Revista Argentina de Clínica Psicológica
2020, Vol. XXIX, N°3, 1201-1206
DOI: 10.24205/03276716.2020.948

Effects of Mir-96 On Proliferation, Apoptosis and Epithelial-Mesenchymal Transition of Oral Tongue Squamous Carcinoma Cells

Xiuqin Zhang[a]*, Zhenhua Liu[b]

Abstract
Objective: This study was designed to figure out the effects of miR-96 on proliferation, apoptosis and epithelial-mesenchymal transition (EMT) of oral tongue squamous cell carcinoma (TSCC) cells.
Methods: The expression levels of miR-96 and EMT related proteins were detected through qRT-PCR and WB, and apoptosis was tested through flow cytometry. miR-96 inhibitor vector was established to transfect TSCC cell lines, and TSCC cell proliferation, migration, invasion, apoptosis and EMT were observed. Results: miR-96 was dramatically down-regulated in TSCC cells, and down-regulating it inhibited proliferation, EMT, migration, invasion and apoptosis of cells.
Conclusion: miR-96 is down-regulated in TSCC cells and down-regulating it can inhibit their biological ability, so it may become a new target for TSCC treatment.
Keywords: miR-96, tongue squamous cell carcinoma, proliferation, apoptosis, epithelial-mesenchymal transition

Introduction

TSCC is the most familiar malignancy in oral cavity (Yu,2017), accounting for 25% to 40% of oral cancer (OC), and it is the main reason for OC death (Kimple et al.,2014). The global morbidity is increasing at a rate of 0.4% to 3.3% per year (J H Ng et al.,2017). Surgical resection and radiotherapy and chemotherapy are currently the primary clinical treatment methods (Yu et al.,2017), but the risk of TSCC developing into secondary or recurrent tumors is still very high (Schwam et al.,2016), with an overall survival rate lower than 65% (Tu et al.,2015). Thus, to study the pathogenesis of TSCC and seek more effective treatment methods is the key to improve the prognosis of patients.

microRNA (miR) is a research hotspot recently. Research shows that (Rupaimoole et al.,2017) most gene transcription in human body can be regulated by miR, which plays a vital role in cell growth, proliferation and apoptosis. miR is a non-coding short-chain RNA with a length of about 22nt. Its main function is to regulate protein expression by completely or incompletely binding and complementing the untranslated region at the 3' end of mRNA of its downstream target gene, thus affecting mRNA stability or inhibiting its translation (Lin et al.,2015; Agarwal et al.,2015; Zhang et al.,2015). Studies have shown that miRNA is related to epithelial-mesenchymal transition (EMT) and cell cycle arrest of OC cells (Fang et al.,2019). Among them, miR-96 is subordinate to miR family that is tied to the development of various malignant tumors, including hepatic cellular cancer (HCC) (Iwai et al.,2019), prostate cancer (Long et al.,2019), bladder cancer (Xu et al.,2018), etc. miR-96-5p is dramatically up-regulated in HCC, and it can induce EMT and promote cancer progression by inhibiting apoptosis of tumor cells (Iwai et al.,2017;Tang, 2019). Guo et al. Guo et al.,2015) found that miR-96 promoted TSCC cell biological ability by inhibiting MTSS1 expression. This suggested that it was also involved in TSCC progression and might be a potential therapeutic target for TSCC. However, at present, the mechanism in TSCC and its effects on the biological ability and EMT of cells are still indistinct.

Therefore, through down-regulating miR-96's expression in TSCC cells, this study studies its effect on the biological ability and EMT, and provides valuable reference for TSCC treatment.

[a,b] Department of stomatology, Zhongguancun Hospital, 100080, Beijing,China
Correspondence Author: Xiuqin Zhang
zhangjiakouzxq@163.com

1 Materials and methods

1.1 Experimental materials

1.1.1 Cell sources

Normal oral mucosa epithelial cells NHOK, TSCC cell lines CAL27, Tca8113 were all bought from ATCC, U.S.A.

