Expressions of miR-590 in Oral Lichen Planus and Oral Squamous Cell Carcinoma

Tissues and Clinical Values

Wanlu Chen1, Yong Zhou2, Zhongxiong Ma3 and Yunde Xie4

1 Department of Oral Mucosal Diseases, School and Hospital of Stomatology, Fujian Medical University, Fuzhou, Fujian Province, China
2 Department of Implantology, School and Hospital of Stomatology, Fujian Medical University, Fuzhou, Fujian Province, China
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Abstract: To detect the expression of miR-590 in oral lichen planus (OLP) tissues and oral squamous cell carcinoma (OSCC) tissues, and to analyze the correlations with clinicopathological characteristics and prognosis of OSCC patients. The oral mucosa tissues were selected from 180 patients with OLP or OSCC. They were divided into OLP group (n=92) and OSCC group (n=88), and 40 healthy volunteers with normal oral mucosa tissues were set as control group. Human tongue squamous cell carcinoma cell line SCC9 and human oral keratinocyte HOK were used. The expressions of miR-590 in tissues and cells were detected using qPCR. SCC9 cells were transfected with small interfering (si)-miR-590 and si-NC plasmids. MTT assay, flow cytometry and Transwell assay were used to detect cell proliferation, apoptosis, migration and invasion abilities, respectively. The expression levels of apoptosis-, migration- and invasion-related proteins were examined using Western blotting. Control, OLP and OSCC groups displayed successively increased expression of miR-590, suggesting that the expression was related to the TNM stage and lymph node metastasis of OSCC patients (P<0.05). Following transfection with si-miR-590, the proliferation, migration and invasion abilities of SCC9 cells were weakened significantly, while the apoptosis rate rose (P<0.05). The expression levels of Bcl-2, N-cadherin and vimentin dropped significantly, whereas those of Bax and E-cadherin increased (P<0.05). MiR-590 is highly expressed in OSCC and SCC9 cells. Silencing miR-590 can suppress the proliferation, migration and invasion and promote the apoptosis of SCC9 cells.

Key words: miR-590, Oral lichen planus, Oral squamous cell carcinoma, Proliferation, Apoptosis, Invasion

Introduction

Oral lichen planus (OLP) is a common oral mucosal disease, which differs widely from normal oral mucosa in appearance. For example, reticular, annular or dendritic white spots appear in the oral cavity of OLP patients. Since the etiology of OLP is complex, the pathogenesis of OLP has not yet been fully elucidated, but clinical statistics show that cellular cancerization easily occurs in OLP patients compared with normal people, so the World Health Organization has listed OLP as the precancerous symptom of oral mucosa cancer. Oral squamous cell carcinoma (OSCC), a common malignant tumor in the oral and maxillofacial region, is prone to lymph node and systemic metastasis, seriously endangering the patients. Hence, it is of great significance to search for therapeutic targets and explore the molecular mechanisms of the occurrence and development of OSCC for improving the prognosis of OSCC patients. Micro ribonucleic acid (miRNA) is a kind of non-coding small-molecule RNA widely existing in eukaryotes, which is not only involved in the processes of cell proliferation, differentiation and apoptosis, but also closely associated with the occurrence, development and prognosis of tumors. MiR-590 is one of the most common miRNAs in recent research, which exerts crucial effects in physiological and pathological processes such as inflammation, angiogenesis and tumorigenesis. Meanwhile, it displays abnormal expressions in various malignant tumors such as liver cancer and renal clear cell carcinoma, which is closely associated with the occurrence, development and prognosis of tumors.

Considering that the expression and significance of miR-590 in OSCC have rarely been studied at present, the possible mechanism involved in the process of transformation from OLP to OSCC was investigated in this study. To this end, real-time quantitative polymerase chain reaction (qPCR) technology was employed to detect the expression of miR-590 in oral mucosal tissues of healthy volunteers, OLP patients and OSCC patients, aiming to analyze the correlations of miR-590 expression with clinicopathological characteristics and prognosis of OSCC patients, to observe the effects of miR-590 overexpression on SCC9 cell proliferation, apoptosis, migration and invasion, and to explore the biological function of miR-590 in OSCC.

