Effect of microRNA-27b on cisplatin chemotherapy sensitivity of oral squamous cell carcinoma via FZD7 signaling pathway

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Abstract. Effect of microRNA-27b (miR-27b) on cisplatin chemotherapy sensitivity of oral squamous cell carcinoma (OSCC) was investigated to provide a reference for clinical prevention of OSCC cell resistance. The clinical tissues of 34 patients with OSCC-resistant cancer and 28 patients with cisplatin-sensitive OSCC in Nanjing General Hospital were collected. The expression levels of miR-27b and Frizzled-7 (FZD7) in cancer tissues were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After a certain gradient of cisplatin was used to induce stable acquired resistance of OSCC cell line Tca8113/CDDP, a dose of miR-27b was added to construct a cell line overexpressing miR-27b in the drug-resistant cell line. The effect of cisplatin on the proliferation of drug-resistant OSCC cells was detected by colony formation assay. In addition, the scratch test and Transwell formation assay was performed to examine the effect of cisplatin drug stimulation on proliferation and migration of Tca8113/CDDP. Flow cytometry and Hoechst 33258 staining were used to detect the effect of miR-27b on apoptosis of OSCC-resistant cells after cisplatin chemotherapy. The expression level of miR-27b in cancer tissues of patients with drug-resistant OSCC was significantly lower than that of patients with OSCC cisplatin sensitivity (P<0.05). After high expression of miR-27b, the number of clones of drug-resistant OSCC cells after adding cisplatin drugs can be significantly inhibited. The proliferation and migration ability of drug-resistant OSCC was significantly decreased after the addition of miR-27b overexpression (P<0.05). miR-27b mimic enhanced the pro-apoptotic ability of cisplatin drugs (P<0.05). The expression of FZD7 in cisplation-resistant patients was significantly higher (P<0.05). miR-27b significantly inhibited the expression of FZD7 and β-catenin proteins. miR-27b can inhibit the resistance of OSCC to cisplatin drugs, increase apoptosis of cancer cells, and inhibit the proliferation of cancer cells. The mechanism may be related to the inhibition of FZD7/β-catenin signaling pathway activation in drug-resistant cell lines by miR-27b.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck cancers, accounting for 90% of the total number of malignant oral tumors (1). The 5-year survival rate for OSCC patients is ~60-80%, and if not diagnosed, the survival rate will be reduced to 50% (2). Cisplatin compounds are widely used in the clinical treatment of OSCC and have a variety of advantages, including reducing distant metastasis rate, improving patient survival and maintaining organ function. The main mechanism is exerting its cytotoxic effect through the formation of intrachain DNA cross-linking in tumor cells (3,4). Most OSCC patients are less sensitive to cisplatin compounds, which is the main reason for the low survival rate of OSCC patients (5). Therefore, to clarify the molecular mechanism of chemotherapy drug resistance in OSCC patients is of great significance for improving the survival and prognosis of patients with OSCC.

miRNAs (microRNAs) are a group of single stranded non-coding RNAs (6) that are 20 to 24 nt in length and exist in eukaryotes. By binding to specific genes, miRNAs can regulate the expression of multiple genes, and thus play an important role in physiological activities such as cell proliferation, differentiation and apoptosis (7,8). Studies have shown that targeted inhibition of miR-27b can increase the sensitivity to doxorubicin of human thyroid cancer, and its mechanism may be related to the activation of PPAR (9). In addition, studies have shown that miR-27b expression levels are significantly reduced in tamoxifen-resistant breast cancer cells, possibly related to methylation of the miR-27b promoter region, whereas overexpression of miR-27b can enhance the sensitivity of breast cancer cells to tamoxifen chemotherapeutic drugs through targeted inhibition of HMGB3 expression (10). However, the effect of miR-27b on OSCC cisplatin-resistant cells has not been reported yet. In this study, we detected the expression level of miR-27b in cancer tissues

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of cisplatin-resistant patients and cisplatin-sensitive patients, and further induced Tca8113/CDDP cells to construct the cisplatin-resistant cell line by using gradient concentration of cisplatin to detect the effect of miR-27b high expression on drug-resistant cell proliferation, apoptosis and invasion and migration, and explored the molecular mechanism of miR-27b affecting OSCC cell resistance.

