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Down-regulation of miR-155 promotes apoptosis of nasopharyngeal carcinoma CNE-1 cells by targeting PI3K/AKT-FOXO3a signaling

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Abstract. – OBJECTIVE: Nasopharyngeal carcinoma is one of the common malignant tumors of the ear, nose, and throat in China. Cell apoptosis is expected to be closely related to prognosis. In recent years, with the development of non-coding RNA function research, it is proposed that miRNA plays an important role in the pathogenesis of nasopharyngeal carcinoma. This study aimed to investigate the role of miR-155 in the apoptosis of nasopharyngeal carcinoma cells.

PATIENTS AND METHODS: Cell transfection was performed to knockdown or overexpress the level of miR-155 or knockdown of the level of FOXO3a. The expression of the related protein was detected by immunoblotting. The Real Time quantitative-PCR was used to detect miR-155 expression. Cell proliferation was assessed by MTT assay. The changes of cell apoptosis were observed by flow cytometry using AV-PI staining and TUNEL staining.

RESULTS: The miR-155 inhibitor and mimics were successfully capable of knocking down or overexpressing miR-155 levels. After knocking down miR-155 level, cell proliferation was significantly attenuated, and apoptosis was significantly increased compared with the sham group ($p < 0.05$). After overexpression of miR-155, opposite results were observed. In addition, in the cells with the knockdown of miR-155 level, further knockdown of FOXO3a level significantly reduced the inhibitory effect of miR-155 on cell apoptosis compared with the control group ($p < 0.05$).

CONCLUSIONS: In nasopharyngeal carcinoma CNE-1 cell line, miR-155 can inhibit the proliferation and promote apoptosis of nasopharyngeal carcinoma cells by targeting PI3K/AKT-FOXO3a signaling. MiR-155 may be a novel target for the treatment of nasopharyngeal carcinoma.

Key Words:
Nasopharyngeal carcinoma, MiRNA, PI3K/AKT, FOXO3a, Apoptosis.

Introduction

Malignant tumors occurring on the top and sidewalls of the nasopharyngeal cavity are collectively referred to nasopharyngeal carcinoma, which is one of the most common malignant tumors in China. The five-year survival rate is only 40-50%, and the incidence rate is the first in otolaryngology malignant tumors[1,2]. Radiation therapy is the preferred approach for the treatment of nasopharyngeal carcinoma, but the surgical resection and drug treatment are indispensable for highly differentiated cancer, or patients in late-stage or recurrence after radiotherapy.

Apoptosis is a basic form of cell death. Some investigations have shown that apoptosis is closely related to the occurrence and development of tumors, treatment, and prognosis. Moreover, they have demonstrated that in patients with recurrent or metastatic death of nasopharyngeal carcinoma, the apoptotic index is significantly reduced compared with that of patients without recurrence[3]. Therefore, the apoptosis-regulating genes have been regarded as a new class of tumor-related genes. The occurrence of tumors is the result of the imbalance of cell proliferation and cell apoptosis. Cell apoptosis plays a negative regulatory role in tumor growth and can inhibit the rapid growth of tumor cells[4].

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In recent years, with the development of disciplines, such as gene sequencing technology, transcriptomics technology, and bioinformatics technology, many cancer-related non-coding RNAs have been discovered, and many microRNAs have been confirmed to participate in the process of cancer development. These non-coding RNAs provide a powerful basis for screening biomarkers and targets for the diagnosis and treatment of nasopharyngeal carcinoma[5,6]. MicroRNA (miR) is a non-coding small-molecule single-stranded RNA with a length of 18-24 bp, which can bind to the 3' untranslated region of the target mRNA, leading to selective degradation or inhibition of the transfection of the downstream target genes under the action of RNA exonuclease[7,8]. MiR regulates the downstream genes mainly by inhibiting mRNA transcription. At the same time, the regulation of mRNA degradation and activation can also affect the function of cells and the activation of oncogenes by downregulating the levels of the related proteins.

By reviewing the literature and bioinformatics databases, we found that there may be potential interactions between miR-155 and PI3K/AKT signals. Therefore, in this study, we investigated the expression of miR-155 and PI3K/AKT signal in nasopharyngeal carcinoma cell proliferation and apoptosis, in order to provide the rationale for the diagnosis and treatment of nasopharyngeal carcinoma-related diagnostic and therapeutic markers.

