Inhibition effect of miR-150 on the progression of oral squamous cell carcinoma by data analysis model based on independent sample T-test

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To explore the influence of mir-150 (M-150) ornithine decarboxylase (ODC) or inhibition in the development of oral squamous cell carcinoma (OSCC), the malignant tumor (MT) textures removed by surgical resection of maxillofacial tumors in patients with OSCC and the normal neighbor oral textures were collected. Then human OSCC cal-27 cell line was cultivated in vitro. The expression differences of M-150 in MT textures, neighbor textures and cal-27 cells were explored by fluorescence polymerase chain reaction (PCR). Cal-27 cells were transfected with M-150 mimic, M-150 inhibitor (M-150-I) and negative control of different concentrations, respectively, to test the transfection rate. After transfection (AF) with the optimum transfection concentration, the migration rate of transfected cells was explored by cell scratch test. Transwell assay was used to detect the change of aggression rate of transfected cells. Finally, independent sample t-test model was used to explore and compare the results between groups. The results manifested that the expression of M-150 (Eom) in MT textures and cal-27 cells was obviously less than that in neighbor normal textures (P < 0.05). Transfection rate results manifested that M-150 mimic of 100 nmol/L and M-150-I of 50 noml/L had the best efficiency. AF, cell migration and aggression (M&A) rates in the M-150 mimic group were obviously less than those in the negative control group (CP) (P < 0.05), while those in the M-150-I group were obviously upper (P < 0.05), which indicates that the over Eom could inhibit the M&A of OSCC cells, and thus play an effect in inhibiting the development of OSCC.

1. Introduction

OSCC is a common malignant tumor of the head and neck (Wang et al., 2015). It is mainly caused by carcinogenesis of luminal mucosal epithelial cells. As a malignant tumor of oral and maxillofacial region, the incidence of OSCC is about 80% (Kim et al., 2013). In recent years, the number of people with OSCC has increased year by year. At the same time, due to unhealthy eating habits, environmental pollution and other external factors, the age of onset of OSCC patients is getting smaller and smaller (Liu et al., 2010). According to incomplete statistics, about 50,000 people worldwide develop OSCC every year. Two-thirds of patients with OSCC are at an advanced stage of diagnosis, seriously threatening the lives of patients (Kawashiri et al., 2009; Deng et al., 2016). In addition, OSCC has a strong invasiveness and metastasis, and it is easy to metastasize to the lymph nodes in the neck. Therefore, in the early stage of MT, oral squamous MT cells are easy to metastasize to the lymph nodes in the neck (Rather et al., 2013; Zhang et al., 2018). In recent years, OSCC patients have a mortality rate of up to 40%, while for advanced MT patients, the mortality rate is as high as 70% (Ma et al., 2018). At present, the main method for the treatment of OSCC is to surgically remove the tumor texture to achieve therapeutic purposes (Cufer et al., 2013). At this stage, people still do not know how the oral squamous cell MT occurs.

In recent years, with the rapid development of life sciences, researchers have begun to turn their attention to a class of micro RNA molecules that regulate gene expression (GE) at the transcriptional level - microRNA (miRNAs) (Hunt et al., 2011). miRNAs are usually a class of non-coding single stranded RNA molecules (NCSSRM) encoded by endogenous genes of approximately 22 nucleotides in length, which are involved in the regulation of...
post-transcriptional GE in eukaryotic cells by binding to the 3′-UTR of the target gene mRNA. (Shibba et al., 2013). With the in-depth study of miRNA molecules, a large number of experimental results show that the abnormal expression (AE) level of miRNA has close tie with the malignant change of textures, and has close tie with the infiltration and metastasis of tumor cells.

The miRNAs are a kind of NCSSRM that can target and bind to the 3′ UTR region of the target gene and then play a biological effect by regulating the expression of the target gene. With the in-depth study of miRNA molecules, many experimental results show that the abnormal miRNA expression level has close tie with the infiltrated and metastasis of tumor cells. The miRNA expression level of the target gene mRNA. (Shiiba et al., 2013). With the in-depth study of miRNA molecules, many experimental results show that the abnormal expression (AE) level of miRNA has close tie with the malignant change of textures, and has close tie with the infiltration and metastasis of tumor cells.