Materials and methods

Tissue specimens. During the period from January 2016 to May 2018, 62 patients with OSCC who underwent surgical resection in Nanjing General Hospital (Nanjing, China) were included, there were 34 patients with cisplatin resistance and 28 patients with cisplatin sensitivity. After all the specimens were removed from the blood by physiological saline, they were cut into EP tubes and stored in a -80˚C refrigerator.

All the above procedures were approved by the Medical Ethics Committee of Nanjing General Hospital. Signed informed consents were obtained from the patients or guardians.

Materials and cells. Tca8113/CDDP cells (cat. no. TC-hxbz-479) were purchased from Shanghai Kanglang Biotechnology Co., Ltd. (Shanghai, China) and gradually induced to become resistant strains by gradient concentration of cisplatin from low concentration to high concentration for 2 months. Fetal bovine serum (FBS) was purchased from Biochrom (Berlin, Germany). RPMI-1640 medium was purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-FZD3 monoclonal antibody (1:1,000; cat. no. ab217032), anti-β-catenin monoclonal antibody (1:5,000; cat. no. ab32572), anti-GAPDH monoclonal antibody (1:5,000; cat. no. ab181602) and sheep anti-rabbit secondary polyclonal antibody (1:5,000; cat. no. ab10183) were purchased from Abcam (Cambridge, UK).

Culture of Tca8113/CDDP resistant cells. Tca8113/CDDP resistant cells were routinely cultured with 10% FBS and a certain amount of penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.; 15140-122) was added. The culture conditions were 37˚C and 5% incubator constant temperature culture. In order to maintain the drug resistance of Tca8113/CDDP-resistant cells, stimulation with cisplatin at a concentration of 5 µmol/l per month was necessary. All the cells used in this experimental study were in log phase growth. In the subsequent experiments, OSCC cells were divided into two groups, the resistant group: DRG; the resistant group + miR-27b mimic group: DRG+miR-27b mimic.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection of related gene expression. i) TRizol method was performed to extract total RNA in cells or tissues (Invitrogen; Thermo Fisher Scientific, Inc.), and then UV spectrophotometer (UV-1800; Shimadzu Corporation, Kyoto, Japan) was used to detect the concentration and purity of the extracted RNA. A260/A280 should reach 1.8-2.0 for further experiment; ii) the mRNA was synthesized into cDNA by reverse transcription and stored in a refrigerator at -80˚C; iii) RT-PCR system: 10X buffer 2.5 µl; cDNA 2 µl; forward primer (20 µmol/l) 0.25 µl; reverse primer (20 µmol/l) 0.25 µl; dNTPs (10 mmol/l) 0.5 µl; Taq enzyme (2x10^6 U/l) 0.5 µl; ddH2O 19 µl. The amplification systems for RT-PCR were identical. The primer sequences of each index in RT-qPCR are shown in Table I. The suppliers of RT and PCR apparatus were Applied Biosystems; Thermo Fisher Scientific, Inc., and Sigma-Aldrich Corporation (St. Louis, Missouri, MO, USA).

