methods

Main Instruments and Reagents

The human oral cancer Tca8113 cell line was preserved in our laboratory and stored in liquid nitrogen. Dulbecco's Modified Eagle's Medium (DMEM) medium, fetal bovine serum (FBS), and cyan chain double antibody were purchased from HyClone (South-Logan, UT, USA). Dimethyl sulfoxide (DMSO), tetrazolium salt (MTT) powder was purchased from Gibco (Grand Island, NY, USA); trypsin-EDTA digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Life Sciences (Washington, NY, USA), ethylenediaminetetraacetic acid (EDTA) was purchased from HyClone (Logan, UT, USA), Western blot and chemiluminescent reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), rabbit anti-human SALL4, E-cadherin and Vimentin Antibody, mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell signaling Corporation (Danvers, USA). The transwell chamber was purchased from Corning (Corning, NY, USA). MiR-103 mimics and miR103 inhibitor were purchased from Shanghai Jikai Gene Chemical Technology Co., Ltd (Shanghai, China). Luciferase assay reagent was purchased from Cell Signaling Corporation (Danvers, MA, USA). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Union City, CA, USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). ABI7900 HT Real Time-PCR was purchased from ABI (Waltham, MA, USA).

Oral cancer Tca8113 Cell Culture and Grouping

After being thawed at 37°C, Tca8113 cells were seeded in 6-well plates, and cultured in 10% S 90% high glucose DMEM medium (containing 100 U/ml penicillin, 100 ug/ml streptomycin) at 37°C, 5% CO_2 incubator. Tca8113 cells were divided into control group, miR-103 mimics, and miR-103 inhibitor group.

Transfection of miR-103 Mimics and MiR-103 Inhibitor into Tca8113 Cells

The miR-103 mimics sequence was 5'-TAGCAGCACCCAAAGAATCATA-3'. The miR-103 inhibitor sequence was 5'-AUUGGUGGACUGCUGGGA-3'. The cell density was fused to 70-80% in 6-well plate; miR-103 mimics and miR-103 inhibitor were separately added into 200 µl of serum-free DMEM medium, mixed well for 15 min incubation. The mixed Lipofectamine 2000 was mixed with miR-103 mimics and miR-103 inhibitor dilutions for 30 min incubation at room temperature. The serum of the cells was removed, PBS was gently rinsed, 1.6 ml serum-free DMEM medium was added, and each system was added to each system, and cultured in a 5% CO_2 incubator at 37°C for 6 hours. The serum DMEM medium was replaced and cultured for 48 hours for experimental research.

Real Time-PCR Detection of MiR-103, MMP-2, and MMP-9 Expression

The total RNA was extracted using TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. The primers were synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Shanghai, China) (Table I). Real Time-PCR reaction conditions: 55°C 1 min, 92°C 30 S, 58°C 45 S, 72°C 35 S, for a total of 35 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn and then the semi-quantitative analysis was carried out using the $2^{-\Delta Ct}$ method.

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGTACCTGTAGTCTGCTGG	TAAACCCGGATGTAGTCTGGT
MiR103	CATGTATCTCTTTGGGACTT	CCTCAGTTGCTCACCAGCTG
SNAL4	ATCTCTCGCTTGTGGTTGTG	CACATGATGGGTATCAA
MMP-2	CTCTCTCGCCACCTTCAAG	TTAGGATGATGGGGTAATT
MMP-9	ATCTCTCACATCAATCAA	GATGTGGAAATTGCG CTGA

MTT Assay to Detect Cell Proliferation

Tca8113 cells were inoculated into a 96-well culture plate with 10% fetal bovine serum DMEM culture medium at a cell number of 5×10^3, and the supernatant was discarded after 24 hours of culture, and three groups were randomly divided according to the above treatment methods. 20 µl of sterile MTT was added at intervals of 24 h, and 3 replicate wells were set at each time point. After 4 hours of continuous culture, the supernatant was completely removed followed by addition of 150 µl/well of dimethyl sulfoxide (DMSO) for 10 min. After the purple crystals were fully dissolved, the absorbance (Absorbance, A) was measured at a wavelength of 570 nm, and the proliferation rate of each group was calculated.

