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MiR-223-3p inhibits proliferation and metastasis of oral squamous cell carcinoma by targeting SHOX2

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-233-3p (miR-233-3p) in the development of oral squamous cell carcinoma (OSCC), and to elucidate the underlying mechanism.

PATIENTS AND METHODS: The expression of miR-233-3p in OSCC tissues and cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The target of miR-233-3p was detected and evaluated by L-test and Western blot assays, respectively. Furthermore, the effects of miR-233-3p on cell proliferation, migration and apoptosis were discussed by cell counting kit-8 (CCK-8), scratch-wound and flow cytometry test.

RESULTS: MiR-233-3p was lowly expressed in OSCC tissues and cells. Short stature homeobox 2 (SHOX2) was predicted and verified as the downstream target gene of miR-233-3p. Inhibiting the expression of SHOX2 could significantly reduce the malignant behaviors of OSCC cells. The proliferation, migration and anti-apoptotic abilities of miR-233-3p overexpressed cells were obviously limited. However, the recovery of SHOX2 counteracted the beneficial effect of miR-233-3p.

CONCLUSIONS: MiR-223-3p acted as a tumor suppressor gene in OSCC by targeting SHOX2. Our findings revealed that miR-223-3p/SHOX2 axis could be a potential therapeutic target for OSCC.

Key Words:
MiR-223-3p, Oral squamous cell carcinoma (OSCC), Short stature homeobox 2 (SHOX2).

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignant tumor of the head and neck, with the highest incidence rate worldwide. Meanwhile, it accounts for about 3% of systemic malignancies[1]. OSCC is mainly found in tongue, cheek, gingiva, hard and soft palate and mouth floor[2]. In recent years, micro-vascular surgery and repair and reconstruction techniques have been widely used in clinical practice. At the same time, breakthroughs have constantly been made in new treatment methods, including immunotherapy, targeted therapy and biological therapy. However, the cure rate of OSCC has not been significantly improved. The overall five-year survival rate of OSCC patients remains less than 50%. Due to the features of high metastasis and recurrence rate, it seriously threatens human health[3,4].

In the early 1990s, Sun et al[5] discovered lin-4 in the nematode for the first time. Lin-4 is a non-coding single-stranded ribonucleic acid (RNA) molecule that can regulate the time sequence of embryonic development. Successively, hundreds of non-coding RNA molecules with similar function have been found in eukaryotes, such as fruit fly, mouse and human. These RNA molecules are called micro RNAs (miRNAs). MiRNAs can regulate the expression of related target genes at the post-transcriptional level, thereby affecting various physiological activities of the body[6]. So far, more than 2,000 human miRNAs have been identified and proved. Each miRNA is capable of regulating the expression of several target messenger RNAs (mRNAs). Moreover, miRNAs are able to regulate over one-third of human genes[7]. Numerous reports demonstrated that miRNAs play key roles in the development and progression of human benign and malignant tumors by modulating cell proliferation, differentiation, apoptosis and angiogenesis[8,9]. Therefore, it is of great significance to investigate the ex-

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pression and mechanism of miRNAs in OSCC for early diagnosis, reasonable therapeutic regimen and prognosis of tumor patients. As a member of the miRNA family, miR-223-3p has shown its unique advantages in the diagnosis and treatment of a variety of malignancies, such as hepatocellular carcinoma, prostate cancer, bladder cancer and ovarian cancer[10-13]. However, few reports focused on the function of miR-223-3p in OSCC. Therefore, the aim of this study was to investigate the exact role of miR-223-3p in the occurrence and development of OSCC and to explore the possible underlying mechanism.

Patients and Methods

OSCC Clinical Samples and Cell Lines

A total of 33 definitely diagnosed OSCC patients who received treatment in Jinan Stomatological Hospital were enrolled as cancer group. All OSCC patients had primary lesions and received no other treatment. Meanwhile, 27 healthy oral mucosa specimens were obtained as control group. Signed written informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Jinan Stomatological Hospital (Jinan, China).