Therefore, in this research, the MT textures and neighbor normal oral tissues of patients with OSCC were collected, and the Cal-27 cells of human OSCC were cultivated at the same time to detect the difference in the Eom. Cal-27 cells were transfected with M-150 mimic, M-150-I and negative control, respectively. The results of this research were designed to provide theoretical basis for studying the mechanism of M-150 in OSCC.

### 2. Materials and methods

#### 2.1. Test sample

In this research, the textures at the lesion site and the neighbor normal oral tissues of patients with OSCC who underwent maxillofacial tumor resection surgery in Xijing hospital of Shaanxi province from July 2018 to September 2018 were collected. The specimens were collected rapidly after excision of the tumor texture, and the texture pieces of 8 mm in size were immediately cut. Immediately, the stains and blood stains on the textures were rinsed twice with pre-cooled PBS. 1 mL of RNAiso for mRNA Tailing Reaction System. Then, the cells were transferred to a 1.5 mL EP tube, gently mixed upside down, and placed at RT for 5 min. Then the cell RNA was extracted by the above method.

#### 2.2. Extraction of total RNA from textures and cells

Texture samples were taken from liquid nitrogen and about 100 mg of texture samples were put into a mortar after liquid nitrogen precooling for grinding. During the process, liquid nitrogen was continuously added until the textures were ground into dry powder. It was transferred to a 1.5 mL centrifuge tube, added lml RNAiso for Small RNA reagent, mixed well and left on ice for 5 min. 200 lL chloroform was added and mixed well. It was let stand at room temperature (RT) for 5 min and centrifuged at low temperature of 12000 rpm for 10 min. The supernatant was discarded, followed by lmL pre-cooled 75% ethanol washing and precipitation prepared by sterilized DEPC, and centrifuged at a low temperature of 12000 rpm for 10 min. The supernatant was discarded, washed for 3 times in total, and let stand at RT until the tube was dry. 0.1% DEPC water of 20 lL was added to dissolve the precipitate. Two lL RNA was used to detect the concentration and purity of the extracted RNA. The rest RNA was stored in the refrigerator at −80 °C.

Therefore, PloyA tails need to be added before reverse transcription of miRNAs. Mir.X miRNA First-Strand Synthesis Kit from Beijing Qingke Xinye Biotechnology Co., Ltd. was used for tailing. Specific miRNA reverse transcription steps were carried out according to the instructions.

The miRNA tailing reaction system was prepare according to Table 1, the mixture was gently shaken and mixed, cultivated at 37 °C for 1 h. heated at 85 °C for 5 min to inactivate the cdNA enzyme, then 90 µL of ddH2Owas added to constant value 100 µL to obtain the cdNA reverse transcription product of miRNA. The sample was then store at −20 °C.

#### 2.4. Fluorescent quantitative PCR (RT-qPCR) detection of M-150

Fluorescence RT-qPCR is a technology developed based on traditional polymerase chain reaction (PCR) technology for qualitative and quantitative analysis of initial amplification templates with high specificity, sensitivity and accuracy, as well less loading quantity of sample, simple operation, and no need of special and expensive reagents and instruments. On account of these advantages, it has been widely used to detect the expression of mRNA and small RNAs such as miRNAs in textures or cells. For the qPCR amplification of M-150, U6 was used as the internal reference gene, and the M-150 and U6 qPCR inverse systems were prepared as manifested in Tables 2 and 3. The process was performed at low temperature on ice, and was protected from light.

#### 2.5. Cell transfection of M-150

Primer sequences of M-150mimic and M-150-I and corresponding negative control sequences were designed by ShengGong Biotechnology (Shanghai) Co., Ltd. The CAL-27 cells (Feng et al., 2017) were seeded in a 6-well plate 24 h in advance to a cell concentration of 2 × 105 cells/well. 500 µL of fresh high-sugar DMEM (HyClone, USA) medium was added, and the medium was gently

| Reagent                  | Volume (µL) |
|--------------------------|-------------|
| mRX Buffer(2X)          | 5           |
| RNA sample               | 3.75        |
| mRX Enzyme               | 1.25        |
| Total                    | 10          |

Table 1

miRNA Tailing Reaction System.
shaken to uniformly plate the cells. The cells were cultivated at 5%
use. Respectively 1
L, 2
L, 3
L, 5
L, 10
L, 20
L, 30
L, 50
L, 100
L, three replicates per
sample. The sample was transfected at 1% O2, 5% CO2, 37
C. At 0 h and 24 h, the change of the
boundary distance between the scratches was observed using an
inverted microscope, and the relative migration rate was calcu-
lated. The effect of M-150 on the migration of human oral squa-
mous carcinoma cells was explored by independent sample T test
model (Weichieh et al., 2014).