Western blot analysis. i) The culture medium was discarded, and washed with PBS 3 times; ii) 1,000 µl RIPA lysis (Beyotime Biotechnology, Shanghai, China) was added per dish and shaken well for 20 min; iii) the brush was used to fully scrape the cells at the bottom of the dish and the cells were collected in EP tubes; iv) the collected cells were lysed by an ultrasonic pyrolyzer for ~15 sec; v) after standing for 15 min, centrifuged at 10,000 x g and 4˚C for 30 min; vi) the supernatant was dispensed into the EP tube, the BCA method (BCA protein concentration kit; Biosharp Life Sciences, Hefei, China) and ultraviolet spectrometry was performed to measure the protein concentration, and the protein of all samples was made up to the same concentration and vii) the samples were put in a -80˚C refrigerator after dispensing. After extracting the total protein of OSCC resistant cells, SDS-PAGE electrophoresis could be performed with 10% separation gel and 10 µl of protein was added to each lane. After electrophoresis, the proteins in the gel were transferred into a PVDF membrane and blocked with non-fat milk (1×TBST), and the primary antibody was incubated at 4˚C overnight, and then incubated in sheep anti-rabbit secondary antibody in the dark for 1 h; prepared ECL mixture (ECL kit; Beyotime Biotechnology) was added; protein bands were scanned and quantified using an Odyssey membrane sweeper (Odyssey, LI-COR, Lincoln, NE, USA), and GAPDH was used to correct the level of protein to be tested. Image Lab 4.0 software (Bio-Rad, Hercules, CA, USA) was used to quantify.

Cloning formation experiments. Each group of cells was cultured to logarithmic growth phase, digested into single cell suspensions with 0.25% trypsin, and the single cell ratio was guaranteed to be >95%. The cell suspension was then inoculated into a 6-well plate with ~500 cells/well. Subsequently, 2 ml of RPMI-1640 medium was added to each well and the solution was changed every 48 h. After 10 days, the cells were fixed with formaldehyde and stained with crystal violet, and the number of cell clones in each well plate was counted.

| Target genes | Primer sequences |
|--------------|-----------------|
| GAPDH        | F: 5'-GACATGCCGCTTGGAGAAAC-3' |
|              | R: 5'-AGCCAGGATGCCCTTTAGT-3' |
| miR-27b      | F: 5'-ACGATGCTAGCTCTGTTTCTT-3' |
|              | R: 5'-CTGTAGCTGTAGCTAGGAAAT-3' |
| FZD7         | F: 5'-CGCTAGCTGATCGATTAACC-3' |
|              | R: 5'-ACGTAGCTAGCGATTTCCC-3' |

F, forward; R, reverse; FZD7, Frizzled-7.
Flow cytometry to detect apoptosis. OSCC cell suspension in logarithmic growth phase were prepared by digesting with 0.25% trypsin-EDTA and inoculated on 6-well plate medium. The apoptosis rate was measured according to the procedure of the apoptosis detection kit Annexin V-FITC PI (Biotime, Shanghai, China).

Hoechst 33258 staining. After adding miR-27b for 24 h, resistant OSCC cells were stained with Hoechst 33258 staining kit (Invitrogen; Thermo Fisher Scientific, Inc.). The specific protocol was as described in the manufacturer's instructions. After the staining, photographs were taken using a fluorescence microscope (XSP-63XD, Shanghai Optical Instrument Factory Co., Ltd., Shanghai, China), and three fields of view were randomly selected for each slide. Finally, Hoechst positive cells were counted and quantified.

Scratch test. Cells in logarithmic growth phase were seeded in 96-well plates to ensure that the number of cells per plate was ~5x10^4 cells. At 24 h, the well was scratched in the middle with a pipette tip. The cells dropped by the scratches were washed with PBS, and replaced with serum-free medium. The cell migration was photographed under a microscope at 24 h.

Invasion experiment. Matrigel was diluted at a ratio of 1:8, coated with Transwell chamber (8 µm), and incubated in a 37°C incubator for 2 h to form a gel. Subsequently, the two groups of cells were separately diluted into a single cell suspension with serum-free medium, and then inoculated into the Transwell upper chamber (density of 5x10^4 cells/100 µl), and the lower chamber was added with 10% FBS medium, and cultured for 24 h. The transmembrane cells were fixed with 5% glutaraldehyde, stained with 0.1% crystal violet and photographed.