Materials and Methods

General data

This study has been approved by the ethics committee of School and Hospital of Stomatology, Fujian Medical University (approval No. SHS201801013), and written informed consent has been obtained from all patients. Oral mucosa tissues were selected from 180 patients who were pathologically diagnosed as OLP or OSCC and treated in our hospital. Patients were divided into OLP group (n=92) and OSCC group (n=88), and 40 healthy volunteers with normal oral mucosa tissues were set as control group. In control group, there were 19 males and 21 females aged from 18-75 years old, with an average age of (57.89±11.34) years old. OLP group consisted of 45 males and 47 females at the age of 18-73 years old ([57.74±11.23] on average). OSCC group included 43 patients aged from 18-73 years old ([57.74±11.23] on average).
males and 45 females aged 34-74 years old, with an average age of (57.7±11.23) years old. No significant differences in general data were found among the three groups (P>0.05). The clinical data, including tumor location, size, pathological grade and tumor-node-metastasis (TNM) stage, of OSCC patients were collected.

Cell lines and main reagents

Cells lines used in this study included human tongue squamous cell carcinoma SCC9 cell line and human oral keratinocyte HOK cell line (Shanghai Institute of Cells, Chinese Academy of Sciences). Roswell Park Memorial Institute (RPMI) 1640 culture medium, fetal bovine serum (FBS) and 0.25% trypsin were purchased from Gibco (USA). TRIzol reagent and Lipofectamine™2000 were bought from Invitrogen (USA). Reverse transcription kit and SYBR Green fluorescence qPCR kit were obtained from TaKaRa (Japan). Transwell assay kit was provided by Beijing Reanta Biotechnology Co., Ltd. (China). Methyl thiazolyl tetrazolium (MTT) assay kit was purchased from Sigma (USA). B-cell associated X protein (Bax), B-cell lymphoma-2 (Bcl-2), vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2), MMP-9, vimentin, N-cadherin, E-cadherin, β-actin antibodies and horseradish peroxidase (HRP)-labeled goat anti-rabbit or mouse immunoglobulin G (IgG) secondary antibody were bought from Abcam (UK).

Cell culture and transfection

SCC9 and HOK cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an incubator with 5% CO2. Next, SCC9 cells in the good logarithmic growth phase were inoculated into a 96-well plate at 1×104 cells/well with RPMI 1640 medium containing no antibiotics. After cell confluence reached about 60%, SCC9 cells were transfected as per the instructions of Lipofectamine™2000 transfection reagent. Later, the cells were divided into miR-590 group (transfected with small interfering (si)-miR-590), miR-negative control (miR-NC) group (transfected with NC) and normal control (NC) group (only added with transfection reagents), and 6 replicate wells were set in each group.

Detection of miR-590 expressions in OSCC tissues and SCC9 cells by qPCR

The total RNA was extracted from healthy tissues, OLP tissues, and OSCC tissues and cells using TRizol kit, and then reversely transcribed into cDNAs according to the instructions of the reverse transcription kit. Later, qPCR was conducted using by qPCR instrument (Applied Biosystems 7600, ABI, USA) under the following conditions: pre-denaturation at 95°C for 1 s, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s and 72°C for 30 s. Finally, the expression level of miR-590 in tissues and cells was calculated using 2^(-ΔΔCt) method, with U6 as an internal reference. Primer sequences are listed below: miR-590-F: 5’-GCUCCGUACCGUGAUGAUU-3’ and miR-590-R: 5’-CAGTG- CAGGGTCCGAGGT-3’; and U6-F: 5’-CTCGCCTTCGCAGCACAA-3’ and U6-R: 5’-AAGGCTTCCAAGATTTGCGT-3’.