2.7. Transwell aggression experiment

The Transwell chamber (Coming Company, US) with a pore size
of 89 M was used in this experiment. The experimental group was
CAL-27 cells transfected with M-150 mimic and M-150-I respec-
tively, and the CP was CAL-27 cells transfected with M-150 mimic
control (MC) and M-150-I control respectively. The matrigel gel
was first placed in a refrigerator at 4 ºC overnight to melt it into
a liquid state. A certain amount of high-sugar DMEM medium
without fetal bovine serum was added to dilute matrigel gel for
ten times, then 60 µL Matrigel gel was added to the pre-cooled
Transwell upper chamber (TUC), and the gel was spread evenly
and cultivated at 37 ºC for 4 h until the gel is solidified. The resid-
ual liquid in the Transwell chamber was then pipetted, and 100 µL
of serum-free high-glucose DMEM medium was added to each
well, and cultivated at 5% CO2 and 37 ºC for 30 min to gelatinize
the matrix. The medium of four groups of CAL-27 cells in the log-
arithmic growth phase that were routinely transfected in a six-well
plate was discarded, then the cells were centrifuged after pancre-
atic digest, and washed once with PBS, and finally resuspended
in serum-free medium with BSA, the cell density was adjusted to
2 x 105 / mL. In a Transwell chamber with matrigel gel, 100 µL
cell suspension was slowly added along the upper chamber wall,
and 600 µL of high glucose DMEM medium containing 10% fetal
bovine serum (Biological Industries Company, Israel) was added
to the less chamber. Set 3 repetitions per group. The Transwell
chamber was cultivated in a 1% O2, 5% CO2, 37 ºC incubator for
24 h. The Transwell chamber, which was air-dried at RT, was then
infiltrated in hematoxylin solution to stain the cells that pene-
trated the basement membrane for 15 min, and carefully eluted
with running water until it is colorless, and air dried. The finally
cut membrane was placed on a glass slide, fixed with a neutral
resin and a cover glass was added, and five fields of view were
selected under a microscope to perform cell counting, and the
average was taken. The influence of M-150 on aggression of human
oral squamous carcinoma cells was then explored based on an
independent sample T-test model (Zhou et al., 2014).

2.8. Statistical analysis

All data in this experiment were put as mean ± standard devia-
tion (x ± s) of three replicates. Statistical analysis was performed
using SPSS 19.0 statistical software. Independent sample T test
was used to measure the transfection efficiency of M-150, and
the metastasis and aggression results of CAL-27 cell AF, P < 0.05
was viewed of statistical significance.

### Table 2
Sample qPCR Reaction.

| Reagent                  | Volume (µL) |
|--------------------------|-------------|
| ddH2O                    | 9           |
| SYBR Advantage Premix(2X)| 12.5        |
| ROX Dye(50x)             | 0.5         |
| miRNA-specific Primer (10 µL)| 0.5     |
| miR23 Primer             | 0.5         |
| cDNA                     | 2.0         |
| In total                 | 25          |

### Table 3
U6 qPCR Reaction.

| Reagent                  | Volume (µL) |
|--------------------------|-------------|
| ddH20                    | 9           |
| SYBR Advantage Premix(2X)| 12.5        |
| ROX Dye(50x)             | 0.5         |
| U6 Forward Primer (10 µL)| 0.5         |
| U6 Reverse Primer (10 µL)| 0.5         |
| cDNA                     | 2.0         |
| In total                 | 25          |

qPCR reaction conditions: denaturation at 95 ºC for
10 s; circulation for 40s; extension at 95 ºC for 5 s;
60 ºC for 20 s; dissolution curve: 95 ºC for 60 s;
55 ºC for 30 s; 95 ºC for 30 s.
3. Results and discussion

3.1. Comparison of expression amount of M-150

In this research, RT-qPCR was used to detect the difference in the Eom in the MT textures and neighbor normal textures of patients with OSCC, and the results were manifested in Fig. 1. According to Fig. 1A, M-150 was obviously less put in the carcinoma textures of patients with OSCC than in the neighbor normal textures (P < 0.05). The expression difference of M-150 in human oral squamous cell line cal-27 cultivated under normal conditions and 1% hypoxic conditions was also explored. According to Fig. 1B, the Eom in cal-27 cells cultivated under hypoxic condition was obviously less than that in normal cultivated cells (P < 0.05). The difference of M-150 expression in normal neighbor textures of OSCC and cultivated cal-27 cells under hypoxic conditions was compared. As manifested in Fig. 1C, the Eom in cal-27 cells cultivated under hypoxic conditions was obviously less than that in the normal neighbor textures of OSCC (P < 0.05).