1.1.2 Main experimental materials

TRIzol kit, transfection reagent Lipofectamine TM2000 (Invitrogen, U.S.A), real-time fluorescence quantitative PCR (BioRad, Berkeley, California, U.S.A), apoptosis kit (Hangzhou Biomiga Medical Technology Co., Ltd.), 10% fetal bovine serum (Shanghai Lianshuo Baowei Biotechnology Co., Ltd.), DMEM medium (Gibco, U.S.A), CCK8 kit (Beijing G-clone Biotechnology Co., Ltd.), ultraviolet spectrophotometer (Shanghai Aucy Scientific Instrument Co., Ltd.), FC500MCL flow cytometry (BD, U.S.A), Trypsin (Hyclone), secondary antibody (goat anti-rabbit, Shanghai Yuanmu Biotechnology Co., Ltd.), Transwell chamber (Shanghai Shengbo Biomedical Technology Co., Ltd.), microplate reader (Beijing Image Trading Co., Ltd.), primer synthesis of miR-NC, miR-96 and U6 (designed and synthesized by General Air, Shanghai, China).

1.2 Detection methods

1.2.1 Cell culture and transfection

NHOK, CAL27, Tca8113 cells were all placed in a DMEM medium with 10% FBS and 1% penicillin-streptomycin, and then cultured at 37℃, 5% CO_2. When the adherent growth and fusion reached 80%-90%, cells were digested by trypsin and then continuously cultured for passage. Those to be transfected were divided into miR-NC group and miR-96-inhibitor group, and corresponding operations were in line with the instructions of Lipofectamine 2000 kit.

1.2.2 miR-96 relative expression is detected by qRT-PCR

The total RNA was extracted with Trizol reagent, and the purity and concentration were detected by ultraviolet spectrophotometer. Next, 5 μg was taken respectively to reverse transcribe cDNA in the light of the instructions of the kit. The reaction parameters were as follows: 37℃, 15 min; 42℃, 35 min; 70℃, 5 min. Afterwards, PCR amplification was conducted and reaction conditions were in conformity with the kit instructions. The test was conducted 3 times. U6 was employed as internal reference, and the data were assessed through $2^{-\triangle\triangle Ct}$.

1.2.3 Cell proliferation is detected by CCK8 method

Transfected cells were prepared into cell suspension and laid on 96-well plates with 100 μL/well, and each well was supplied with 3 repeated holes. CCK8 reagent was supplemented at 24, 48, 72 and 96 h, respectively, and the cells were cultured 2 h at 37℃, 5%CO_2. The absorbance of each well was detected at 490 nm wavelength and the growth curve was drawn.

1.2.4 Cell migration and invasion are detected by Transwell method

Cell concentration was adjusted to 2×10^5 cells/ml. Altogether 500 μl 10% FBS medium was added to the lower culture hole, and 100 μl cell were supplemented to the upper chamber. Next, the upper chamber was put into the lower culture hole and cultured for 24 h. The cells in the upper chamber were wiped off, and those migrated to the lower chamber were stained with 4% paraformaldehyde and 0.1% crystal violet. After they were dried, a membrane was made and sealed, and the cells penetrating it were observed under an microscope and their migration was counted. Invasion was counted from 5 fields of view randomly, and we observed the number of stained cells microscopically and counted it.

1.2.5 E-cadherin, N-cadherin and Vimentin expression are detected through WB method

The total protein was extracted by RIPA lysis method and separated by 10%SDS-PAGE electrophoresis, and then it was transfered to PVDF membrane. Subsequently, it was closed 2 h with 5% defatted milk powder. After universal secondary antibody was added, it reacted 2 h at indoor temperature and rinsed 3 times with PBS. It developed in a dark room using ECL reagent.

1.2.6 Apoptosis is detected through flow cytometry

After digestion, cells were cleaned twice with PBS. Next, they were prepared into 1×10^6 cells/mL suspension, and AnnexinV-FITC and PI were supplemented in turn. After that, they were incubated 5 min in the dark. We tested them through FC500MCL flow cytometry.

