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Original Article

Expression, Proliferation and Apoptosis of miR-92b in Oral Squamous Cell Carcinoma

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Abstract

Background: Expression of miR-92b in oral squamous cell carcinoma (OSCC) rat tissue and its effect on the OSCC CAL-27 cells were investigated.

Methods: The study was performed in Qingdao Stomatological Hospital, Qingdao, China on December 2018. Thirty Wistar rats were used to construct models of oral squamous cell carcinoma. CAL-27 cells trascfected by Lipofectamine 2000 were divided into miR-92b inhibitor, miR-NC and blank groups. RT-qPCR was used for the detection of the expression level of miR-92b, and MTT and flow cytometry were carried out for the detection of the effect of miR-92b on the proliferation and apoptosis of CAL-27 cells, respectively.

Results: The expression level of miR-92b was significantly higher in tumor tissues than that in normal tissues (P<0.001). The miR-92b inhibitor group had significantly lower proliferation ability but higher apoptosis rate of CAL-27 cells than the miR-NC and blank groups. After miR-92b was downregulated by trans-fecting cells, the expression level of miR-92b was significantly lower in the miR-92b inhibitor group than that in the miR-NC and blank groups.

Conclusion: miR-92b inhibitor can inhibit the proliferation of CAL-27 cells and promote apoptosis, which provides certain references for clinical treatment. It is expected to be a potential target for treating OSCC.

Keywords: miR-92, Rat; Oral squamous cell carcinoma; Cell proli-feration; Apoptosis

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the mouth (1). In 2012, it caused 145,000 deaths worldwide, and accounted for 77% of the burden in underdeveloped areas (2). The 5-year survival rate of OSCC patients is ~50%. Lymph node metastasis and distant metastasis are the main causes of the high mortality of OSCC (3, 4). The biological function of cells plays an important role in the pathogenesis of OSCC (5).

miRNAs, a group of non-coding RNAs consisting of 19-25 nucleotides in length, regulate many key biological functions, including cell proliferation,

differentiation and apoptosis (6-8). Therefore, the abnormal expression of miRNA may be related to the occurrence and development of tumors. miRNAs can be functionally classified into proto- or anti-oncogenes, which are abnormally expressed in different cancers. These cancer miRNAs promote uncontrolled cell proliferation and survival, inhibit differentiation or induce invasion by promoting inappropriate cellular processes, thereby participating in the occurrence and development of tumors (9). miR-92b is highly expressed in hepatocellular carcinoma, osteosarcoma and glioma, indicating that it plays a role in promoting cancers (10-12). However, there are only few studies on the expression of miR-92b in OSCC and its effect on the biological function of cells.

Therefore, in this study, the expression of miR-92b in tumor and normal tissues of OSCC rats was investigated. The effect of miR-92b on the proliferation and apoptosis of CAL-27 cells was explored by MTT and flow cytometry, to provide a basis for further studying the regulation mechanism of miR-92b in OSCC, to understand the pathogenesis, as well as provide some references for the treatment and prognosis of OSCC.

Materials and Methods

Rat grouping and modeling

The study was performed in Qingdao Stomatological Hospital, Qingdao, China on December 2018. A total of 30 healthy Wistar rats aged 6-8 weeks, including 15 males and 15 females, were randomly divided into the OSCC group (n=15) with an average body weight of 213.23±10.96 g, and the normal control group (n=15) with an average body weight of 208.23±12.31 g. Rats were purchased from Cavens Lab Animal Co., Ltd. (Changzhou, China), with the animal certificate number of SCXK (Su) 2011-0003. They were separately reared in cages at a room temperature of 23-25 ˚C and a humidity of 55-62%, and were free to drink. Modeling experiments were performed on them after 1 week of adaptive feeding.

The study was approved by the Ethics Committee of Qingdao Stomatological Hospital (Qingdao, China).