Materials and Methods

Main Materials and Preparations

The nasopharyngeal carcinoma cell line CNE-1 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium-12 (DMEM-F12) medium, penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum, and the Phosphate-Buffered Saline (PBS) buffer were purchased from HyClone (San Angelo, TX, USA). POLD deliver 3000, Opti-MEM (Minimal Essential Medium) medium was purchased from Invitrogen (Carlsbad, CA, USA). The β-actin internal reference antibody was purchased from Kangcheng Biological Company (Shanghai, China). AKT, p-AKT, FOXO3a antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit Anti-Mouse IgG (H+L), Rabbit Anti-Mouse IgG (H+L) was purchased from Proteintech Co., Ltd. (Wuhan, China). Si-FOXO3a MiR-155 inhibitors and mimetics and the corresponding negative controls were purchased from Gima Gene Co., Ltd. (Shanghai, China).

Main Instruments

The ultra-clean workbench is supplied by Boxun Industry Co., Ltd. (Shanghai, China), the gel imaging system UVP Multispectral Imaging System (Upland, CA, USA), and the PS-9 semi-dry transfer electrophoresis instrument is purchased from Jingmai Co., Ltd. (Dalian, China), a carbon dioxide incubator, and the Thermo-354 microplate reader was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Nasopharyngeal Carcinoma Cell Line Culture

The nasopharyngeal carcinoma line (CNE-1) was cultured in containing 10% inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2.

MTT Assay for Cell Proliferation

After the cells were transfected with miR-155 inhibitors and mimics, the cells in the logarithmic growth phase were collected, adjusted to a cell concentration of 5-10×10^4/mL, and seeded in 96-well plates with 100 μL per well and 10 replicate wells per group. After cultured for 24 hours, 10 μL MTT was added to each well. After 4 hours, the upper culture medium was carefully aspirated, and the triple solution was added with 100 μL per well. When the crystal was fully dissolved, the cells were further cultured for 12-15 hours followed by measurement of the OD value at 450 nm. The cell proliferation efficiency of each group was calculated by using 100% of the normal control group.

$$\text{Cell survival rate (\%)} = \frac{\text{(Experimental group OD - Blank group OD)}}{\text{(Control group OD - Blank group OD)}}$$

Cell Transfection

MiR-155 Antagomir and Agomir were synthesized by Jima. The cell line was passaged one day before transfection, and the confluence was 30-50% in a 24-well plate. 1.25 μL siRNA mother solution (20 μM) was dissolved in 100 μl Opti-MEM medium which was defined as A solution. 1 μL Lipofectamine 2000 or Lipofect-

amine™ RNAiMAX was dissolved in the Opti-MEM medium and defined as B solution. After mixing B solution for 5 minutes, A and solution B were mixed and allowed to stand for 20 min, and then added to the cell culture plate. After 4 h of incubation, the cells were changed to the cell growth medium, and the transfection efficiency was observed under a fluorescence microscope.

One day before the transfection, the cells were seeded on 6-well plates, diluted to 100 μL according to 10 pmol of miR-155 inhibitor per well, with corresponding mock or corresponding negative control, 5 μL of POLDdeliver 3000, and mixed for 5 min. The above mixture was uniformly added dropwise to the cells of a 6-well plate, gently shaken, and cultured for 48 hours after transfection, and the proliferation rate and the expression of protein and miR were determined, according to the experiment.

Cellular Protein Extraction

After absorbing the culture medium, the cells were washed three times with PBS, and 10 μL of 100 mM PMSF was added to each 1 mL of the lysate, and 100 μL of the lysate was added to each well in a six-well plate, and lysed on ice for 5-10 min, using a scraping bar. The cells were hung on one side of the well, and the cell debris and lysate were transferred to a pre-cooled clean Eppendorf (EP) tube with a pipette and centrifuged at 12000 rpm for 5 min at 4°C. The supernatant was collected as a whole protein solution.