1.3 Statistical analysis

The collected data were analyzed via SPSS20.0 (IBM, Armonk, NY, USA), and the pictures were drawn via GraphPad 7. The inter-group comparison was analyzed through independent-samples T test, multi-group comparison was assessed through one-way analysis of variance, and pairwise comparison

was analyzed through LSD-t test. There were statistical differences when P < 0.05.

2 Results

2.1 miR-96 relative expression in TSCC cells

By detecting the miR-96 expression in TSCC cell lines, we found that compared with NHOK cells, the relative expression in TSCC cells was dramatically up-regulated. (Figure 1)

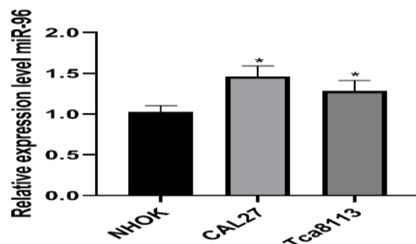

Figure 1. **miR-96 relative expression in TSCC cells**

Compared with NHOK cells, the miR-96 expression in CAL27 and Tca8113 cells is dramatically up-regulated. * means P < 0.05 compared with NHOK cells.

2.2 Effect of down-regulating miR-96 expression on proliferation of TSCC cells

In order to investigate miR-96's effect on TSCC cell proliferation, we found that transfecting miR-NC, miR-96-inhibitor to CAL27, Tca8113 cells respectively could remarkably reduce the miR-96 relative expression in TSCC cells compared with transfecting miR-NC. By plotting the growth curve, we found that the growth of TSCC cells between miR-NC and miR-96-inhibitor had no statistical difference at 24 h (P > 0.05), while the growth of TSCC cells in transfected miR-96-inhibitor group was dramatically lower than that of miR-NC group at 48-96 h (P < 0.05). (Figure 2)

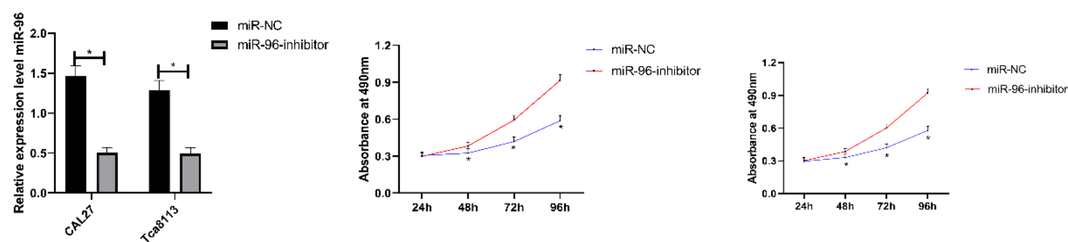

Figure 2. **TSCC cell growth at different periods after being transfected with miR-NC and miR-96-inhibitor**

A. By transfecting miR-NC and miR-96-inhibitor into CAL27 and Tca8113 cells, we discovered that compared with the former, transfecting miR-96-inhibitor could dramatically lower the miR-96 relative expression in CAL27 and Tca8113 cells. * indicates P < 0.05.

B/C. At 24 h, there was no statistical difference in the growth of CAL27 and Tca8113 cells between miR-NC and miR-96-inhibitor, while at 48-96 h, the growth in miR-96-inhibitor group was dramatically lower than miR-NC group. * indicates P<0.05 compared with miR-NC group.

2.3 Effect of miR-96 expression on EMT of TSCC cells

Transfecting miR-96-inhibitor could reduce E-cadherin expression and increase N-cadherin and Vimentin expression in CAL27 and Tca8113 cells. (Figure 3)

