

ORIGINAL ARTICLE

Biological functions of miR-183 on chemosensitivity of laryngeal cancer cells

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Summary

Purpose: The purpose of this study was to investigate the effect of miR-183 on the sensitivity of laryngeal cancer cells to 5-fluorouracil (5-Fu) and its mechanism, so as to provide certain references for the clinical prevention of drug resistance in laryngeal cancer cells.

Methods: Cell proliferation was determined via 5-ethynyl-2'-deoxyuridine (EdU) staining. Colony formation assay was applied to test the colony-formation ability in each group of cells. In addition, the expression levels of apoptosis-related proteins were measured through Western blotting. Wound-healing assay and Transwell assay were performed to examine the migratory and invasive abilities. Furthermore, the tumor-forming ability in vitro was detected by subcutaneous tumor formation assay.

Results: MiR-183 declined remarkably in 5-Fu-RES group compared with that in Control group. After overexpressing miR-183, the DNA replication and colony forming abilities of the resistant human primary laryngeal cancer cells were weakened notably. MiR-183 overexpression could obviously

up-regulate Bax and inhibit Bcl-2 in the resistant human primary laryngeal cancer cells. Moreover, the overexpression of miR-183 was able to repress the migratory and invasive abilities of the resistant human primary laryngeal cancer cells. Further, overexpression of miR-183 restrained the in vitro tumor-forming ability of the resistant human primary laryngeal cancer cells markedly. Finally, it was revealed that TBX3 in the resistant human primary laryngeal cancer cells was suppressed distinctly, while that of PTEN was up-regulated evidently after overexpressing miR-183.

Conclusions: The overexpression of miR-183 can inhibit the drug resistance of the human primary laryngeal cancer cells to 5-Fu, promote cancer cell apoptosis and inhibit their invasive and migratory abilities at the same time, whose mechanism may be associated with the targeted regulation of the TBX3/PTEN signaling pathway by miR-183.

Key words: miR-183, laryngeal cancer, drug resistance, 5-fluorouracil, TBX3

Introduction

Laryngeal cancer is a common malignant tumor of the head and neck originating from squamous epithelial cells in the larynx, which may occur at any site of the larynx [1]. Smoking and alcoholism are ubiquitous risk factors for laryngeal cancer. The number of patients with laryngeal cancer was up to 142,000 in 2000 around the world. It is also estimated that there are approximately

12,500 new cases of laryngeal cancer every year. Simple radiotherapy, chemotherapy, operation or combination therapy can be applied to treat laryngeal cancer [2]. However, the 5-year overall survival rate of laryngeal cancer patients after treatment remains very low over the past 20 years. The organ preservation and survival rate of laryngeal cancer patients treated with chemotherapy, an im-

portant therapeutic approach for the disease, are improved significantly compared with those of patients undergoing traditional operative schemes after radiotherapy [3]. Nevertheless, the resistance to chemotherapy drugs in the early stage of laryngeal cancer is a vital cause of treatment failure [4]. Therefore, further clarifying the molecular mechanism of the resistance of laryngeal cancer cells to chemotherapy drugs is of important significance for improving the chemotherapy efficiency and survival of laryngeal cancer patients.

Micro ribonucleic acids (miRNAs/miRs), a group of single-stranded non-coding RNAs with a length of 20-24 nucleotides, exist in eukaryotes and have regulatory functions [5]. They can regulate the expression of many life genes by binding to specific genes in a targeted manner, thus playing vital roles in cell proliferation, differentiation, invasion, apoptosis and other behavioral activities [6]. Large quantities of clinical and basic studies have revealed that miRNAs have crucial effects on the drug resistance of tumor cells. For example, miR-181a enhances the sensitivity of resistant breast cancer cells to mitoxantrone through targeted inhibition of breast cancer resistance protein (BCRP/ABCG2) [7]. Besides, epigenetic silencing of miR-493 increases the resistance to cisplatin in lung cancer by repressing TCRP1 in a targeted manner [8]. Long non-coding RNA PCAT1 promotes cell migration and invasion in human laryngeal cancer by sponging miR-210-3p [9]. However, the role and mechanism of miR-183 in the drug resistance of laryngeal cancer cells to 5-fluorouracil (5-Fu) have not been reported yet.