Transwell Chamber to Detect Cell Invasion

Serum-free DMEM medium was replaced ... cording to the kit instructions. A... h, the bo... tom and membrane upper c... ace was coated with a 1:5 50 mg/l ... trigel ... ion and air dried at 4°C. 100 µl ... or ce... was prepared by addi... 10 ... DMEM ... dium, and no seru... ee DM... medium was added to the cha... and 3 repli... ells were set in each gro... chamber w... laced in a 24-well plat... Each ... group was cultured in a tran... chamber ... t Matrigel. After 48 hou... culture, the tra... l chamber was wash... ith ph... ate-buffered saline (PBS), the cel... the m... brane were removed, fixed in ice ethan... er stai... with crystal violet, the ... num... the l... layer was counted.

W... ern Blo... alysis of SALL4, E... in and Vimentin Expression

...e protein of each group of TCA8113 ...lls was extracted: radioimmunoprecipitation as... (RIPA) lysate containing protease inhibitor was de... 50 mM NaCL; 1% NP-40; 0.1% SDS; 2 µg/ml Aprotinin; 2 µg/ml Leupeptin; 1 mM PMSF; 1.5 mM EDTA; 1 mM NaVanadate), we lysed the cells on ice for 15-30 min, 5 s × 4 sonication, centrifuged at 4°C, 10 000 × g for ... in, tra... ferred th... pernatant to a new tub... antifie... bicinchonin... ic acid (BCA) assa... an... ...m at ...C for Western blot exp... ents. ... plated ... in was separated on a... sodium d... ...ate-polyacrylamide ... rophoresis (... AGE), and the gel w... ansfe... to a polyvinylidene difluoride (PVDF) membra... blocked with 5% skim mill... for 2 h. Th... membrane was in... c... d with 1:1000, 1:2000, 1:1000 dilution of pri... antibody SA... L4, E-cadherin, and Vimentin ... oclonal antib... shaker, 4°C, overnight. After P... washing, ... 00 sheep anti-rabbit secondary anti... was... d and incubated for 30 min under dar... n washed with PBST, followed by ...ddition of chemiluminescence, X-ray exposure im... X-film and strip density measurements were ...pa... y scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4).

Dual-Luciferase Report Assay

Before the experiment, the Luciferase assay buffer II and the Luciferase assay substrate were thoroughly mixed and configured with Luciferase assay reagent II (LARII), stored at -80°C, and taken out to room temperature before use. The cells were seeded on a 24-well culture plate for overnight and the cells were transfected; after 48 hours of transfection, the cells were lysed on ice for 15 min followed by centrifugation at 12,000 rpm for 2 min and transfection of the supernatant to a new tube on ice. Then, 50 µL LARII and 10 µL of cell lysate was added and Luciferase activity was measured.

Statistical Analysis

Data were shown as mean ± standard deviation (SD). The mean values of the two groups were compared using the Student's *t*-test test, analyzed by SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA), and the comparison of the differences among multiple groups were assessed by analysis of variance (ANOVA) with Bonferroni post-hoc analysis. $p<0.05$ was indicated as a significant difference.

Results

Expression of MiR-103 in Tca8113 Cells of Oral Cancer Group

The transfection of miR-103 mimics can significantly promote the expression of miR-103 compared with the control group ($p<0.05$). MiR-103 expression was significantly downregulated after transfection with miR103 inhibitor ($p<0.05$) (Figure 1).

Effect of MiR-103 on the Proliferation of Tca8113 Cells

The upregulation of miR-103 expression after transfection of miR103 mimics significantly inhibited the proliferation of Tca8113 cells ($p<0.05$). The transfection of miR-103 inhibitor reduced the expression of miR-103 and significantly promoted the proliferation of Tca8113 cells compared with the control group ($p<0.05$) (Figure 2).