Western Blot

The extracted whole protein solution was quantified by bicinchoninic acid (BCA) method, and then calibrated to a uniform concentration. After adding the sample buffer, it was denatured in boiling water for 5 min. The proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane at 300 mA for 1 hour, and incubated with p53 antibody (1:1000) at 4°C overnight. After Tris-Buffered Saline and Tween-20 (TBST) washing for three times, the secondary antibody (1:1000) was added and incubated at 37°C for 2 h. After that, the strip was developed by chemiluminescence.

TUNEL Assay for Cell Apoptosis

The cells were counted after digestion, and 2 × 10^5 cells were seeded and plated in a 24-well plate. After transfection, each group of cells was cultured for 24 hours, and then fixed with paraformaldehyde. After immersing in 3% hydrogen peroxide methanol for 10 min, incubating with 0.2% Triton for 5 min, washing twice with PBS, according to the instructions, we added 50 μL of TUNEL reaction solution to each well, and incubated them at 37°C for 1 h under the dark. After PBS wash for three times, some photographs were taken under a fluorescence microscope. Five fields were randomly selected from each well, and the number of positively stained cells was observed for statistical analysis.

AV-PI Staining

After transfection, the cells were digested with EDTA-free trypsin, washed three times with ice PBS, then 400 μL of AV binding solution was added, and 400 μL of AV binding solution was added after mixing. After resuspending the cells, 5 μL SAV staining solution was added to each sample. After incubating for 15 min under the dark, 10 μL PI staining solution was added for 5 min incubation under the dark, and then cell apoptosis was detected by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

The experimental data were processed by the Statistical Product and Service Solution SPSS 10.0 (SPSS Inc., Chicago, IL, USA) and expressed by mean ± standard deviation (SD). One-way analysis of variance with the Newman-Keuls multiple comparison post-hoc analysis was used for comparison between the groups. SNK-Q test was used for further comparison between groups. $p < 0.05$ indicated a statistical significance.

Results

MiR-155 Levels After Transfection of MiR-155 Inhibitors and Mimics

To investigate the role of miR-155 in the proliferation and apoptosis of nasopharyngeal carcinoma cells, miR-155 inhibitor and mimic transfection were performed. After transfection of the miR-155 inhibitor, real time quantitative-PCR detected a significant decrease in the expression of miR-155 (Figure 1A). Conversely, the expression of miR-155 expression levels was significantly increased after transfection of miR-155 mimics (Figure 1B). The above results indicate that the transfection of miR-155 inhibitors and mimetics was successful.

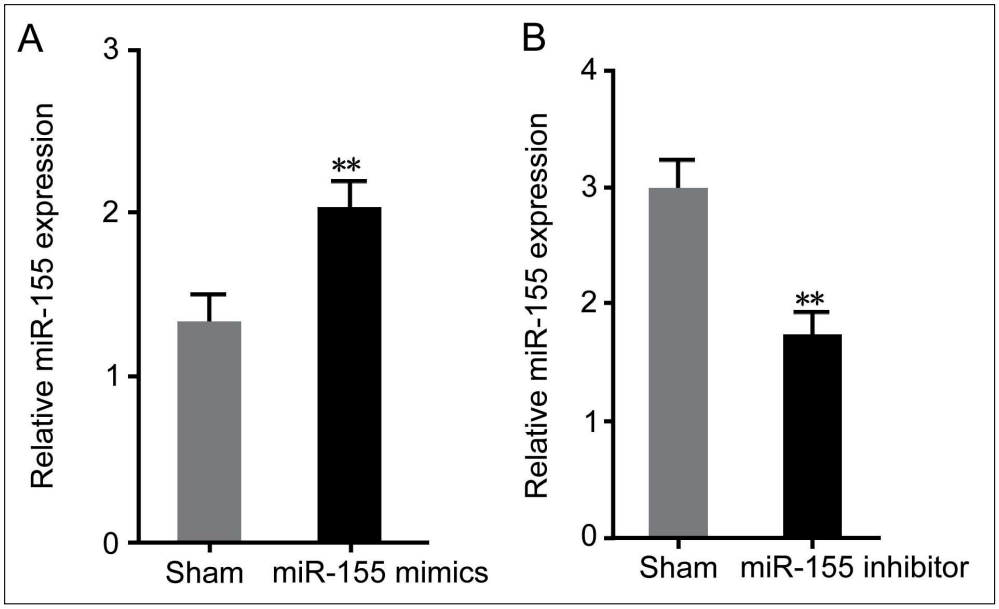

Figure 1. MiR-155 expression compared with the sham group, $^{**}p < 0.05$.