With human primary laryngeal cancer cells as the objects in this research, the expression of miR-183 in non-resistant and resistant laryngeal cancer cells was detected first. Meanwhile, the expression of miR-183 in resistant laryngeal cancer cells was up-regulated to further investigate the function of miR-183 in the resistance of human laryngeal cancer cell lines to 5-Fu and its mechanism, so as to provide certain references for preventing clinical resistance in laryngeal cancer patients in the future.

Methods

Materials and cells

Human primary laryngeal cancer cells were purchased from Shanghai Bei Nuo Biotechnology Co., Ltd. (Shanghai, China). Then they were induced into resistant strains using 5-Fu with a low-to-high concentration gradient for 2 consecutive months. Fetal bovine serum (FBS) was provided by Biochrom (Holliston, MA, Germany), and Roswell Park Memorial Institute (RPMI)-1640 medium was bought from Gibco (Rockville, MD, USA).

Induction of resistant human primary laryngeal cancer cells

The cancer tissues obtained from patients receiving resection of nephroblastoma in our hospital were cut into blocks, soaked in phosphate-buffered saline (PBS) containing dual antibodies for 5 min and cut into pieces by a pair of ophthalmic scissors. Later, the tissues were added with 0.2% type III collagenase and 50 μ L of hyaluronidase, shaken at a constant temperature for 1 h and centrifuged after filtration, followed by discarding of the supernatant. Next, the tissues were cultured with the RPMI-1640 medium supplemented with 10% FBS in an incubator. After cell adherence, the medium was discarded, and a fresh medium was added. Then the cells were digested and subcultured when the fusion reached 70-80%. The human primary laryngeal cancer cells obtained were routinely cultured with 10% FBS containing certain quantities of dual antibodies in the incubator under 5% CO₂ at 37°C. The human laryngeal cancer cells were stimulated by 5 μ mol/L 5-Fu every month to maintain their drug resistance. Only the cells in the logarithmic growth phase were used in this experiment.

Detection of miR-183 expression via reverse transcription-polymerase chain reaction (RT-PCR)

First of all, the total RNAs were extracted from the laryngeal cancer cells in each group through TRIzol method (Invitrogen, Carlsbad, CA, USA). Next, the concentration and purity of the RNAs extracted were measured qualified by an ultraviolet spectrophotometer, with $A_{260}/A_{280}=1.8-2.0$, so as to implement the following procedures. Later, the RNAs were reversely transcribed into complementary deoxyribonucleic acids (cDNAs) which were stored in a refrigerator at -80°C. Subsequently, the RT-PCR assay was performed in a system containing 2.5 μ L of 10 \times Buffer, 1 μ L of cDNAs, 0.5 μ L of forward primer (20 μ mol/L), 0.5 μ L of reverse primer (20 μ mol/L), 10 μ L of LightCycler® 480 SYBR Green I Master (2 \times) and 5.5 μ L of ddH₂O. The RT-PCR amplification system was the same.

Cell transfection

The laryngeal cancer cells were seeded into a 6-well plate supplemented with medium and cultured in a constant-temperature incubator for 36 h. When the cell density in the culture dish reached 70-80%, the cells were transfected with miR-183 nonsense sequence (NS) or miR-183 mimic in the medium by reference to the instructions of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Finally, the cells were cultured again in the constant-temperature (37°C) incubator.

5-ethynyl-2'-deoxyuridine (EdU) staining

At 48 h after transfection of laryngeal cancer cells with miR-183 mimic, a Click-iT EdU staining kit (Invitrogen, Carlsbad, CA, USA) was utilized to stain the human laryngeal cancer cells according to the procedures in the kit instructions. After that, 3 randomly selected fields of vision on each slide were photographed under a fluorescence microscope. Finally, the EdU-positive cells were counted and quantified.

Colony formation assay

The cells in each group were cultured into the logarithmic growth phase and then digested into single-cell suspension with 0.25% trypsin, ensuring a proportion of single cells >95%. Next, the cell suspension was inoculated into the 6-well plate, with about 500 cells in each well as the best. Subsequently, each well was added with 2 mL of RPMI-1640 medium, and the liquid was replaced every 48 h. 10 d later, the cells were fixed in formaldehyde and stained with crystal violet, and the number of colonies in each well plate was recorded.

Wound healing assay

The cells in the logarithmic growth phase were seeded into 96-well plates, ensuring that the number of cells in each plate was about 5×10^4 . Later, a wound was scratched in the middle of the well plate using the pipette tip, the scratched cells were washed away with PBS, and the medium was replaced with a serum-free medium. Thereafter, the cell migration status was photographed and recorded under a high-power microscope at 0 and 48 h.