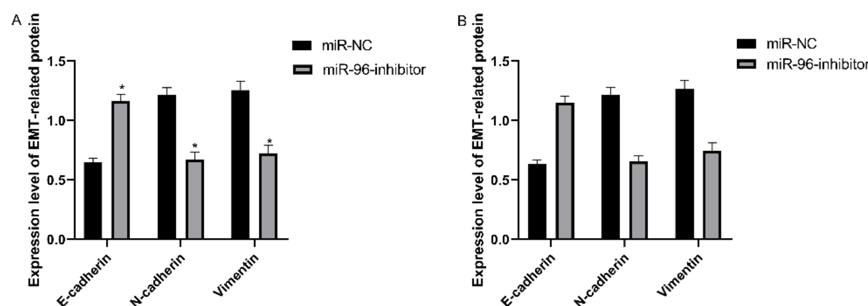

Figure 3. **Effect of miR-96 expression on EMT of TSCC cells**

A/B. Transfecting miR-96-inhibitor can reduce E-cadherin expression, but increase N-cadherin and Vimentin expression in CAL27 and Tca8113 cells. * indicates P < 0.05.

2.4 Effect of down-regulating miR-96 expression on migration and invasion of TSCC cells

The numbers of CAL27 and Tca8113 cells transfected with miR-96-inhibitor were remarkably lower than those with miR-NC. (Figure 4)

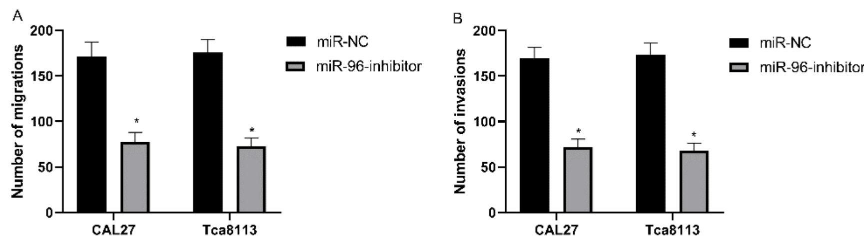

Figure 4. **Effect of down-regulating miR-96 expression on TSCC cell migration and invasion**

A/B. The migration and invasion numbers of CAL27 and Tca8113 cells transfected with miR-96-inhibitor are dramatically lower than those with miR-NC. * indicates P < 0.05.

2.5 Effect of down-regulating miR-96 expression on apoptosis of TSCC cells

CAL27 and Tca8113 cells transfected with miR-96-inhibitor had markedly higher apoptosis rate than those with miR-NC. (Figure 5)

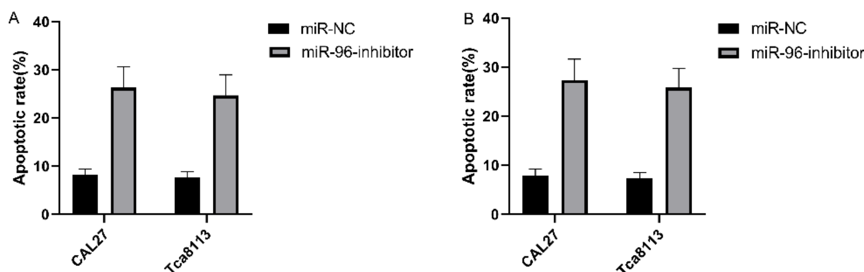

Figure 5. **miR-96's effect on apoptosis of TSCC cells**

CAL27 and Tca8113 cells transfected with miR-96-inhibitor have markedly higher apoptosis rate than those with miR-NC. * indicates P < 0.05.

Discussion

TSCC is the most familiar OC. The incidence is gradually younger and rising, the risk of local recurrence is very high, and the survival rate is lower than that of the general population (Paderno et al.,2018). Early TSCC patients have a good prognosis and a high cure rate, while late patients have a poor overall survival period (Mroueh et al.,2019). Nearly 16,400 new tongue cancer cases were reported in the U.S. during 2017, of which 2,400 died (Siegel et al.,2017). Therefore, it is extremely important to explore TSCC pathogenesis and find effective treatment methods to improve the prognosis of patients.