Detection of proliferation ability of SCC9 cells by MTT assay

Cells transfected in each group were inoculated into a 96-well plate, with 3 replicate wells set for each well, and each well was added with 100 μl of fresh medium, which was replaced after 24, 48, 72 and 96 h of culture, respectively. After that, 20 μl of MTT reagent was added to each well for further 4 h of cell culture in the incubator. Next, after MTT solution was discarded, 150 μl of DMSO was added and fully dissolved by shaking, and the optical density (OD) value was detected at the wavelength of 490 nm using a microplate reader. At last, the growth curve was plotted with OD value as ordinate and time as abscissa.

Detection of migration and invasion abilities of SCC9 cells using Transwell assay

Cell migration assay: The cells receiving transfection were seeded into the upper Transwell chamber at 1×105 cells/ml, and RPMI 1640 culture medium with 10% FBS was added into the lower Transwell chamber. Following incubation in an incubator with 5% CO2 at 37°C for 24 h, the chambers were taken out, washed with phosphate buffered saline (PBS) and fixed in absolute ethanol for 20 min. After staining with 0.1% crystal violet for 15 min, the cells that did not pass through the upper chamber were wiped off with a cotton swab, and the number of cells that passed through the membrane was counted in five fields of view randomly selected under an inverted microscope (×200).

Cell invasion assay: Matrigel gel was diluted with RPMI 1640 culture medium before the assay, then paved evenly in the chamber and dried in the air. Cell invasion assay was carried out according to the instructions of Transwell assay, and other procedures were the same as those in cell migration assay.

Measurement of apoptosis of SCC9 cells by flow cytometry

After digestion of cells transfected for 48 h in each group, the cells were resuspended in binding buffer, mixed evenly, added with 5 μl of Annexin V, dyed in the dark for 10 min, and then reacted with 50 μg/ml PI dye solution in the dark at room temperature for 5 min. Finally, the proportion of Annexin V-positive cells was detected by flow cytometry (Becton Dickinson, U.S.) to determine the apoptosis rate.

Detection of expressions of proteins related to apoptosis, migration and invasion in SCC9 cells by Western blotting

The total protein was extracted from cell total protein extraction kit, whose concentration was determined by BCA method. After that, the protein was subjected to SDS-PAGE, transferred onto membranes, and then sealed with PBS solution containing 5% skimmed milk powder at 4°C. Later, qPCR was conducted using by qPCR instrument (Applied Biosystems 7600, ABI, USA) under the following conditions: pre-denaturation at 95°C for 1 s, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s and 72°C for 30 s. Finally, the expression level of miR-590 in tissues and cells was calculated using 2^(-ΔΔCt) method, with U6 as an internal reference. Primer sequences are listed below: miR-590-F: 5’-GCUCCGUACCGUGAUGAUU-3’ and miR-590-R: 5’-CAGTG-CAGGGTCCGAGGT-3’; and U6-F: 5’-CTCGCCTTCGCAGCACAA-3’ and U6-R: 5’-AAGGCTTCCAAGATTTGCGT-3’.

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Detection of migration and invasion abilities of SCC9 cells using Transwell assay

Cell migration assay: The cells receiving transfection were seeded into the upper Transwell chamber at 1×105 cells/ml, and RPMI 1640 culture medium with 10% FBS was added into the lower Transwell chamber. Following incubation in an incubator with 5% CO2 at 37°C for 24 h, the chambers were taken out, washed with phosphate buffered saline (PBS) and fixed in absolute ethanol for 20 min. After staining with 0.1% crystal violet for 15 min, the cells that did not pass through the upper chamber were wiped off with a cotton swab, and the number of cells that passed through the membrane was counted in five fields of view randomly selected under an inverted microscope (×200).