OSCC cell line SCC-9 was purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). Normal oral mucosal keratinocyte cell line HOK was bought from Sciencell (Carlsbad, CA, USA). Cells were cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator at 37°C, 5% CO_2 and saturated humidity. Next, SCC-9 cells in the logarithmic growth phase were seeded into 6-well plates at a density of 1.5×10^5 cells/well for subsequent experiments.

Luciferase Reporter Gene Assay

Targeted binding sites of miR-223-3p to short stature homeobox 2 (SHOX2) were predicted by bioinformatics online sites (TargetScan and miRDB). On this basis, the binding sequence of miR-223-3p at the 3'-end of SHOX2 was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Subsequently, wild (pGL3-SHOX2-WT) or mutant (pGL3-SHOX2-MUT) plasmid and miR-223-3p or negative control (NC) were co-transfected into SCC-9 cells. After 48 h, luciferase activity was determined according to the instructions of dual luciferase assay kit (Thermo-Fisher Scientific, Waltham, MA, USA).

Cell Transfection

MiR-223-3p mimics, LV-SHOX2 and NC mimics were designed and synthesized by GenePharma (Shanghai, China). Subsequently, they were transfected into SCC-9 cells in accordance with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Three groups of cells were established, including: miR-NC group (negative control), miR-233-3p mimics group (SCC-9 cells transfected with miR-233-3p mimics) and mimics + SHOX2 group (SCC-9 cells co-transfected with miR-233-3p mimics and LV-SHOX2)

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from tissues or cells according to the instructions of TRIzol Reagent (TaKaRa, Otsu, Shiga, Japan). The purity and concentration of extracted RNA were then detected. Next, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit. With cDNA as a template, fluorescent qRT-PCR assay was carried out by PRISM7000 (Applied Biosystems, Foster City, CA, USA) according to relevant instructions. Specific reaction conditions were: 94°C for 10 min, and 94°C for 30 s, 60°C for 30 s and 72°C for 40 s for a total of 40 cycles, followed by extension at 72°C for 10 min. U6 was used as an internal reference. The relative expression level of miR-233-3p was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: miR-233-3p, F: 5'-GAAGCTGTACCTAACATACCGTG-3', R: 5'-GATTGGTCGTGGACGTGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot Analysis

SCC9 cells were first centrifuged and collected, followed by lysis with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) to obtain total protein. Extracted total protein was stored in a refrigerator at -80°C for use. The concentration of the protein sample was determined by the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Before electrophoresis, the concentration of protein was

adjusted to be consistent. Protein samples were separated by 10% gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk powder at room temperature, the membranes were incubated with primary antibodies on a shaker at 4°C overnight. Next, the membranes were washed with phosphate-buffered saline and Tween 20 (PBST) for 3-5 times, followed by incubation with a secondary antibody at room temperature for 2 h. The membranes were washed again with PBST for 3-5 times. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) (ThermoFisher Scientific, Waltham, MA, USA). Finally, ImageJ was utilized to calculate the gray value in each group.

Cell Proliferation

After transfection for 48 h, SCC-9 cells were inoculated into 96-well culture plates (100 μL/well) at a density of 5×10^3 cells/well. Next, 10 μL Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to each well at different time points (24 h, 48 h, 72 h and 96 h), respectively, followed by incubation at 37°C and 5% CO_2 for 2 h. Optical Density (OD) at 450 nm was detected by a microplate reader (SpectraMax 190, Molecular Probes, Eugene, OR, USA). 5 duplicate wells were set for each well, and a blank control group was set as well.

Cell Migration

After transfection for 48 h, cells were first inoculated into 6-well plates. After that, they were cultured overnight until they were confluent in 6-well plates. A 20 μL pipette tip was perpendicular to the plate to streak the middle of wells, and cast-off cells were washed away with phosphate-buffered saline (PBS). Next, the culture medium was replaced by complete medium containing 1% serum. Thereafter, 3-6 positions were selected, photographed and labeled as 0 h. 24 h, 36 h and 48 h later, the labeled positions were photographed.