3.2. Transfection efficiency results of M-150 at different concentrations

M-150 mimic with final concentration of 0 nmol/L (control), 25 nmol/L, 50 nmol/L and 100 nmol/L were transfected into cal-27 cells, respectively. After 48 h, the expression difference of M-150 in the cells was explored by rt-qpcr (Jingsong et al., 2018), as manifested in Fig. 2A. It can be concluded in Fig. 2A that AF with 25 nmol/L, 50 nmol/L and 100 nmol/L concentrations mimic, the Eom in cells was obviously upper than that of 0 nmol/L (P < 0.01). AF with 50 nmol/L and 100 nmol/L concentration mimic, the Eom in cells was obviously upper than that of 25 nmol/L (P < 0.01). AF with a concentration of 100 nmol/L mimic, the Eom in cells was obviously upper than that of 50 nmol/L (P < 0.01). M-150-I with a final concentration of 0 nmol/L (control), 25 nmol/L, 50 nmol/L and 100 nmol/L were transfected into cal-27 cells, respectively. After 48 h, the expression difference of M-150 in the cells was explored by rt-qpcr, as manifested in Fig. 2B. According to Fig. 2B, AF with inhibitor concentration of 25 nmol/L, 50 nmol/L and 100 nmol/L, the Eom in cells was obviously less than that of 0 nmol/L (P < 0.01), and there was no remarkable difference between 25 nmol/L, 50 nmol/L and 100 nmol/L concentration of M-150 (P > 0.05). However, the Eom in cells transfected with 50 nmol/L was the lowest. Therefore, the M-150 mimic of 100 nmol/L and M-150-I of 50 nmol/L were selected for subsequent tests.

3.3. Results of cell scratch experiment

Cal-27 cells were transfected with 100 nmol/L M-150 mimic and 50 nmol/L inhibitor respectively. With 0 h as the CP, the mobility of each group was measured after 24 h of scratch treatment, and the results were manifested in Fig. 3. According to Fig. 3A, 24 h after scratch treatment, transfection of M-150 mimic group obviously inhibited cell migration. According to the calculation of cell migration, it can be concluded from Fig. 3B that the migration rate of cells transfected with M-150 mimic was 13.98 ± 2.52% and 43.03 ± 8.66% respectively in the CP, and the migration rate of cells...
Fig. 2. The transfection efficiency of M-150 in oral squamous cell CAL-27. Note: Figure A: expression differences of M-150 mimic AF in cells with different concentrations. Figure B: expression difference of M-150 in cells AF with different concentrations of M-150-I. **Indicated that there was an extremely remarkable difference compared with 0 nmol/L, \( P < 0.01 \). After comparison with 25 nmol/L, there was an extremely remarkable difference, \( P < 0.01 \). Bb indicated that there was an extremely remarkable difference after comparison with 50 nmol/L, \( P < 0.01 \).

Fig. 3. CAL-27 cell migration ability before and after M-150 mimic, M-150-I, and control transfection through cell scratch test. Note: figure A manifested the cell migration AF of M-150 mimic and MC, and the cell migration at 0 h and 24 h after the sterile nozzle was applied to the cultivate medium. Figure B manifested the relative migration speed of cells AF with M-150 mimic and MC. Figure C manifested cell migration AF with M-150-I and inhibitor control (IC) at 0 h and 24 h after a sterile spear was applied to the cultivate medium. Figure D manifested the relative migration rate of cells AF with M-150-I and IC.
in the CP was obviously upper than that in the M-150 mimic group (P < 0.05). According to Fig. 3C, 24 h after the treatment of scratches, the transfection of M-150-I group obviously promoted cell migration. The cell migration rate was calculated, and it can be concluded from Fig. 3D that the cell migration rate of the M-150-I group was 64.54 ± 9.67%, compared with 36.13 ± 2.77% of the CP. The cell migration rate of the M-150-I group was obviously upper than that of the CP (P < 0.05).