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Detection of expressions of proteins related to apoptosis, migration and invasion in SCC9 cells by Western blotting

The total protein was extracted using cell total protein extraction kit, whose concentration was determined by BCA method. After that, the protein was subjected to SDS-PAGE, transferred onto membranes, and then sealed with PBS solution containing 5% skimmed milk powder at room temperature for 1 h. Later, the protein was incubated with Bax, Bcl-2, VEGF, MMP-2, MMP-9, vimentin, N-cadherin, E-cadherin and β-actin primary antibodies (diluted at 1:1,000) overnight at 4°C. The next day, the membrane was rinsed, and the protein was incubated with HRP-labeled goat anti-rabbit or mouse immunoglobulin G (IgG) secondary antibody (1:2,000) at room temperature for 2 h, followed by luminescence using ECL in a dark room and image analysis by ImageJ software (National Institutes of Health, Bethesda, USA). In the end, the relative expression of target proteins was presented by the ratio of gray value of target protein bands to that of internal reference β-actin bands.

Statistical analysis

QPCR, MTT assay, flow cytometry, Transwell assay and Western blotting were all repeated three times. SPSS 26.0 software (IBM, Armonk, USA) was employed for statistical analysis. The measurement data normally distributed were expressed as (x±s). One-way analysis of variance was used for comparison among groups, while LSD-t test was utilized for comparison between groups. Numerical data were examined by the χ² test. P>0.05 represented a statistically significant difference.

Results

High expression of miR-590 in OSCC tissues and SCC9 cells

The relative expression of miR-590 exhibited a gradual elevation in control group, OLP group and OSCC group. In detail, the relative ex-
expression of miR-590 was (0.82±0.11), (1.92±0.23) and (2.98±0.45) in the oral mucosa of patients in control group, OLP group and OSCC group, respectively. It was observed that the expression of miR-590 in the oral mucosa of patients was increased significantly in OSCC group and OLP patients compared with that in control group, and it significantly rose up in OSCC group compared with that in OLP group (P<0.05) (Fig. 1). Besides, SCC9 cells expressed significantly more miR-590 than HOK cells (P<0.05) (Fig. 2).

Correlations of miR-590 expression with clinicopathological characteristics of OSCC patients

OSCC patients were assigned into high-expression group (M>2.087) and low-expression group (M≤2.087) based on the median expression of miR-590 (M=2.087). According to the results of the one-way analysis of variance, the miR-590 expression showed no correlations with age, gender, tumor size, tumor location and differentiation degree of OSCC patients (P>0.05), but displayed associations with TNM stage and lymph node metastasis (P<0.05) (Table 1).

Table 1. Correlations of miR-590 expression with clinicopathological characteristics of OSCC patients

| Clinicopathologic factor | N  | Expression of miR-590 | $\chi^2$ | P   |
|--------------------------|----|-----------------------|---------|-----|
|                          |    | Low (n=44) | High (n=44) |     |   |
| Age (t/a)                |    |            |            | 0.182 | 0.669 |
| ≤60                      | 46 | 24         | 22         |     |
| >60                      | 42 | 20         | 22         |     |
| Gender                   |    |            |            | 0.046 | 0.831 |
| Male                     | 43 | 21         | 22         |     |
| Female                   | 45 | 23         | 22         |     |
| Tumor size (d/cm)        |    |            |            | 2.933 | 0.087 |
| ≤3                       | 40 | 24         | 16         |     |
| >3                       | 48 | 20         | 28         |     |
| Tumor site               |    |            |            | 0.075 | 0.963 |
| Tongue                   | 34 | 17         | 17         |     |
| Gum                      | 25 | 13         | 12         |     |
| Other                    | 29 | 14         | 15         |     |
| Differentiation degree   |    |            |            | 3.237 | 0.072 |
| Low                      | 30 | 11         | 19         |     |
| Medium + High            | 58 | 33         | 25         |     |
| TNM stage                |    |            |            | 8.116 | 0.004 |
| I+II                     | 50 | 30         | 18         |     |
| III+IV                   | 38 | 12         | 26         |     |
| Lymph node metastasis    |    |            |            | 7.543 | 0.006 |
| Yes                      | 60 | 36         | 24         |     |
| No                       | 28 | 8          | 20         |     |

Figure 3. Expression of miR-590 in SCC9 cells after transfection with si-miR-590.
Successfully constructed SCC9 cell line with silencing of miR-590

At 24 h after transfection of si-miR-590, the qPCR results revealed that si-miR-590 group exhibited a significantly lower expression level of miR-590 in SCC9 cells than NC group and si-NC group (P<0.05). Meanwhile, the expression level of miR-590 in si-NC group did not significantly differ from that in NC group (P>0.05) (Fig. 3). The above results presented that the SCC9 cell line with silencing of miR-590 was successfully established.