Cell Apoptosis

After 48 h of transfection, SCC-9 cells were trypsinized, washed twice with PBS and collected into a centrifuge tube. After adding a binding buffer, cell suspension with a concentration of 1×10^6 cells/mL was prepared. Next, cell suspension was placed in a 5 mL flow tube and added with Annexin V-FITC (fluorescein isothiocyanate) and Propidium Iodide (PI) in sequence. After that, the reaction was performed in a dark place at room temperature for 5 min. Finally, cell apoptosis was detected by flow cytometry, and the apoptosis rate of SCC-9 cells was calculated.

Statistical Analysis

Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis. Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p<0.05$ was considered statistically significant.

Results

MiR-223-3p was Down-Regulated in both OSCC Tissues and Cells

We first detected the expression of miR-223-3p in 33 pathologically confirmed OSCC tissue samples by qRT-PCR. Compared with 27 normal oral epithelial tissue samples, the expression of miR-223-3p in OSCC tissues was significantly down-regulated (Figure 1A). Meanwhile, we examined the expression of miR-223-3p in OSCC cell line SCC-9 as well. Compared with normal oral mucosal keratinocyte cell line HOK, we found that the expression of miR-223-3p in SCC-9 cells was significantly suppressed (Figure 1B), which was consistent with the results of clinical samples. Therefore, SCC-9 cells were chosen for later *in vitro* experiments.

SHOX2 was a Direct Target of miR-223-3p

Target Gene Prediction Software predicted that SHOX2 was a supposed target of miR-223-3p (Figure 2A). More importantly, we found that miR-223-3p could significantly inhibit the luciferase activity of wide-type SHOX2 3'-UTR. However, no obvious changes were found in the luciferase activity of Mut-type SHOX2 3'-UTR. This indicated that miR-223-3p had a targeting effect on SHOX2 in SCC-9 cells (Figure 2B). Transfection efficiency was first confirmed by qRT-PCR (Figure 2C).

MiR-223-3p Decreased the Expression Level of SHOX2

Based on the above findings, we examined the expression of SHOX2 in cells after transfection. Consistent with our hypothesis, the up-regulation of miR-223-3p significantly decreased the protein expression of SHOX2 in SCC-9 cells. At the same

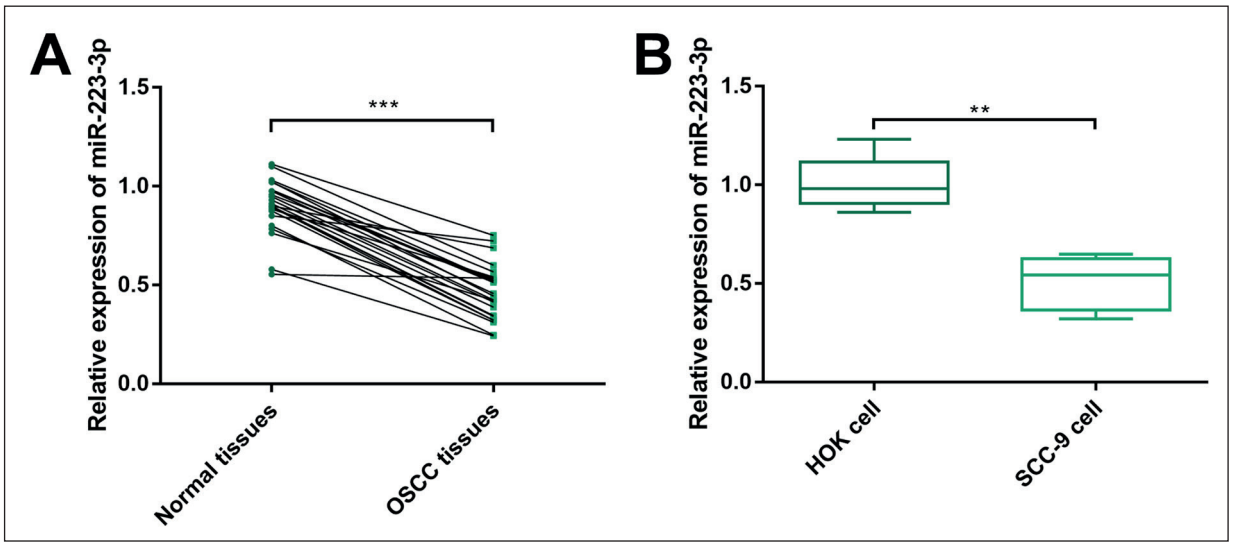


Figure 1. The expression of miR-223-3p in OSCC tissue samples and cells. ***A,*** Difference in the expression of miR-223-3p between OSCC tissues and normal tissues (***p<0.001). ***B,*** The expression of miR-223-3p in OSCC cells (SCC-9) and normal oral mucosal keratinocyte cells (HOK) (**p<0.01).