Statistical analysis. All data were analyzed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation, and data comparison between the two groups was performed by t-test. P<0.05 represents a statistically significant difference.

Results

Expression of miR-27b and Frizzled-7 (FZD7) mRNA in OSCC cisplatin-sensitive and drug-resistant patients. As shown in Fig. 1, by detecting the expression of miR-27b and FZD7 in oral cancer tissues of OSCC-resistant patients and OSCC-cisplatin-sensitive patients, we found that the expression
level of miR-27b in cisplatin-sensitive OSCC patients was significantly higher (P<0.05), and the expression of FZD7 in cisplatin-resistant patients was significantly higher (P<0.05). The results suggest that the expression of miR-27b and FDZ7 may be associated with cisplatin resistance in OSCC patients.

Effect of miR-27b overexpression on the formation of resistant OSCC cell clones. To investigate the effect of miR-27b overexpression on the proliferation of drug-resistant OSCC cells, we first examined the difference in the ability of forming cell clones in the two groups. The results are shown in Fig. 2. The number of clones on the 10th day of the DRG and DRG+miR-27b mimic groups were 133.23±12.78 vs. 28.89±7.12, respectively (P<0.05). It was demonstrated that miR-27b can increase the sensitivity of OSCC cells to cisplatin by inhibiting the proliferation of cancer cells.

Effect of miR-27b overexpression on migration of drug-resistant OSCC cells. The effect of miR-27b overexpression on the migration ability of drug-resistant OSCC cells was also investigated. The results of the scratch test showed (Fig. 3) that miR-27b inhibited the migration ability of drug-resistant OSCC cells by ~2.67-fold (P<0.05).

Effect of miR-27b overexpression on invasion of drug-resistant OSCC cells. In addition, we also used Transwell chambers to assess the invasive ability of OSCC-resistant cells. The results showed (Fig. 4) that miR-27b significantly inhibited the invasive ability of drug-resistant cancer cells. The number of invasive cells of OSCC-resistant cells in the DRG and DRG+miR-27b mimic groups was 91.13±1.45 vs. 23.34±4.52 (P<0.05).

Effect of miR-27b overexpression on apoptosis of drug-resistant OSCC cells. Considering that tumor cell apoptosis is an important indicator for assessing the sensitivity of cancer cells to chemotherapeutic drugs, we further tested the apoptosis rate of the two groups by flow cytometry. The results are shown
in Fig. 5. The apoptosis rates of the DRG and DRG+miR-27b mimic groups were 1.42±0.39 vs. 19.56±1.92%, respectively (P<0.05).

Hoechst 33258 staining results of two groups of cells. We further evaluated the apoptosis of the two groups of cells using Hoechst 33258 staining. The results showed that the number
Effect of miR-27b overexpression on FZD7/β-catenin signaling pathway in drug-resistant OSCC cells. Finally, we further examined the effect of miR-27b overexpression on the expression of FZD7/β-catenin protein in the classical pathway of tumor cell resistance. The results showed that the expression levels of FZD7 and β-catenin were inhibited after overexpression of miR-27b (P<0.05). Therefore, we speculated that the mechanism of miR-27b increasing cisplatin sensitivity in OSCC cells may be related to the suppression of FZD7/β-catenin signaling pathway. The results are shown in Fig. 7.