All rats were banned from water and fasted for 10 h before modeling. Rats in the control group were provided with food and sufficient water. Rats in the OSCC group were modeled with 4-nitroquinoline 1-oxide (4-NQO) (Shanghai Yubo Biotechnology Co. Ltd., Shanghai, China; item no. ZSG-442683). Sterile distilled water was used to dilute the 4-NQO to a concentration of 5 g/l. The 4-NQO solution (1 ml) was applied to the oral jaw mucosa every 2 days for 28 weeks.

The body weight, mental and activity status of rats in the two groups were observed and recorded during the modeling. After 28 weeks, all rats were tumorigenic. Rats were injected with 10% chloral hydrate at a dose of 3 ml/kg for anesthesia. They were anesthetized with ether and sacrificed by cervical dislocation. Their tumors were removed to obtain the tumor and normal tissues. The expression level of miR- 92b in the tissues was detected.

Cell culture and transfection

OSCC cell line CAL-27 (Shenzhen Biowit Biotech Co., Ltd., Shenzhen, China; item no. C0039) was placed in DMEM culture liquid (Shanghai Xinyu Biotechnology Pharmaceutical Co., Ltd., Xinyu, China; item no. 19-0040-100) containing 10% fetal bovine serum. Culture conditions were 5% CO_2 and 37 ˚C. Passage was carried out when the cells were adherently grown to 80%-90%. Lipofectamine 2000 (Shanghai Hengfei Biotechnology Co., Ltd., Shanghai, China; item no. 11668027) was used for trans-fecting cells in the logarithmic growth phase. The specific steps followed were strictly in accordance with the protocol. CAL-27 cells were divided into miR-92b inhibitor, miR-NC and blank groups.

RT-qPCR detection of miR-92b expression level in tumor and normal tissues of rats

Rat tumor tissues (100 mg) were taken and homogenized. TRIzol lysate (1 ml) (LabGene, Guangzhou, China; item no. LGTQ-001-x) was

added to separate total RNA from tissues. After extraction, 1.5% agarose gel electro-phoresis was used for analyzing RNA integrity, and ultraviolet spectrophotometer (BMD Labservice, Nanjing China; item no. GeneQuant 1300) was used for detecting the purity and concentration of the extracted RNA. A260/A280 value between 1.8 and 2.0 was considered to meet the experimental requirements. Total RNA (2 μg) was taken to synthesize cDNA in accordance with the instructions of Takara SYBR® PrimeScript™ RT- PCR kit (PCR fluorescence quantification kit and RT kit were purchased from Takara Biotechnology Co., Ltd., Dalian, China). Then, 2 μl of products were subjected to PCR cycle according to the manufacturer's instructions. Conditions were pre-denaturation at 94°C for 10 min, denaturation at 94 °C for 30 sec, denaturation at 55 °C for 45 sec, extension at 72 °C for 45 sec, for 40 cycles, and then extension at 72 °C for 5 min after the cycles. U6 was used as a reaction internal reference. The procedure was repeated 3 times. 2-ΔCq was used to calculate the expression level of miR-92b in tumor and normal tissues of rats (13). Primers were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) (Table 1).

Table 1: miR-92b primer and internal reference sequences

Genes	*upstream primers*	*Downstream primers*
miR-92b	5'-CCAAGGTGAACCCAACTCCCCA GC-3'	5'-ATGCCCGAAGTCCTCCCAGACC-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

MTT detection of cell proliferation

After transfection (48 h), CAL-27 cells were prepared into single-arranged cell suspension, and routinely inoculated with a 96-well cell culture plate. When they grew adherently, 15 μl of MTT solution (Beijing Huamaike Biotechnology Co., Ltd., Beijing, China; item no. T107801) at a concentration of 5 g/l were added to each well and continuously cultured at 37°C for 4 h, with the supernatant containing impuri-ties aspirated. Dimethyl sulfoxide (150 μl) (Shanghai Shifeng Biotechnology Co., Ltd., Shanghai China; item no. A5852) formulation was added, placed on a horizontal shaker and shaken for 15 min. A CLARIOstar microplate reader (Hong Kong Boqi Technology Co., Ltd., Hong Kong, China) was used to measure the OD value at a wavelength of 570 nm, at 0, 12, 24, 48 and 72 h, and the growth curve was plotted.