3.4. Transwell aggression assay results

Fig. 4 is a photograph of the migration of CAL-27 cells AF of M-150 mimic and inhibitor by Transwell chamber assay (Zhao et al., 2016). It can be obtained that the two groups of cells of M-150 mimic and M-150 MC were added to the TUC, and the number of cells penetrating matrigel after 24 h was (36 ± 0.2) and (96 ± 3.6) respectively, the difference was statistically remarkable based on independent sample T test model analysis (P < 0.05). It can be observed from Fig. 4A that after the cells of M-150-I and M-150 IC were added to the TUC, the number of cells penetrating matrigel after 24 h was (197 ± 0.9) and (98 ± 2.8) respectively, the difference was statistically remarkable based on independent sample T test model analysis (P < 0.05). Therefore, it can be concluded that M-150 has a remarkable inhibitory effect on the invasive ability of CAL-27 cells in vitro.

4. Discussion

OSCC is one of the most common oral malignancies, and the incidence of OSCC is on the rise year by year (Chen, 2012). At present, the main treatment for OSCC is surgical excision of MTous textures, combined with comprehensive treatment program of adjuvant chemotherapy, etc. However, OSCC has a high recurrence rate and metastasis rate, so the current clinical treatment effect for OSCC is not ideal, and the survival rate of OSCC patients within 5 years is only 60%. The miRNAs are a kind of RNA molecules that can target and bind target mRNAs so as to play biological functions. Many analyses have manifested that the AE of miRNAs has close tie with the appearance and development of tumors. However, many analyses have proved that the AE of miRNA is related to the appearance and development of OSCC. For example, Weichieh et al. found that ODC of mir-391-5p could inhibit the migration of OSCC cells and the ability of lung metastasis, while Zhou et al. manifested that ODC of mir-21 could affect the propagation and aggression ability of OSCC cells by activating STAT3. Therefore, the differential expression of miRNA is of great significance for the study of the mechanism of OSCC appearance and development. However, some analyses have manifested that AEOM has close tie with the development of MT. Yu et al. found that the Eom-5p decreased in prostate MT textures, while overEom-5p could inhibit the propagation and aggression ability of prostate MT cells. Zhao et al. found that M-150 was obviously upregulated in prostate MT cells, and M-150 could obviously promote the propagation and aggression of prostate MT cells by targeting p53. In this research, the expression levels of M-150 in OSCC, neighbor normal textures and in vitro cal-27 cells were quantitatively explored by RT-qPCR. Under the analysis of independent sample t-test model, it was concluded that M-150 was obviously decreased in OSCC MT textures (P < 0.05), which coincided with the results of Chen et al. ’s study that the Eom in non-small-cell lung MT textures was obviously less than that in neighbor normal textures, suggesting that M-150 may be involved in the appearance and development of OSCC disease. Subsequently, the hypoxic environment of MT textures in vitro was simulated and 100 nmol/L M-150 mimic and 50 nmol/L M-150-I into cal-27 cells, respectively, were trans-
fected to research the influence of M-150 on the M&A of OSCC cells. After processing the experimental data of cell M&A of the independent sample t-test model, it was found that M-150 had a remarkable antagonistic effect on the M&A of cal-27 cells in OSCC. Chen et al. found that M-150 could target ZEB1 and inhibit tumor growth in the transplanted model of esophageal squamous cell carcinoma in mice, which prompts that Mir - 150 could inhibit OSCC cell M&A ability, and play a effect in inhibiting the progress of OSCC disease. With the above results, it can be inferred that M-150 functions as a tumor suppressor gene by inhibiting the metastasis and infiltration of tumor cells. Mutations in the M-150 gene may lead to the development of OSCC. Therefore, M-150 can be used as a target site to inhibit the M&A of tumor cells, thereby antagonizing the spread of tumor cells to achieve the purpose of treating MT. Compared with traditional tumor resection, this targeted treatment can reduce the recurrence rate and greatly increase the survival rate of patients. The action pathway of M-150 is still unknown. However, the study of M-150 in OSCC in this thesis provides a theoretical basis for the future treatment of OSCC with M-150 as a target site.

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