Discussion

A variety of chemotherapy drugs for the treatment of OSCC have emerged, and these chemotherapy drugs have achieved good results in the treatment of OSCC (11). However, the prognosis and survival of patients with OSCC are still not optimistic. On the one hand, early lymph node metastasis of tumor cells is difficult to detect, and on the other hand, there is OSCC resistance (12). In the early 1980s, cis-diamino-dichloroplatinum (II) (CDDP) and 5-fluorouracil were first used to treat OSCC, and the initial reported cancer cell response rate was only 30-40% (13). Currently, cisplatin-based chemotherapy drugs can be used to treat a variety of malignant tumors including OSCC. In vitro OSCC resistance experiments have revealed that cancer cells can fight against chemotherapeutic drugs with a highly complex phenotypic defense mechanism, such as reducing apoptosis, enhancing repair after DNA damage, changing cell cycle checkpoints and destroying cytoskeleton assembly to increase self-resistance (14,15). Changes in the cytoskeleton can disrupt the transport of cellular proteins and redistribute transporters on the cell membrane surface, even making cancer cells permanently resistant to cisplatin (16).

Studies have shown that Wnt/β-catenin signaling pathway is abnormally activated in various human tumors and is closely related to cancer cell proliferation, differentiation, invasion, angiogenesis and metastasis (17). The classical Wnt/β-catenin signaling cascade can be activated through the binding of Wnt ligand and the seven-pass transmembrane FZD7 receptor protein and co-receptors of Lipoprotein receptor-related protein 5/6 (LRP5/6), and this interaction can lead to the stable transfer of intracellular β-catenin into the nucleus, thereby regulating the expression of related genes (18,19). FZD7 is located on human chromosome 2q33 and is one of the most abundant proteins in the frizzled protein family (19). FZD7 is highly expressed in breast cancer, hepatocellular carcinoma and colon cancer, thus promoting cancer cell proliferation and invasion by activating Wnt signaling pathway (20). Recent studies have found that FZD7 can increase the resistance of cancer cells by activating Wnt signaling. For example, FZD7 silencing can promote the sensitivity of hepatoma cells to 5-fluorouracil by blocking Wnt signaling and downregulation of P-gp expression. Quercetin can reverse the resistance of hepatoma cells to 5-fluorouracil by inhibiting the activation of the FZD7/β-catenin signaling pathway (21). This study first examined the mRNA expression of miR-27b and FZD7 in clinically cisplatin-resistant OSCC patients and cisplatin-sensitive OSCC patients. It was found that cisplatin-sensitive OSCC patients had a higher expression of miR-27b in cancer tissues, but the level of FDZ7 expression was lower. Therefore, we further stimulated the cisplatin-resistant OSCC cell line by gradient concentration of cisplatin, and added miR-27b to upregulate the expression level of miR-27b. It was found that when the expression level of miR-27b was increased, the sensitivity of OSCC cells to cisplatin drugs was significantly increased, mainly manifestations were significantly inhibited in cell proliferation ability, significantly promoted apoptosis, and significantly inhibited cell proliferation and migration ability. These results suggested that miR-27b can improve the resistance of OSCC cells to cisplatin drugs to a certain extent. Finally, by detecting the relevant protein signaling pathways affecting drug-resistant cells, we found that the expression levels of FDZ7 and β-catenin were inhibited by miR-27b, which partially explained the inhibitory effect of miR-27b on cell resistance. However, there are still some limitations in this study: i) Only one cell line was used in this study; ii) Only the expression of FDZ7/β-catenin signaling pathway was detected in this study, but the mechanism of cisplatin resistance in OSCC cells is complex with many signaling pathways. Further experiments are needed to verify whether high expression of miR-27b also affects other drug-resistance-related pathways.

In conclusion, this study reveals for the first time that miR-27b can increase the sensitivity of OSCC cells to cisplatin drugs, significantly inhibit OSCC cell proliferation, promote cell apoptosis, and inhibit cell invasion and migration, which may be related to the inhibition of FDZ7/β-catenin signaling pathway by miR-27b.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

BL drafted the manuscript. BL and TG were responsible for the design of the study, Hoechst 33258 staining and scratch test. GC and ZD collected the specimens, and performed the experiments. All authors read and approved the final manuscript.
Ethics approval and consent to participate

The study was approved by the Ethics Committee of Nanjing General Hospital (Nanjing, China). Signed informed consents were obtained from the patients or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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