Flow cytometry detection of apoptosis

Annexin V- FITC/PI apoptosis kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). After transfection (48 h), CAL-27 cells were prepared into single -arranged cell suspension, and washed 2-3 times with pre-cooled PBS (Shanghai Lianmai Bioengineering Co., Ltd., Shanghai, China; item no. LM0221A). After centrifugation, at 200 x g for 5 min at 20°C, 500 μl of 1X combination buffer were added to resus-pend the cells. Then, 5 μl of Annexin V-FITC and 5 μl of PI staining solution were added, and incubation followed for 15 min in the dark, after mixed evenly. FACSCanto™ II flow cytometer [Exsson (Beijing) Technologies Co., Ltd., Beijing, China] and Kaluza 2.0 software (Beckman Coulter, Atlanta, USA) was used to detect the apoptosis rate. The experiment was repeated 3 times.

Statistical analysis

SPSS 19.0 softeware (IBM Corp., Armonk, NY, USA) was used for the statistical analysis of the experimental data. Measurement data were expressed as mean ± standard deviation. Independent t-test was used for the comparisons between two groups. One-way analysis of variance, followed by Bonferroni post hoc test, was carried out for the comparisons among multiple groups. GraphPad Prism 6 (GraphPad Software, Inc., La

jolla, CA, uSA) was used to generate the pictures. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Modeling results

There were no significant differences in the sex and age of rats between the two groups before modeling. Thirteen rats were successfully modeled in the OSCC group, while the remaining 2 rats died unexpectedly due to scuffle during the modeling. The modeling success rate was 86.67%. At the beginning of modeling, there was no significant difference in the body weight of rats between the two groups. In the late stage of modeling, rats in the OSCC group had low spirits, accompanied by hair loss, slow movement, sudden slow weight gain, and even weight loss. Rats in the control group grew well. During the modeling, there was no abnormal change in the oral cavity of rats in the control group, with pink, soft and elastic tongue. At the 8th to 15th weeks, some rats in the OSCC group showed white spots on their tongue, which gradually increased, and the back of the tongue became rough. At the 17th-18th weeks, most of rats in the OSCC group developed mucosal edema, white patches on the tongue accompanied by blood and loose teeth. At the 20th week, all rats in the OSCC group had different degrees of ulceration and new organisms began to appear. At the 24th week, all rats were sacrificed, and the ulceration area and new organisms increased in size. The ulceration was mainly concentrated in the base of the tongue of rats, but also appeared in other sites in the tongue.

Expression level of miR-92b in tumor and normal tissues

The results of RT-qPCR showed that the expression level of miR-92b was significantly higher in tumor tissues than that in normal tissues, with a statistically significant difference ($P<0.001$) (Table 2 and Fig. 1).

Table 2: Expression level of miR-92b in tumor and normal tissues

Group	*n*	*miR-92b*
Tumor tissues	13	3.46±1.21
t		7.685
P		<0.001

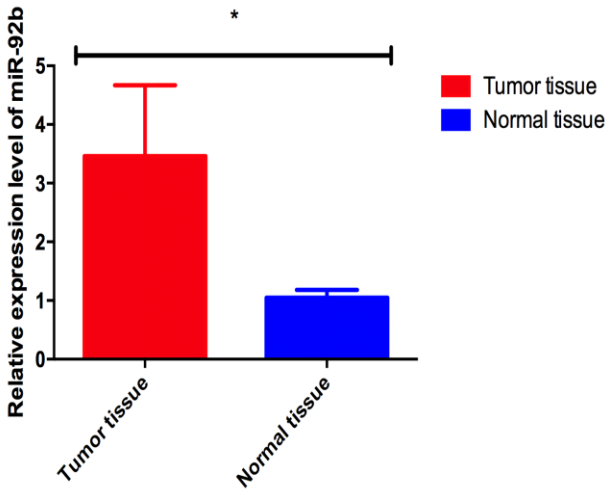

Fig. 1: Expression level of miR-92b in tumor and normal tissues. The results of RT-qPCR showed that the expression level of miR-92b was significantly higher in tumor tissues than that in normal tissues (*$P<0.001$)