time, the transfection of LV-SHOX2 plasmid also increased the protein expression of SHOX2 in SCC-9 cells (Figure 2D-2E). Therefore, we explored the effect of miR-223-3p/SHOX2 axis on the function of SCC-9 cells.

MiR-223-3p Suppressed the Proliferation of OSCC Cells

CCK-8 assay was used to evaluate the proliferative capacity of cells after transfection. As shown in Figure 3, the proliferation ability of

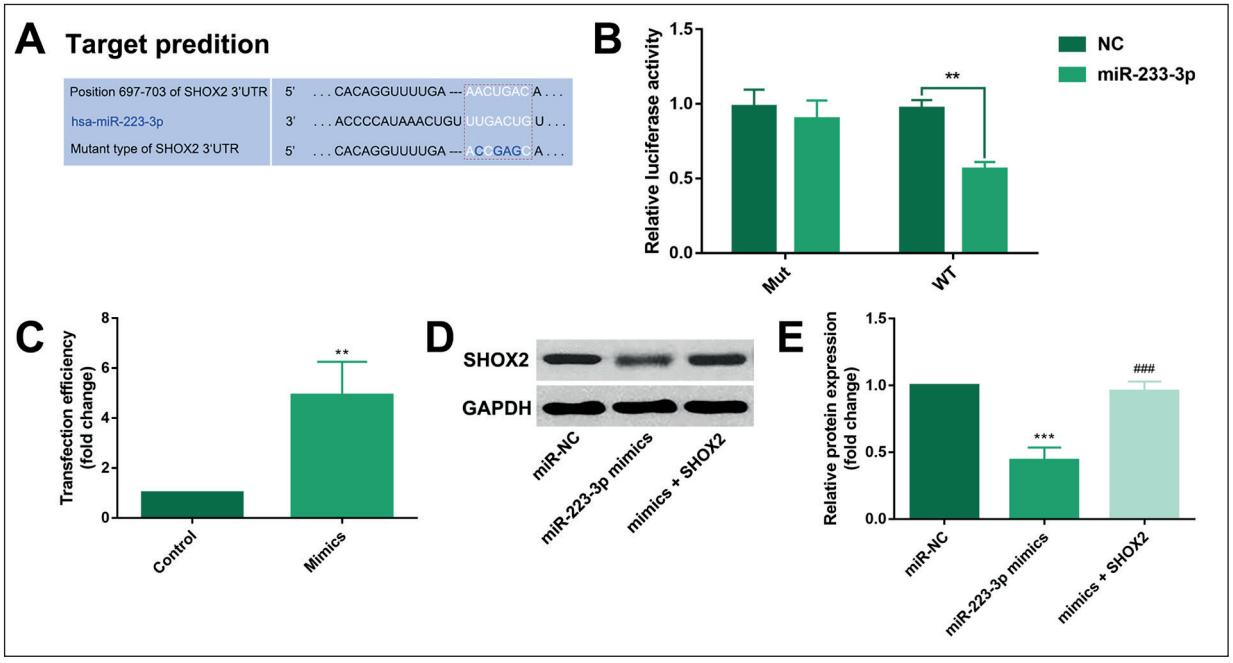
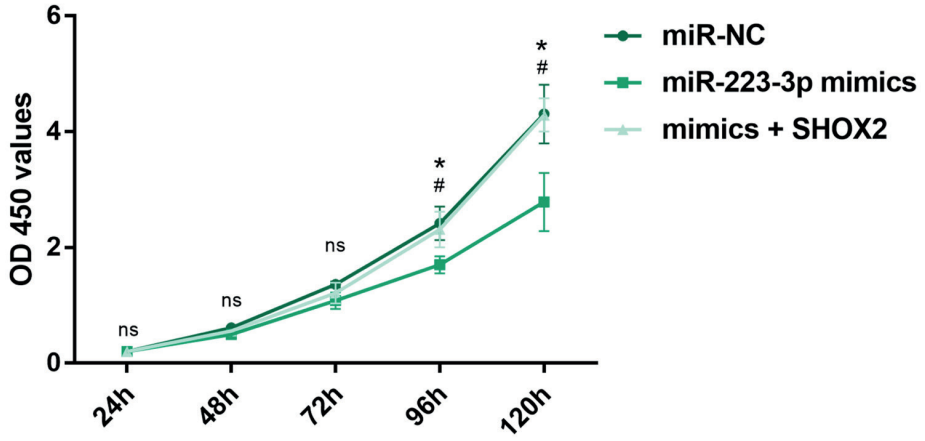