Effect of MiR-103 on Invasion Ability of Tca8113 Cell

The transwell chamber assay was performed to detect the effect of miR-103 mimics and i... itor on the invasive ability of Tca8113 ce... results showed that the upregulation of mi...03 expression after transfection of miR-103 r... ics significantly inhibited Tca8113 cell invas... ($p<0.05$). Transfection of miR-... ibitor in... Tca8113 cells significantly re... pression... of miR-103 and promoted ...8113 c... nvasion ($p < 0.05$) (Figure 3).

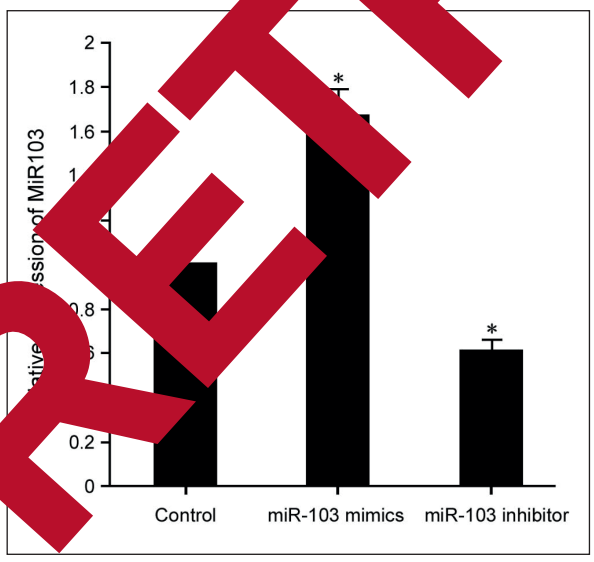
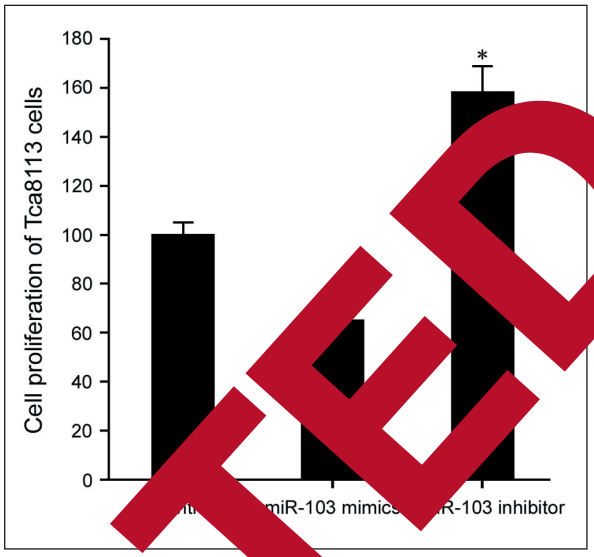


Figure 1. Expression of miR103 in Tca8113 cells of oral cancer group. Compared with the control group, *$p<0.05$.



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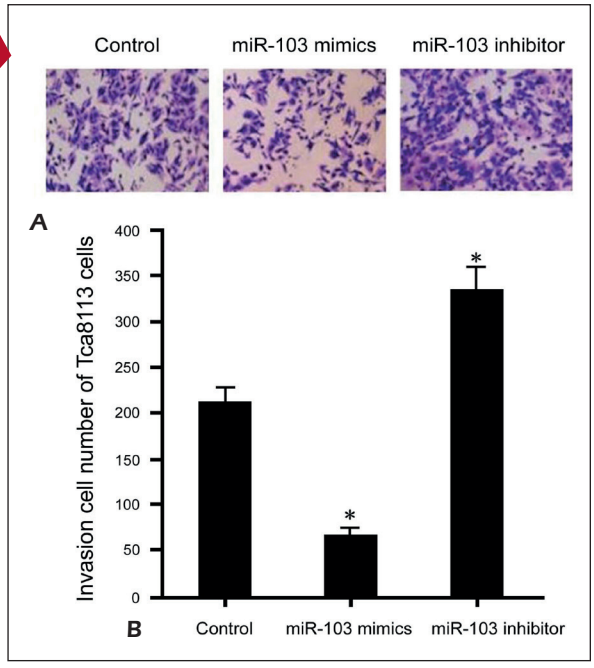