Transwell assay

The transwell chambers (8 μ m) were coated with Matrigel diluted at 1:8 and then incubated in the incubator at 37°C for 2 h for gelation. Later, the two kinds of cell lines were diluted into single-cell suspension by serum-free medium and then seeded into the upper transwell chamber (5×10^4 cells/100 μ L). The RPMI-1640 medium containing 10% FBS was added into the lower chamber, followed by 48 h of culture. Finally, the transmembrane cells were fixed in 5% glutaraldehyde, stained with 0.1% crystal violet and photographed.

Western blotting assay

Firstly, the solution in the medium was discarded, and the medium was rinsed by PBS for 3 times. Secondly, 1,000 μ L of cell lysate was added into each dish and shaken sufficiently for 10 min. Then the cells at the bottom of the dish were fully scrapped using a brush and put into prepared Eppendorf (EP) tubes. Thirdly, the cells collected were lysed in an ultrasonication instrument for no more than 15 s (1-2 s/time), placed for 15 min and centrifuged at 12,000 r/min for 0.5 h. Fourthly, the supernatant was aspirated and subpackaged into the EP tubes. Later, the protein concentration was determined through bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometric assay, and all the sample proteins were maintained at a constant volume and equal concentration. Fifthly, the proteins were subpackaged and preserved in the refrigerator at -80°C. Subsequently, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the proteins, after which the proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated with primary antibodies at 4°C overnight and with goat-anti-rabbit secondary antibodies in the dark for 1 h. Finally, the protein bands were scanned and quantified using an Odyssey scanner (Seattle, WA, USA), with glyceraldehy-

de 3-phosphate dehydrogenase (GAPDH) as an internal reference.

Subcutaneous tumor formation assay in nude mice

A total of 30 nude mice were randomly divided into 3 groups, identical to the grouping of cells. Matrigel and PBS were prepared into mixture at 1:1, and the 3 groups of laryngeal cells were resuspended into single-cell suspension. After the nude mice in the 3 groups were anesthetized by diethyl ether, the 3 groups of cells (10^7) were inoculated into the loose skin on the lateral side of right forelimb. 6 weeks later, the mice were killed by cervical dislocation, and the tumor masses were isolated and measured.

Statistics

All the data were analyzed using SPSS 22.0 software (IBM, Armonk, NY, USA). The measurement data were presented as mean \pm standard deviation, and t-test was performed for comparison of data between two groups. $P < 0.05$ suggested that the difference was statistically significant.

Results

Expression of miR-183 in each group of laryngeal cancer cells

The cells in the logarithmic growth phase in Control group and 5-Fu-RES group were applied to extract the total RNAs, and the content of miR-183 was measured (Figure 1). The results of RT-PCR indicated that the expression level of miR-183 in cells in 5-Fu-RES group was remarkably lower (about 0.39 times) than that in Control group ($p < 0.05$), suggesting that miR-183 may be related to the decreased sensitivity of laryngeal cancer cells to 5-Fu.

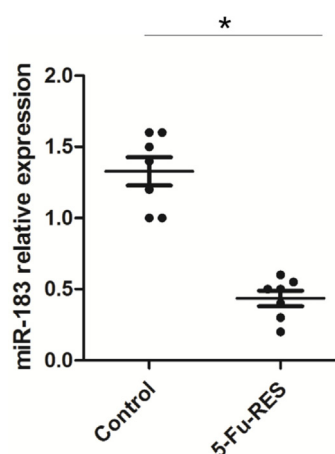


Figure 1. Expression of miR-183 in each group of cells. Control: non-resistant laryngeal cancer cell group, 5-Fu-RES: resistant human laryngeal cancer cell group. * $p < 0.05$ vs. Control group, with a statistical difference.

Effect of miR-183 overexpression on proliferation of resistant laryngeal cancer cells

It was shown in the results of EdU staining (Figure 2) that the number of EdU-positive resistant human laryngeal cancer cells was reduced notably after the overexpression of miR-183 ($p < 0.05$), implying that miR-183 overexpression is able to inhibit the proliferation ability of resistant human laryngeal cancer cells.

Effect of miR-183 overexpression on colony-formation ability of resistant laryngeal cancer cells

According to the results of colony formation assay (Figure 3), 5-Fu-RES + miR-183 mimic group had a markedly smaller number of colonies formed than 5-Fu-RES group [(45.12 ± 2.08) vs. (202 ± 4.09)] ($p < 0.05$).