Figure 2. SHOX2 was a direct and functional target of miR-223-3p. ***A,*** Diagram of putative miR-223-3p binding sites of SHOX2. ***B,*** Relative activities of luciferase reporters (**p<0.01). ***C,*** Transfection efficiency of miR-233-3p mimics tested by qRT-PCR (**p<0.01). ***D-E,*** The protein expression of SHOX2 in SCC-9 cells. Data were presented as means ± standard deviations (***p<0.001 *vs.* NC group; $^{###}p$<0.001 *vs.* Mimics group).

A

Proliferation (CCK-8)

	24 h	48 h	72 h	96 h	120 h
miR-NC	0.202 ± 0.003	0.622 ± 0.043	1.312 ± 0.177	2.492 ± 0.406	4.373 ± 0.401
miR-223-3p mimics	0.201 ± 0.011	0.530 ± 0.039	0.998 ± 0.245	1.571 ± 0.311 *	2.702 ± 0.464 *
mimics + SHOX2	0.202 ± 0.008	0.607 ± 0.067	1.202 ± 0.219	2.372 ± 0.352 #	4.502 ± 0.316 #



B

Migration (Scratch-wound)

	0 h	24 h	36 h	48 h
miR-NC	0	0.232 ± 0.019	0.527 ± 0.031	0.883 ± 0.037
miR-223-3p mimics	0	0.193 ± 0.008	0.332 ± 0.040 **	0.634 ± 0.028 ***
mimics + SHOX2	0	0.246 ± 0.023	0.499 ± 0.024 ##	0.917 ± 0.033 ###

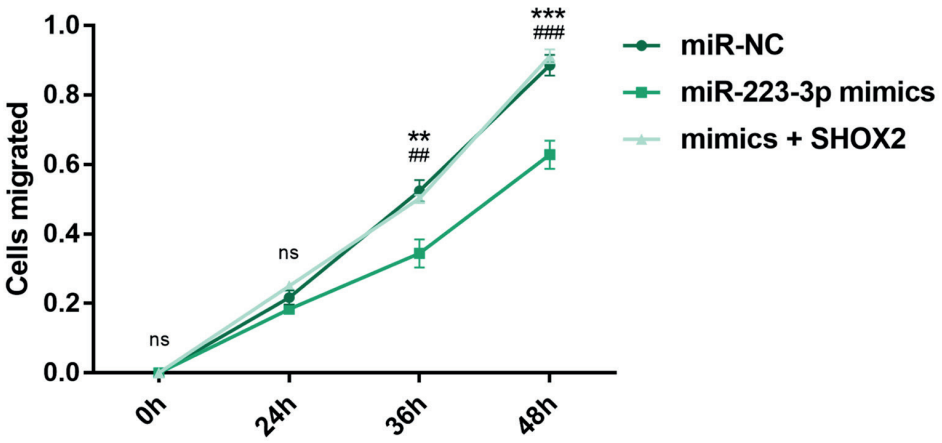


Figure 3. ***A,*** MiR-223-3p inhibited the proliferation of OSCC cells (*p<0.05 *vs.* NC group; #p<0.05 *vs.* Mimics group). ***B,*** MiR-223-3p inhibited the migration of OSCC cells (**p<0.01, ***p<0.001 *vs.* NC group; ##p<0.01, ###p<0.001 *vs.* Mimics group).

cells after transfection with miR-223-3p mimics was significantly weakened. Significant differences were found between experimental group and control group 96 h after cell transfection. However, the proliferation ability of cells in mimics + SHOX2 group was basically the same as that of control group (Figure 3A).