Proliferation ability of SCC9 cells inhibited by silencing miR-590 expression

It was discovered from MTT assay results that the proliferation ability of SCC9 cells in si-miR-590 group was significantly weaker than that in miR-NC group and NC group at 48, 72 and 96 h (P<0.05), but there was no significant difference between si-NC group and NC group (P>0.05), suggesting that silencing miR-590 significantly suppresses the...
Expression promotes the apoptosis of SCC9 cells. NC group (P>0.05) (Fig. 8). It could be concluded that silencing miR-590 expression in SCC9 cells were not significantly different between si-NC group and NC group, si-miR-590 group displayed a significantly expression level of Bcl-2 in SCC9 cells.

Western blotting results manifested that compared to si-NC group and NC group, si-miR-590 group had significantly decreased expression levels of VEGF, MMP-2 and MMP-9 in cells (P<0.05). Additionally, si-miR-590 group exhibited significantly reduced expression levels of N-cadherin and vimentin (P<0.05), but a significantly raised expression level of E-cadherin (P<0.05) (Fig. 6). The above results indicated that silencing miR-590 expression dramatically suppresses the migration and invasion abilities of SCC9 cells.

Migration and invasion abilities of SCC9 cells suppressed by miR-590 overexpression

Transwell assay illustrated that the number of migrating and invading cells in si-miR-590 group was significantly smaller than that in si-NC group and NC group (P<0.05) (Fig. 5). According to Western blotting results, in comparison with si-NC group and NC group, si-miR-590 group had significantly decreased expression levels of VEGF, MMP-2 and MMP-9 in cells (P<0.05). Additionally, si-miR-590 group exhibited significantly reduced expression levels of N-cadherin and vimentin (P<0.05), but a significantly raised expression level of E-cadherin (P<0.05) (Fig. 6). The above results indicated that silencing miR-590 expression dramatically suppresses the migration and invasion abilities of SCC9 cells.

SCC9 cell apoptosis promoted by miR-590 overexpression

It was found from the results of flow cytometry that the apoptosis rate of SCC9 cells in si-miR-590 group was significantly higher than that in si-NC group and NC group (P<0.05), but no significant difference was found between si-NC group and NC group (P>0.05) (Fig. 7). Western blotting results manifested that compared to si-NC group and NC group, si-miR-590 group displayed a significantly expression level of Bax (P<0.05) but a significantly reduced expression level of Bel-2s in SCC9 cells (P<0.05). Moreover, the expression levels of Bax and Bel-2 in SCC9 cells were not significantly different between si-NC group and NC group (P>0.05) (Fig. 8). It could be concluded that silencing miR-590 expression promotes the apoptosis of SCC9 cells.

Discussion

OSCC is a malignant tumor of the head and neck, showing the highest incidence rate and accounting for about 3% of all malignant tumors in the body, and it is also prone to lymph node metastasis. In spite of great progress in the diagnosis and treatment methods of OSCC in recent years, the 5-year survival rate of OSCC patients is still lower than 50%. Hence, searching for novel molecular targets with high specificity is highly valuable for improving the diagnosis, treatment and prognosis of OSCC patients.

Until now, the value of TNM staging for predicting the prognosis of OSCC patients has seldom been reported. A large number of molecular markers have been involved in the pathogenesis of OSCC. Particularly, the expression levels of miRNAs often abnormally increase or decrease, which play crucial roles in the progression of OSCC. Some miRNA families have been verified to participate in the occurrence and development of cancers, as potential markers for diagnosis. Additionally, cancers have been effectively treated by regulating mRNA levels. Clarifying the relationship between miRNA expression profiles and prognosis of OSCC patients may help understand the molecular mechanism of OSCC progression and identify new targets for treatment.