Effect of miR-183 overexpression on apoptosis of resistant laryngeal cancer cells

Furthermore, the expression levels of apoptosis-related proteins [B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax)] in each group of cells were detected via Western blotting assay. As shown in Figure 4, miR-183 overexpression could obviously up-regulate the expression of pro-apoptotic protein Bax and inhibit that of anti-apoptotic protein Bcl-2 in the laryngeal cancer cells ($p < 0.05$), illustrating that miR-183 is capable of inducing the apoptosis of resistant laryngeal cancer cells.

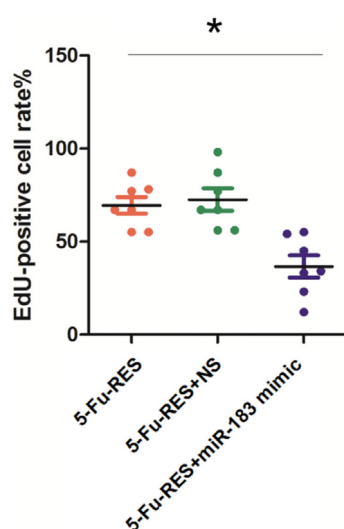


Figure 2. Effect of miR-183 overexpression on proliferation of resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

Effect of miR-183 overexpression on migratory ability of resistant laryngeal cancer cells

The results of wound-healing assay revealed that the overexpression of miR-183 restrained

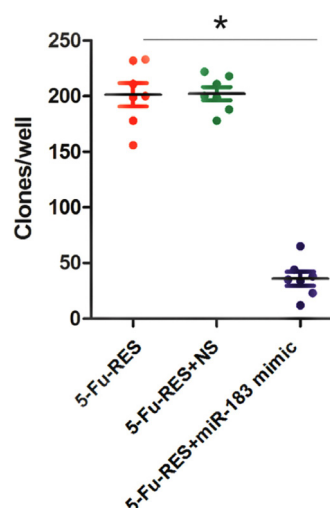


Figure 3. Effect of miR-183 overexpression on colony-formation ability of resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

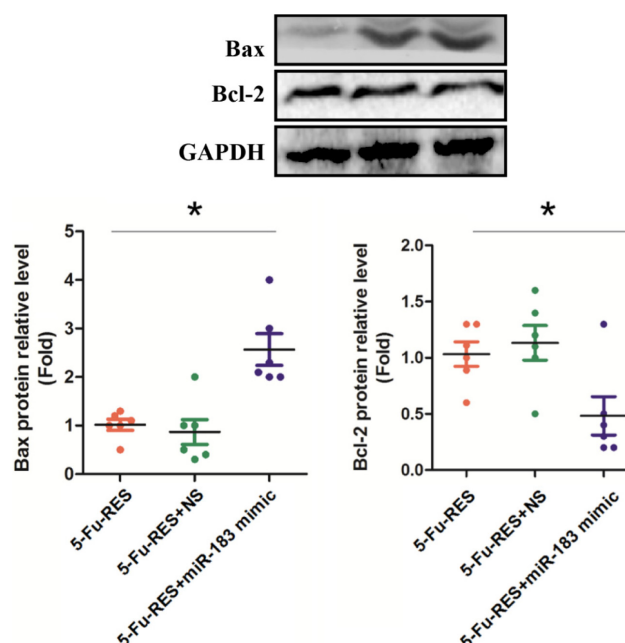


Figure 4. Effect of miR-183 overexpression on apoptosis of resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

the wound healing ability of the resistant laryngeal cancer cells distinctly at 48 h after scratching ($p < 0.05$) (Figure 5).

Effect of miR-183 overexpression on invasive ability of resistant laryngeal cancer cells

It was displayed in the results of Transwell assay that the invasion and metastasis abilities of

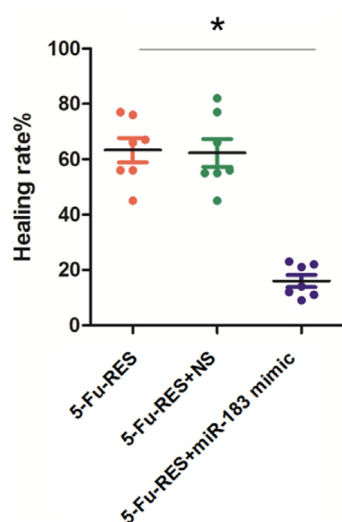


Figure 5. Effect of miR-183 overexpression on migratory ability of resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