MiR-223-3p Inhibited the Migration of OSCC Cells

Scratch-wound assay showed that miR-233-3p significantly slowed down the migration rate of SCC-9 cells. As shown in Figure 3B, we might clearly find the inhibitory effect of miR-233-3p on cell migration. However, no significant

difference in cell migration rate was observed between mimics + SHOX2 group and miR-NC group.

MiR-223-3p Promoted the Apoptosis of OSCC Cells

Flow cytometry assay indicated that the apoptotic rate of SCC-9 cells was positively correlated with the expression of miR-233-3p. The apoptotic rate of SCC-9 cells with high expression of miR-233-3p remained high. Meanwhile, SHOX2 significantly inhibited the apoptotic rate of SCC-9 cells. When the expression of SHOX2 was restored, the anti-apoptotic ability of SCC-9 cells was demonstrated (Figure 4).

Discussion

OSCC is a common malignancy with the highest incidence rate in the head and neck, ranking eighth among systemic malignant tumors worldwide[14]. If OSCC is diagnosed at its early stage, its five-year survival rate can reach 80%. This is significantly higher than that of patients at mid- or advanced stages[15]. Hence, early detection, diagnosis and treatment are critical for the improvement of survival rate in patients with malignant tumors. As a result, how to detect the factors with high specificity and sensitivity in OSCC, and to achieve early diagnosis and reasonable treatment of OSCC patients are hot spots in medical research. These findings may provide a theoretical basis for improving the survival rate of patients.

MiRNAs are a kind of important discoveries in the field of molecular biology in recent years. They have rapidly become hot spots competitively studied by researchers from various countries[16]. MiRNAs are a class of endogenous short-chain small-molecule non-coding RNAs widely expressed in eukaryotic cells. They can regulate the expression of various genes, leading to the degradation or translational inhibition of target mRNAs. Finally, these changes can modulate the physiological functions of cells[17,18]. In addition, abnormal expression of miRNAs can be detected in all malignant tumors of the human body[9,19,20]. Due to their tissue specificity, the expression of miRNAs is different in different types of tumors. Some miRNAs promote cancerization, while others serve as tumor suppressors. Similarly, related studies[21,22] have proved that specific expression of related miRNAs is found in the development and progression of OSCC. Chen et al[23] have confirmed that 7 miRNAs (such as miR-21, -7, -155, -130b, -223 and -34b) are over-expressed in oral squamous cells, while 7 miRNAs (including miR-100, -99a, -125b and -375) are inhibited. Ramdas et al[24] verified the expression of miRNAs in OSCC tissues *via* cDNA microarray and qRT-PCR assays. They have found that there are around 20 miRNAs with specific expression in comparison with normal oral tissues, including 16 over-expressed miRNAs and 4 down-regulated miRNAs.

Furthermore, miRNAs are important for the regulation of life activities in organisms. Current researches have indicated that this large class of non-coding RNAs can only exert their functions