Expression level of miR-92b in miR-92b inhibitor, miR-NC and blank groups

CAL-27 cells were transfected with miR-92b inhibitor. The results of RT-qPCR showed that there was a statistically significant difference in the expression level of miR-92b among miR-92b inhibitor, miR-NC and blank groups ($P<0.05$). There was no significant difference in the expression level of miR-92b between miR-NC and blank groups.

The expression level of miR-92b was significantly lower in the miR-92b inhibitor group than that in the miR-NC and blank groups ($P<0.05$) (Table 3 and Fig. 2).

Table 3: Expression level of miR-92b in each group of cells after transfection

Group	*miR-92b*
Blank group	3.46±1.01
miR-NC group	3.32±1.15
miR-92b inhibitor group	1.01±0.37a,b
F	6.871
P	0.028

P<0.05, compared to the blank group; [b]*P*<0.05, compared to the miR-NC group

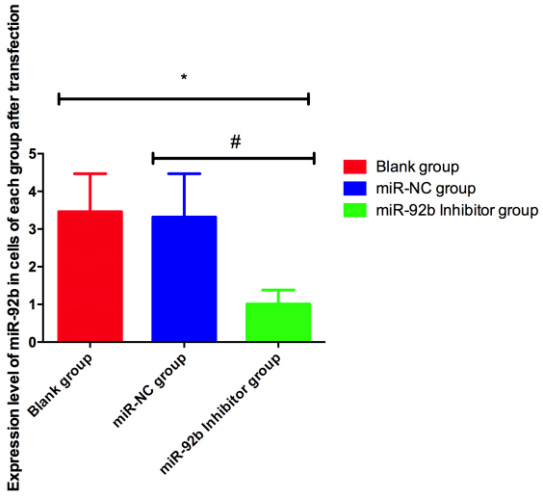

Fig. 2: Expression level of miR-92b in blank, miR-NC, and miR-92b inhibitor groups. The expression level of miR-92b was significantly lower in the miR-92b inhibitor group than that in the miR-NC group (#*P*<0.05) and blank group (**P*<0.05)

Effect of miR-92b on proliferation of CAL-27 cells

The results of MTT showed that the proliferation ability of CAL-27 cells was significantly lower in the miR-92b inhibitor group than that in the miR-NC and blank groups (*P*<0.05) (Fig. 3).

Effect of miR-92b on apoptosis of CAL-27 cells

The results of flow cytometry showed that there was a statistically signifi-cant difference in the apoptosis rate of CAL-27 cells among the miR-92b inhibitor, miR-NC and blank groups (*P*=0.026).

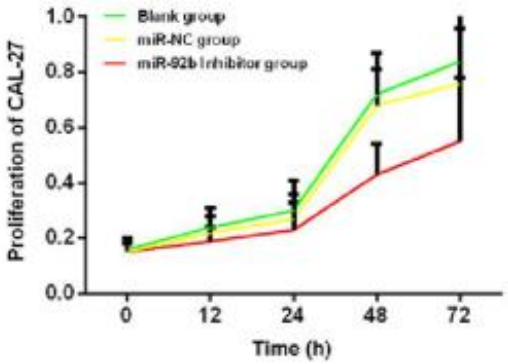

Fig. 3: Effect of miR-92b on proliferation of CAL-27 cells
The proliferation ability of CAL-27 cells was significantly lower in the miR-92b inhibitor group than that in the miR-NC group and the blank group

There was no significant difference in the apoptosis rate of CAL-27 cells between miR-NC and blank groups. The apoptosis rate of CAL-27 cells was significantly higher in the miR-92b inhibitor group than that in the miR-NC and bl ank groups, with a statistically significant difference (*P*<0.001) (Table 4 and Fig. 4).