Down-Regulation of MiR-155 Levels Inhibits the Proliferation of Nasopharyngeal Carcinoma Cell Lines and Promotes Cell Apoptosis

After transfection of miR-155 inhibitor, the changes of cell proliferation were detected by MTT assay, and the changes of apoptosis were observed by AV-PI and TUNEL staining. As shown in Figure 2A, cell viability was significantly reduced after transfection with miR-155 inhibitor, and there was a significant difference compared with the sham group ($p < 0.05$). At the same time, the effect of miR-155 on cell apoptosis was observed by AV-PI staining and TUNEL staining. As shown in Figures 2B and 2C, after transfection of miR-155 inhibitor, the number of TUNEL-positive cells was significantly increased, and also the apoptosis rate was remarkably increased. There was a significant difference ($p < 0.05$) compared with the sham group.

Overexpression of MiR-155 Levels Promotes the Proliferation of Nasopharyngeal Carcinoma Cell Lines and Inhibits Their Apoptosis

The changes in cell proliferation and apoptosis were also examined after transfection of miR-155 mimics. In contrast to the action of miR-155, the cell proliferation was significantly increased after miR-155 mimics treatment, and there was a significant difference compared with the sham group ($p < 0.05$) (Figure 3A), and the number of the apoptotic cells was also significantly decreased ($p < 0.05$) (Figure 3B and 3C).

Effect of MiR-155 on PI3K/AKT-FOXO3a Signaling Pathway

In order to verify the effect of miR-155 on PI3K/AKT-FOXO3a signaling, the expressions of AKT, p-AKT, and FOXO3a were detected by immunoblotting. After knocking down of miR-155, the expression of p-AKT was significantly increased, the PI3K/AKT signaling pathway was activated, and the expression of FOXO3a was significantly decreased (Figure 4A). In contrast, after the overexpression of miR-155, the PI3K/AKT signaling pathway was inhibited and the FOXO3a expression level was significantly increased (Figure 4B).

SiRNA Knocked Down FOXO3a Levels and Altered Apoptosis

The above results indicate that miR-155 can indeed affect the PI3K/AKT-FOXO3a signaling pathway, accompanied by cell apoptosis, and whether miR-155 affects apoptosis through the FOXO3a pathway remains unclear. Therefore, after we down-regulated miR-155, we simultaneously knocked down FOXO3a levels and