Figure 3. Effect of miR103 on invasion ability of Tca8113 Cells. **A**, Transwell chamber assay regulates the effect of MiR103 on Tca8113 cell invasion (×100). **B**, Invasive ability analysis, compared with the control group, * $p<0.05$.

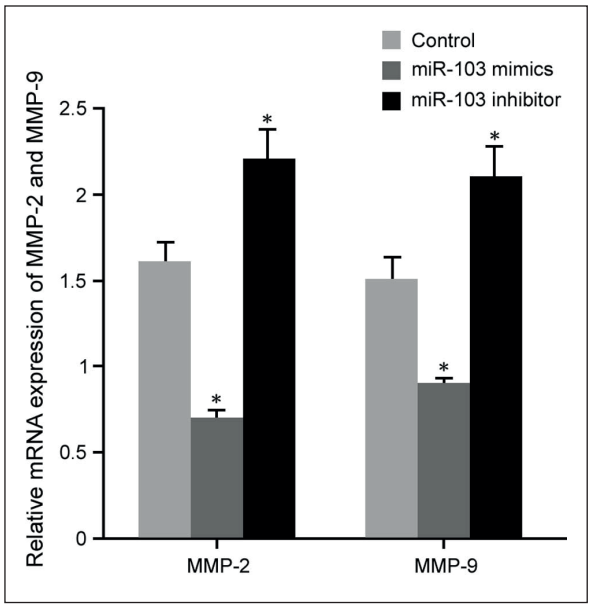

Figure 4. Effect of miR103 on the expression of MMP-2 and MMP-9 in Tca8113 cells. Compared with the control group, * $p<0.05$.

in Tca8113 cells ($p<0.05$). Transfection of miR-103 inhibitor into Tca8113 cells significantly promoted MMP-2 and MMP-9 expression in Tca8113 cells ($p < 0.05$) (Figure 4).

Effect of MiR-103 on SALL4 Expression in Tca8113 Cells

The upregulation of the expression of miR-103 significantly inhibited SALL4 mRNA and the protein expression in Tca8113 cells.

with the control group ($p<0.05$). The transfection of miR-103 inhibitor into Tca8113 cells significantly promoted SALL4 mRNA and protein expression in Tca8113 cells ($p<0.05$) (Figure 5).

Effect of MiR-103 on EMT Protein in Tca8113 Cell

The transfection of miR-103 inhibitor upregulated miR-103 expression in Tca8113 cells increased E-cadherin protein expression, decreased Vimentin expression. The transfection of miR-103 inhibitor in Tca8113 cells decreased the E-cadherin protein expression and increased Vimentin expression after silencing miR-103 expression (Figure 6).

Analysis of Targeting Effect of MiR-103 on Scal4 in Tca8113 Cells

Dual-luciferase reporter assay analysis of the regulation of miR-103 targeting SALL4 in Tca8113 cells indicated that the transfection of miR-103 mimics regulated miR-103 expression and negatively targeted SALL4 (Figure 7).