Table 4: Effect of miR-92b on apoptosis of CAL-27 cells

Group	*Apoptosis rate (%)*
Blank group	6.68±1.49
miR-NC group	7.17±1.47
miR-92b inhibitor group	13.32±3.61a,b
F	7.077
P	0.026

[a]*P*<0.001, compared to the blank group; [b]*P*<0.001, compared to the miR-NC group

Discussion

Oral cancer is the sixth most common cancer in the world (14), with >500,000 new patients diagnosed each year (15). >90% of oral tumors are

OSCC (16). Despite the advances in treatment, the incidence and mortality of OSCC has not been significantly improved over the past 20 years (17).

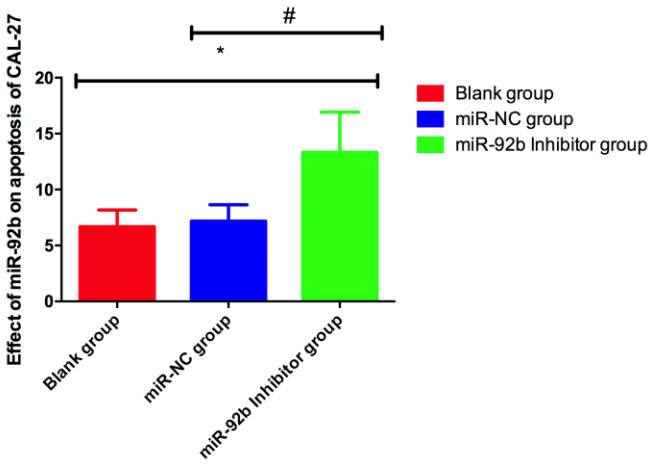

Fig. 4: Effect of miR-92b on apoptosis of CAL-27 cells. The apoptosis rate of CAL-27 cells was significantly higher in the miR-92b inhibitor group than that in the miR-NC group (#P<0.05) and blank group (*P<0.05)

The ingestion of alcohol, tobacco and betel nut and the infection of human papillomavirus (18-20) are considered risk factors for the abnormal expression of genes, and the occurrence and development of OSCC (21, 22). In recent years, biomedical research has increasingly focused on the relatively small amount of miRNAs (23).

In this study, an OSCC rat model was established through the 4-NQO method. The expression level of miR-92b was significantly higher in tumor tissues of rats than that in normal tissues, with a statistically significant difference. The expression level of miR-92b is significantly higher in human OSCC tumor tissues than that in adjacent normal tissues, which is basically consistent with our findings (24). In this study, the expression of miR-92b in CAL-27 cells was downregulated by transfecting miR-92b inhibitor. The expression level of miR-92b and the prolif-eration ability of CAL-27 cells were significantly lower in the miR-92b inhibitor group than those in the miR-NC and blank groups, but the apoptosis rate of CAL-27 cells was significantly higher in the miR-92b inhibitor group than that in the miR-NC and blank groups. miR-92b inhibitor can inhibit the proliferation, migration and invasion of glioma cells, and promote their apoptosis (25). miR-92b significantly inhibits the proliferation, migration and invasion of NSCLC cells (26), consistent with our research results.

In the present study, downregulation of miR-92b significantly inhibited the proliferation ability of CAL-27 cells, and significantly increased their apoptosis ability. However, due to limited fund support in this study, only one cell line was studied, with a lack of research on miR-92b-related signaling pathways. This investigation has clearly defined the direction of future research study. The specific pathogenesis of miR-92b on OSCC remains to be further studied.

Conclusion

MiR-92b is highly expressed in OSCC. After miR-92b is downregulated by transfecting cells, the expres-sion level of miR-92b is significantly lower in the miR-92b inhibitor group than that in the miR-NC and blank groups. miR-92b inhibitor can inhibit the proliferation of CAL-27 cells and promote their apoptosis, which provides certain references for clinical treatment and is expected to be a potential target for treating OSCC.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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No funding was received in this study.

Conflict of interests

The authors declare that there is no conflict of interest.

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