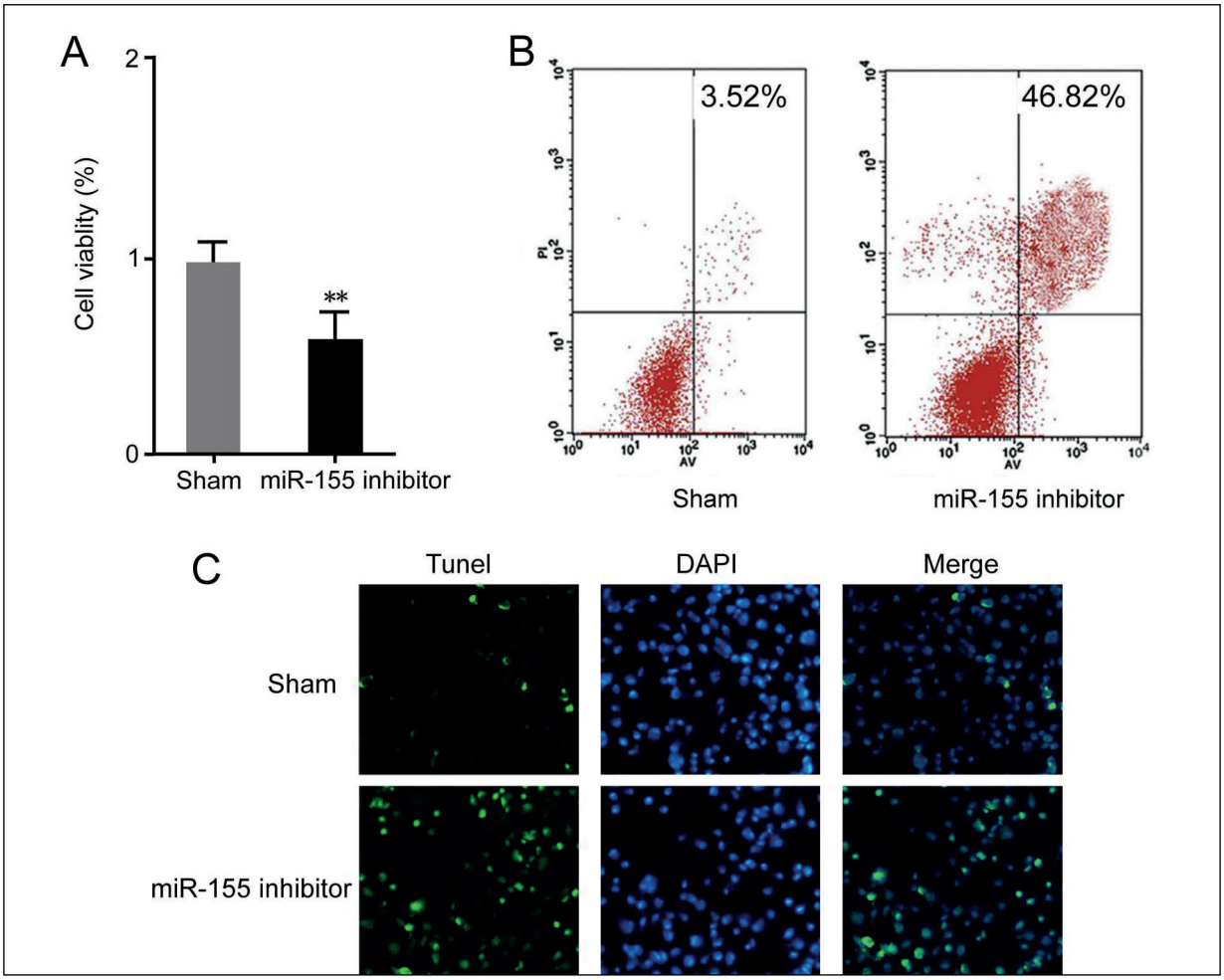

Figure 2. Effect of down-regulation of miR-155 on proliferation and apoptosis of nasopharyngeal carcinoma cells. ***A,*** MTT assay. ** $p < 0.05$ compared with the sham group. ***B,*** AV-PI staining. ***C,*** TUNEL staining (× 40).

observed cell apoptosis. As shown in Figure 5A, the expression of FOXO3a protein was significantly decreased after knocking down of FOXO3a, demonstrating that the knockdown was successful. The level of cell apoptosis was significantly reduced as compared to the miR-155 inhibitor group alone ($p < 0.05$) (Figure 5B). This result suggests that the knockdown of miR-155 levels-induced cell apoptosis was dependent on FOXO3a expression.

Discussion

Nasopharyngeal carcinoma is a malignant tumor that occurs on the top and sidewalls of the nasopharyngeal cavity. It can be treated with radiation in the early stage. However, for highly differentiated diseases and postoperative recurrence, drug therapy is an indispensable approach[9]. MiRNAs are newly discovered non-coding RNAs with a length of 20 to 24 bases, which bind to the 3' untranslated region of mRNA encoding proteins, and regulate mRNA expression at post-transcriptional levels[10]. The regulation of protein synthesis by MiR at the post-transcriptional level is very important under physiological conditions. The abnormality of miR regulation is related to the initiation and course of various human cancers, including thymic carcinoma, liver cancer, cholangiocarcinoma, etc[11]. Different miRs play an important role in malignant tumors in different sites. Thus, we speculated that miRNAs have a far-reaching future as a new target for cancer therapy.