Discussion

During the occurrence of OSCC, due to the rich blood supply and rich lymphoid tissue, patients are prone to metastasis at an early stage, and the development speed is fast, leading to low survival rate and difficulty in the treatment of OSCC patients[17]. MiRNAs regulate the normal

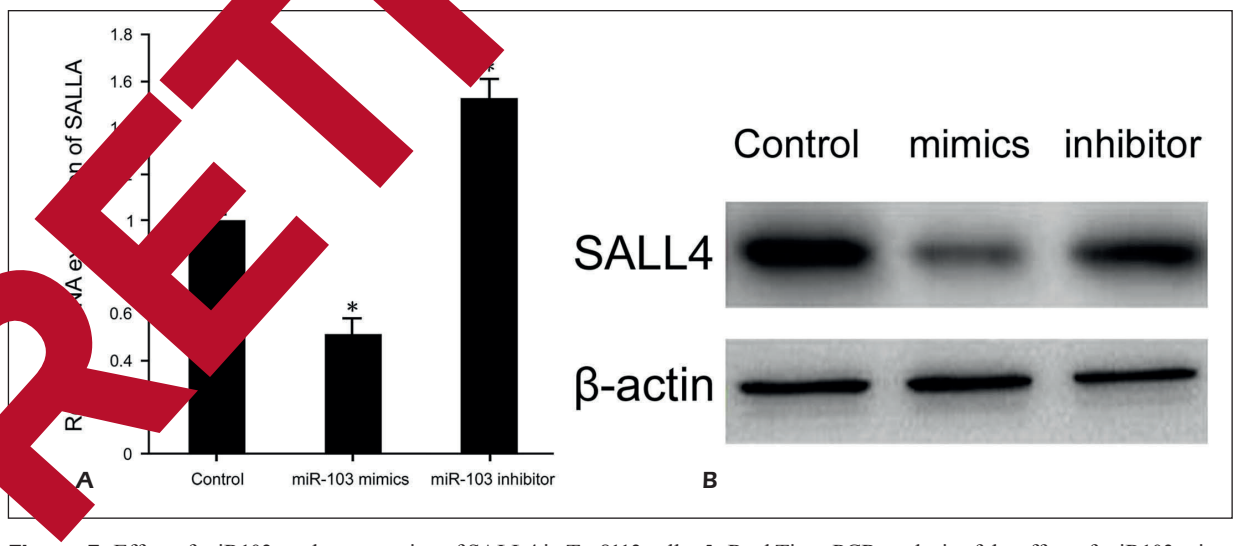

Figure 5. Effect of miR103 on the expression of SALL4 in Tca8113 cells. **A**, Real Time-PCR analysis of the effect of miR103 mimics and miR103 inhibitor on the expression of SALL4 mRNA in Tca8113 cells, compared with the control group, *$p<0.05$. **B**, Western blot analysis of miR103 mimics and MiR103 inhibitor respectively after Tca8113 effect of cell SALL4 protein expression.

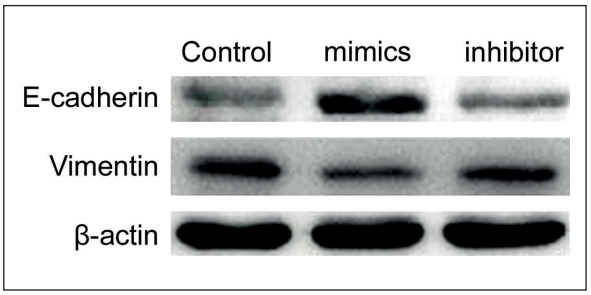

Figure 6. Regulation of miR103 on EMT protein in Tca8113 cells.