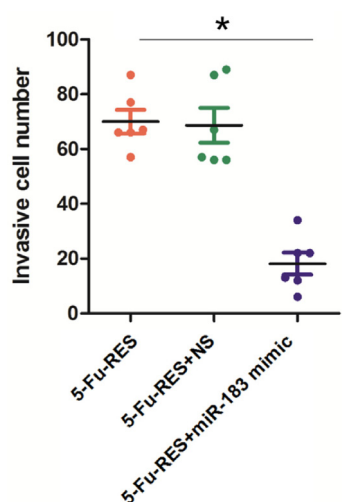


Figure 6. Effect of miR-183 overexpression on invasion ability of resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

the resistant laryngeal cancer cells were repressed evidently at 48 h after the overexpression of miR-183 ($p < 0.05$) (Figure 6).

Effect of miR-183 overexpression on subcutaneous tumor-forming ability of resistant laryngeal cancer cells

Moreover, the allogenic subcutaneous tumor-forming ability of the 3 groups of resistant laryngeal cancer cells was observed by means of subcutaneous tumor formation assay in nude mice. The results (Figure 7) elucidated that the volume of subcutaneous tumors was prominently smaller in 5-Fu-RES + miR-183 mimic group than that in 5-Fu-RES group at 6 weeks ($p < 0.05$), suggesting that miR-183 has the potential to inhibit the tumor-forming ability of the resistant laryngeal cancer cells.

Effect of miR-183 overexpression on T-box 3 (TBX3)/phosphatase and tensin homolog deleted on chromosome ten (PTEN) signaling pathway in resistant laryngeal cancer cells

Previous studies have demonstrated that TBX3 is a potential target gene of miR-183, and the TBX3-mediated inhibition of PTEN is one of the key mechanisms of drug resistance in tumor cells. Hence, the protein expression levels of TBX3 and PTEN in the 3 groups of cells were determined, which revealed that overexpressing miR-183 could clearly suppress the protein expression of TBX3 and up-regulate that of PTEN simultaneously ($p < 0.05$) (Figure 8).

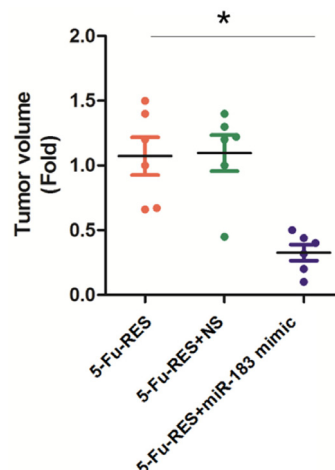


Figure 7. Effect of miR-183 overexpression on subcutaneous tumor-forming ability of resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

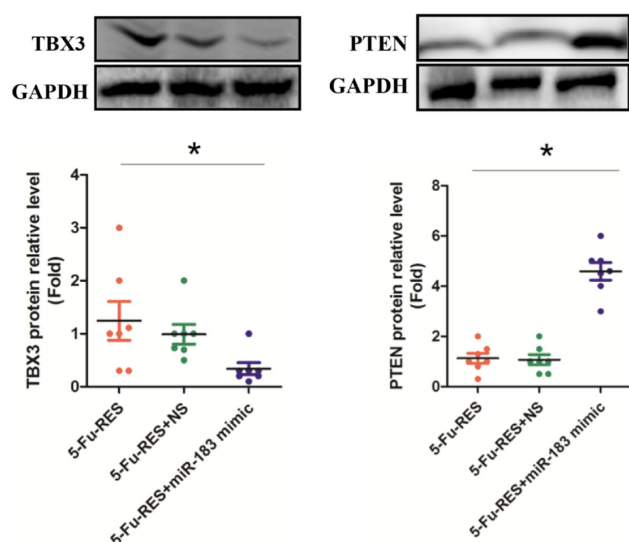


Figure 8. Effect of miR-183 overexpression on the TBX3/PTEN signaling pathway in resistant laryngeal cancer cells. 5-Fu-RES: resistant human laryngeal cancer cell group, 5-Fu-RES + NS: resistant human laryngeal cancer cells + nonsense sequence group, 5-Fu-RES + miR-183 mimic: resistant human laryngeal cancer cells + miR-183 overexpression group. * $p < 0.05$ vs. 5-Fu-RES group, with a statistical difference.