Although TSCC diagnosis and treatment have made great progress, the pathogenesis is still vague (Christopherson et al.,2017 ;Keshavarzi et al.,2017). Studies have shown that miR is abnormally expressed in TSCC and plays a vital role in its occurrence, development, metastasis, chemical radiation resistance and relapse. Its stable presence in plasma, cells and saliva is recommended as a biomarker for TSCC diagnosis and prognosis (Karatas et al.,2017). Research shows that miR-96 takes part in cell proliferation and migration, so we study its mechanism in cells. Firstly, we detected the miR-96 expression in TSCC cells. Compared with normal oral mucosa cells, the expression in TSCC cells was up-regulated, and miR-96 was differentially expressed in TSCC, which was expected to be a diagnostic marker. miR-96 is subordinate to miR-183 family and is identified as a useful prognostic marker and therapeutic target for various cancers (Zhang et al.,2013). Some studies have shown that miR-96-5p expression in oral squamous cell carcinoma increases dramatically, and it takes part in the proliferation, invasion and EMT (Wang et al.,2013) of tumor cells, similar to our research. In order to study miR-96's effect on the biological ability of TSCC cells, we constructed miR-96 inhibitors and transfected them into TSCC cell lines CAL27 and Tca8113. We discovered that the growth ability of CAL27 and Tca8113 cells was inhibited, indicating that down-regulating miR-96

could inhibit TSCC cell growth. Studies have shown that (Zhou et al.,2015) miR-96 is remarkably correlated with the expression of transcription factors and death related proteins, which may play a central role in TSCC regulation. Hence, we suspect that miR-96, as a carcinogenic gene of TSCC, promotes TSCC cell proliferation by regulating growth-related factors and inhibits cell death-related proteins to reduce apoptosis rate.

EMT is a cellular program, which is known to be crucial to embryo formation, wound healing and malignant tumor progression. If there are malignancies, EMT can increase proliferation, metastasis and invasion of cancer cells (Dongre et al.,2019). And it also increases the resistance of chemotherapy and immunotherapy. So, studying the molecular and cellular mechanisms may be a new idea for cancer treatment (Lu et al.,2019). During EMT, cell surface markers E-cadherin are down-regulated, and intercellular matrix markers N-cadherin and Vimentin are up-regulated, leading to cancer progression (Serrano-Gomez et al.,2019). Thus, we discussed miR-96's role in EMT of TSCC cells by detecting the expression levels of EMT related proteins. Our research results manifested that the E-cadherin expression was dramatically up-regulated and the N-cadherin and Vimentin expression levels were remarkably down-regulated in TSCC cells after being transfected with miR-96 inhibitor, which indicated that down-regulating miR-96 could inhibit EMT process in TSCC cells and provided a new idea for TSCC treatment. Next, we examined the migration and invasion of TSCC cells after being transfected with miR-96 inhibitor. The results manifested that the migration and invasion decreased dramatically, indicating that down-regulating miR-96 could inhibit their migration and invasion and inhibit cancer progression. Wei et al. (Wei et al.,2019) reported that miR-96-5p increased obviously in non-small cell lung cancer, and down-regulating it could inhibit EMT process. Some studies have shown that miR-96 takes part in the EMT process of head and neck cancer and is relevant to cancer development (Domingues et al.,2018). This is similar to our research results: Down-regulating miR-96 can inhibit EMT process, thus inhibiting the migration of cancer cells and slowing down cancer progression. In the end, we found that the apoptosis rate increased dramatically, which indicated that down-regulating miR-96 expression could promote apoptosis. The above results show that down-regulating miR-96 can inhibit the biological ability of TSCC cells, further confirming its possibility as a therapeutic target.

Although this study shows that miR-96 plays a crucial part in regulating the biological ability of TSCC cells and affects its EMT process, there are still limitations. For example, the downstream signaling pathway of miR-96 is still vague, and we do not know how it regulates their biological ability. Studies show that it is found in breast cancer and non-small cell lung cancer to regulate the proliferation and apoptosis of tumor cells by regulating FOXO1 and FOXO3 respectively. Therefore, we will add specific downstream signaling pathways of miR-96 regulating cell proliferation and apoptosis to further confirm our research results.

To sum up, this study proves that miR-96 is down-regulated in TSCC cells and down-regulating it can inhibit the biological ability of cells, so it is expected to become a new target for TSCC treatment.

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