Numerous recent studies have indicated that miRNAs are crucial regulators in vital life activities such as cell development, metabolism, aging and death. Research on miRNAs contributes to clarifying the mechanism of disease occurrence and development, by which new therapeutic targets can be provided. Meanwhile, miRNAs are stably present in body fluids at a concentration usually consistent with that at the focus. Therefore, the determination of miRNA concentration variations is often extremely crucial for the early diagnosis and prevention of diseases such as tumors.

MiR-590 is located near the long arm of chromosome 7 of human genome, and its function remains elusive. Existing research has reported that miR-590 shows abnormal expressions in various tumors and diseases. For example, Cai et al. identified that miR-590-5p was able to inhibit the mRNA expression of its target gene signal transducer and activator of transcription 3 (STAT3) through the luciferase reporter system, and that STAT3 was only expressed in osteosarcoma tissues and cells, exhibiting great effects in the invasion and infiltration of osteosarcoma cells. The study of Cai et al. also proved that miR-590-5p was able to suppress the migration and invasion of osteosarcoma 143B cells, providing a new research direction for clinical treatment of osteosarcoma. The study of Xu et al. demonstrated that in addition to osteosarcoma, miR-590 had the ability of reducing the resistance of tongue squamous cell carcinoma to cisplatin and blocking the immune escape of tumor cells by inhibiting the expression of SCC-3 Fas ligands. In this study, the relative expression level of miR-590 in control group, OLP group and OSCC group displayed a gradual up trend, and it was associated with TNM stage and lymph node metastasis in OSCC.
Biological behaviors of tumor cells, such as proliferation, apoptosis, migration and invasion, are the main affecters for tumor progression. Hence, the influences of miR-590 on the biological behaviors of OSCC cells were investigated, so as to explore the correlation between miR-590 and OSCC in the current study. In liver cancer cells, miR-590 also exhibits a high expression, and down-regulating miR-590 can trigger cell cycle arrest, suppress the growth of liver cancer cells and increase the chemotheraphy sensitivity of cells. Meanwhile, miR-590 also acts as an oncogene in ovarian cancer. Currently, the relationship between miR-590 and OSCC at the cell level has rarely been researched, and their mechanism of action needs more exploration. The results of this study revealed that the expression level of miR-590 in SCC9 cells was significantly higher than that in HOK cells. After silencing miR-590 level in SCC9 cells, the apoptosis rate rose up significantly, whereas the abilities of proliferation, migration and invasion declined significantly. Meanwhile, corresponding variations also occurred in the expressions of apoptosis-related proteins (Bcl-2 and Bax) and migration- and invasion-related proteins (VEGF, MMP-2 and MMP-9), which indicated that miR-590 exerts anti-tumor effects probably by participating in the regulation of the biological processes of SCC9 cells. Epithelial-mesenchymal transition (EMT) is the main mechanism of invasion and metastasis of tumor cells, and the enhancement of EMT of tumor cells is the main reason of malignant behaviors such as cell migration and invasion, and the metastasis and diffusion of cancer cells. It was discovered from the results of this study that silencing miR-590 can down-regulate the EMT marker protein N-cadherin but up-regulate the expression levels of E-cadherin and vimentin, indicating that silencing miR-590 can inhibit the EMT of SCC9 cells to play roles in the migration and invasion process of OSCC cells.

In summary, miR-590 shows a high expression in OSCC tissues, which is closely associated with TNM stage, lymph node metastasis and poor prognosis. Additionally, silencing the miR-590 expression is able to suppress the proliferation, invasion and migration and promote apoptosis of SCC9 cells, suggesting that miR-590 can act as a potential target for OSCC treatment.

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Competing Interests

The authors declare that they have no competing interests.

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