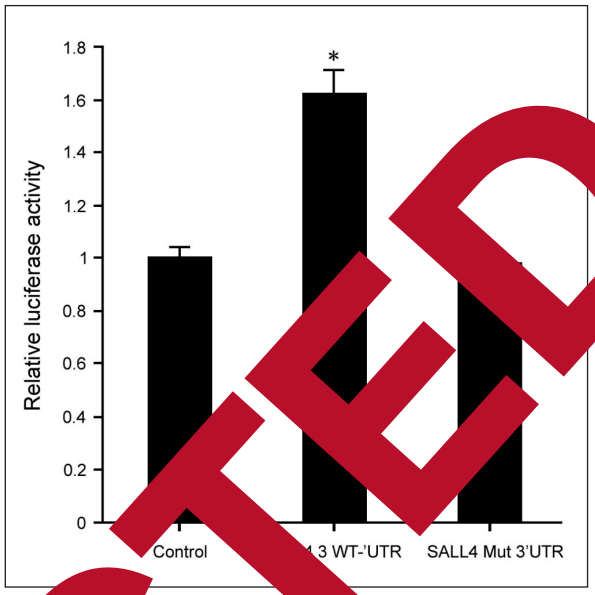

Figure 7. Analysis of targeting effect of miR103 on Scal4 in Tca8113 cells. Compared with the control group, * $p<0.05$.

and pathological state of cells, including growth, proliferation, cell cycle, and apoptosis; therefore, miRNAs are important regulators of the occurrence and development of human diseases[18]. The role of miRNAs in tumors has attracted the attention of scholars. Scholars[19,20] have found that lncRNA, as a transcriptional and post-transcriptional regulator, has a potential as a therapeutic target and can be used as one of the prognostic indicators of tumors. Therefore, identification of miRNAs targeting OSCC can help elucidate the mechanism of oral cancer and establish ther tic targets[21]. MiR-103 is abnormally expre a variety of tumors[15,16]. The present study a to assess miR-103's role in the regulation of O cells. The results indicated that the transfection miR-103 mimics can promote t xpressio of miR-103 in OSCC cells an l prolif- eration and invasion. Conv y, the sfection of miR-103 inhibitor can ificant the expression of miR-10 n cells and promote the prolife n and n of OSCC cells ability. The ts suggest t e upregulation of miR- inhibitory t on the occurrence an develop of OSCC.

SALL family of Z nger transcription factors d in *C. elegans*. amily has been foun vertel s in recent years and usually les f SALL genes, of which SALL4 plays human ysiological pathology[22].

SA gene located on chromosome 13-q lays a role in early embryoni evelopm maintaining embryonic stem ce newal and pluripotency, and reducing ing human tissue and organ matu- ion, but in oral cancer, its abnormal expression ssociated with tumor progression[23,24]. This st showed that the upregulation of miR-103 expression significantly inhibited SALL4 mRNA and the protein expression in Tca8113 cells. The transfection of miR-103 into Tca8113 cells de- cre e mi 3 expression, which promoted SALL and protein expression, and further confirmed that miR-103 negatively regulates pression of SALL4. Metalloproteinases participate in the development and progression of tumors. As important members of the MMP family, MMP-2, and MMP-9 are gelatinases and their activation and tumor metastasis and infiltration are closely related[25,26]. The occurrence of EMT is associated with tumorigenesis and development. EMT can lead to reduced E-cadherin expression and elevated Vimentin expression, and promotes tumor progression[27]. Further analysis in this study disclosed that by upregulating miR-103 expression, SALL4 level was decreased, MMP-9 and MMP-2 mRNA level was downregulated with elevated E-cadherin expression and reduced Vimentin expression. The downregulation of miR103 expression increased the expression of SALL4, MMP-9, and MMP-2 mRNA, decreased E-cadherin expression, increased Vimentin expression, and promoted proliferation and invasion of Tca8113 cells. This result suggests that miR-103 further regulates MMP-9 and MMP-2 expression by regulating SALL4, thereby altering the biological characteristics of oral cancer squamous cell Tca8113 cells. In further study, we will analyze the expression and related regulatory mechanism of miR-103 in clinical OSCC patients, and provide a theoretical reference for miR-103 as a research target in OSCC.

Conclusions

The overexpression of miR-103 can promote the expression of MMP-9 and MMP-2 through negative regulation of SALL4 and promote the proliferation and invasion of oral squamous cell carcinoma Tca8113 cells, leading to the occurrence of EMT.

Conflict of Interests

The authors declare no conflict of interest.

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