Discussion

The head and neck malignancy in humans is not only the eighth most common cancer in the USA but also the sixth most common cancer in the world, accounting for 10-15% of all the malignant tumors and leading to 4-5% of the cancer-related deaths [10]. It is estimated that there are about 650,000 patients diagnosed with head and neck carcinoma every year worldwide, and 350,000 of them die of the disease [11]. Although clinicians have conducted in-depth studies and made improvements in multi-modal therapies such as operation, radiotherapy and chemotherapy, the 5-year survival rate of the patients with head and neck malignancy remains at 50-60% [12]. Early laryngeal cancer, one of the most prevalent head and neck malignancies, usually can be treated by means of radiotherapy or operation, with satisfactory efficacy. For advanced laryngeal cancer, however, the therapeutic effect of conventional chemoradiotherapy or operation is not conspicuous, of which the generation of multi-drug resistance in tumor cells is a crucial cause [13,14]. Therefore, further identifying the molecular mechanism of the multi-drug resistance in laryngeal cancer cells is of important significance for improving the chemotherapy efficiency in laryngeal cancer patients.

As a kind of endogenous non-coding small RNAs with 20-24 nucleotides in length, miRNAs are capable of regulating the expressions of vari-

ous genes in organisms at the post-transcriptional level. Specifically, miRNAs can bind to the 3'UTR of target genes to inhibit the translation of corresponding proteins [15]. MiR-183, a member of the miRNA family, plays pivotal roles in the proliferation, differentiation, apoptosis, invasion, migration and drug resistance of tumor cells. In the case of synovial sarcoma, miR-183 is able to facilitate the migration of cancer cells through targeted inhibition of transcription factor EGR1 [16]. Besides, miR-183 down-regulates the expression of PDCD4 to inhibit the apoptosis of human hepatocellular carcinoma cells induced by TGF-beta 1 [17]. Additionally, miR-183 regulates the autophagy of gastric cancer cells and promotes the occurrence and development of gastric cancer *via* the MALAT1/miR-183/SIRT1 axis and the PI3K/AKT/mTOR signal [18]. All these 3 studies have elaborated that miR-183 has latent pro-oncogenic effects. Interestingly, miR-183 can restrain the metastasis of cancer cells through down-regulating the protein expression of Ezrin in F5M2 osteosarcoma cells, implying that miR-183 exerts an anti-cancer effect [19]. It is worth noting that miR-183 also serves as a promising diagnostic biomarker for tumors. The expression level of miR-183 is raised obviously in the cancer tissues of patients with urothelial carcinoma, and it is positively correlated with the grade and pathological stage of tumor. After surgical resection, the expression level of miR-183 in the urine is reduced significantly [20]. In terms of the drug resistance of tumor cells, the inhibited miR-183/96/182 cluster enhances the sensitivity of glioma cells to temozolomide *via* the ROS-mediated apoptotic pathways [21]. On the contrary, it was found in this research that the expression level of miR-183 declined markedly in the resistant laryngeal cancer cells, while the up-regulated miR-183 strengthened the sensitivity of laryngeal cancer cells to 5-Fu distinctly, indicating the potential anti-cancer effect of miR-183.

Both TBX2 and TBX3 are the members of the T-box transcription factor family, which are vital players in cell proliferation, development, survival and characteristics. TBX2 and TBX3, as transcription factors, share a >90% homology of DNA binding domain, indicating that they probably regulate similar target genes [22]. A study has revealed that the expressions of TBX2 and TBX3 are up-regulated in multiple tumors, including ovarian cancer, breast cancer, cervical cancer and melanoma [23]. In the case of head and neck squamous cell carcinoma, the expression level of TBX3 is elevated prominently, which notably enhances the malignant biological behaviors of cancer cells by inhibiting the gene expression of PTEN [24]. In this research, it was revealed that the expression level of TBX3 rose

markedly, but that of PTEN declined evidently in the resistant laryngeal cancer cells. However, the overexpression of miR-183 was able to repress TBX3 expression clearly and activate the protein expression of PTEN, finally increasing the killing effect of 5-Fu on cancer cells.

Conclusions

In summary, this research uncovers for the first time that miR-183 expression is lowered in

5-Fu-resistant human laryngeal cancer cells, and up-regulating miR-183 can prominently strengthen the sensitivity of the resistant laryngeal cancer cells to 5-Fu, whose mechanism may be correlated with the targeted regulation of TBX3/PTEN by miR-183.

Conflict of interests

The authors declare no conflict of interests.

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