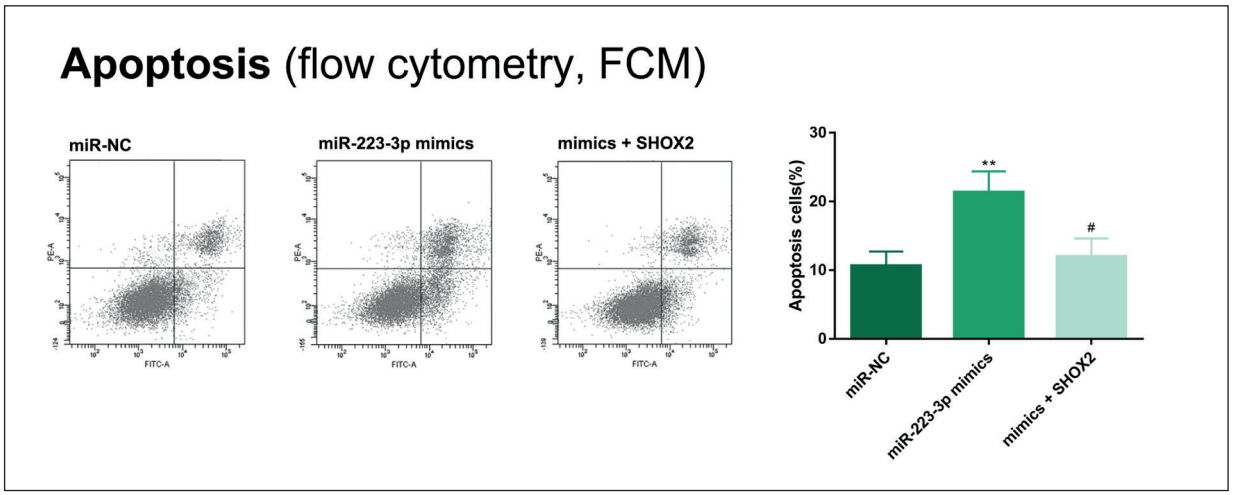


Figure 4. MiR-223-3p promoted the apoptosis of OSCC cells. Data were presented as means ± standard deviations (**p<0.01 *vs.* NC group; #p<0.05 *vs.* Mimics group).

by regulating target mRNAs and affecting the protein expression of these mRNAs. MiRNAs show efficient and complex mechanisms in regulating tumors. One miRNA molecule can simultaneously regulate multiple effector molecules, while a single effector molecule is capable of being simultaneously regulated by multiple miRNAs. Eventually, an intricate regulatory network is formed[7,25]. More and more scholars have paid attention to the functions of miRNAs. With the improvement and development of bioinformatics technology, target genes and binding sites of certain miRNA molecules can be predicted and analyzed through online databases and prediction software[26,27]. If a target gene of a miRNA is predicted *via* many databases, it is likely that this mRNA will become the target gene of miRNAs. Meanwhile, the success rate of subsequent experiments may be greatly increased. In this study, two databases were used simultaneously to predict the target genes of miR-223-3p. The results revealed that SHOX2 was a potential target gene of miR-223-3p.

SHOX2 is a homologous gene of SHOX, which is located on the long arm of human chromosome 3 with about 10,000 bp in length[28]. SHOX2 mainly acts as a transcriptional regulatory molecule in vertebrate embryogenesis[29]. Researches shown that SHOX2 regulates the development and progression of malignant tumors. Dietrich et al[29] discovered in 2010 that SHOX2 methylation could better distinguish between benign and malignant lung tumors. Afterwards, they have detected blood and clinical pathological tissue samples of patients with lung cancer and found that the methylation of SHOX2 can be used as a biochemical indicator for early diagnosis of lung cancer. Meanwhile, it is also an independent predictor for the prognosis of non-small cell lung cancer[30]. Other researches have manifested that over-expression of SHOX2 is closely correlated with the development of liver cancer. High expression of SHOX2 enhances the invasion and proliferation of hepatoma carcinoma cells *in vitro*[31]. Besides, it is also considered that SHOX2 is involved in the development of tumor formation including breast cancer, gastric cancer and thyroid cancer[32-34].

In our study, after up-regulating the expression of miR-223-3p in OSCC cells, the biological functions such as cell proliferation, migration and anti-apoptosis were significantly inhibited. However, co-transfection of SHOX2 restored the protein expression of SHOX2 in OSCC cells with a high expression of miR-233-3p. Meanwhile, it neutralized the regulatory function of miR-233-3p in cells. The above results indicated the inhibition of miR-223-3p on the ability of OSCC cells by targeting SHOX2 expression.

Conclusions

This study preliminarily revealed the molecular mechanism of miR-233-3p in OSCC. Our findings provided a theoretical basis for exploring the pathogenesis of OSCC and reference value for further developing the biological targeted gene therapy for OSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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