The transcript miR-155 is a widely expressed microRNA in a variety of human tissues. MiR-155 is encoded by the cell integration cluster

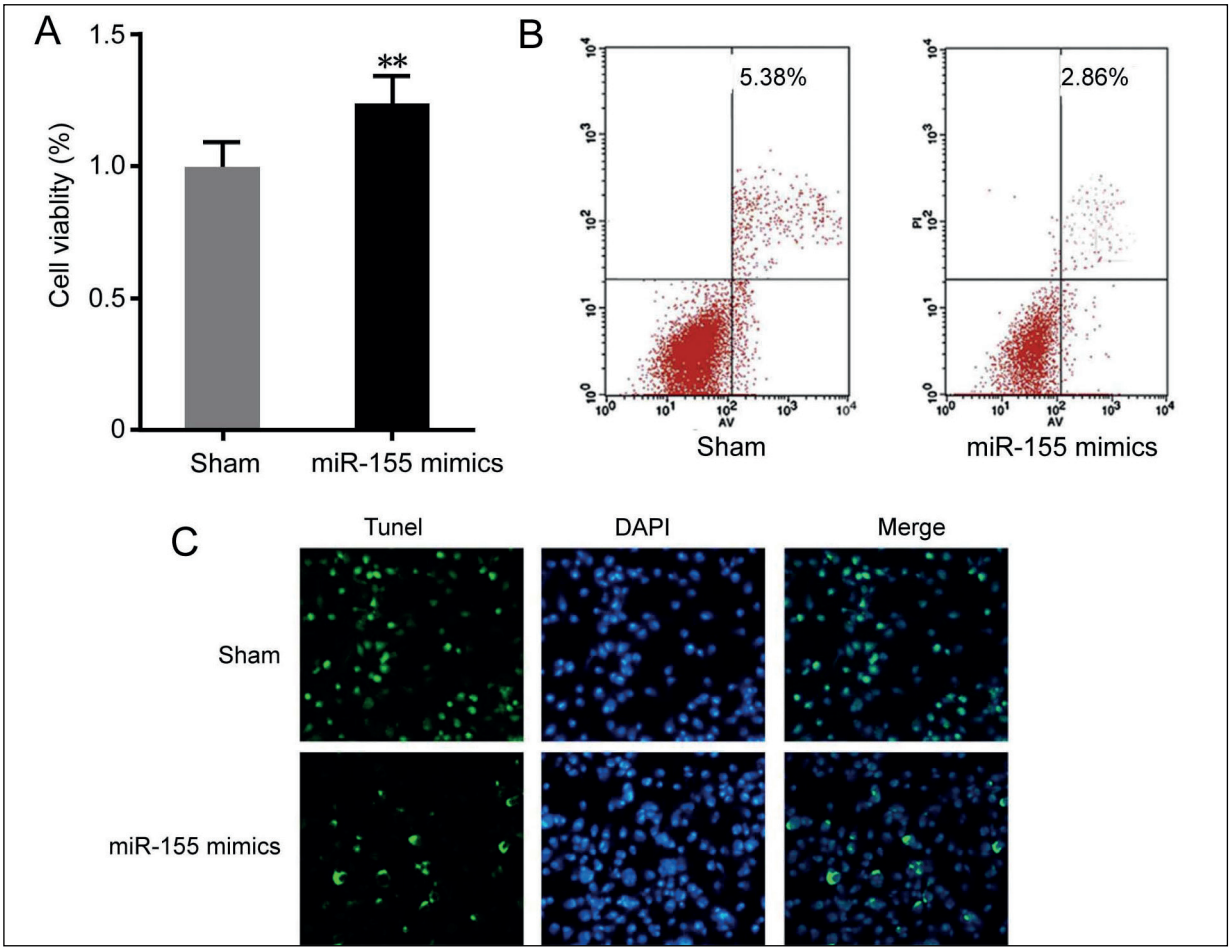

Figure 3. Effect of up-regulation of miR-155 on proliferation and apoptosis of nasopharyngeal carcinoma cells. ***A,*** MTT assay. ** $p < 0.05$ compared with the sham group. ***B,*** AV-PI staining. ***C,*** TUNEL staining (× 40).

(BIC) gene[12]. Wan et al[13] have shown that miR-155 plays an important role in hematopoietic cell production, inflammatory response, immune response, and tumorigenesis. High expression of miR-155 has been reported in various malignant tumors such as breast cancer, giving love, colon

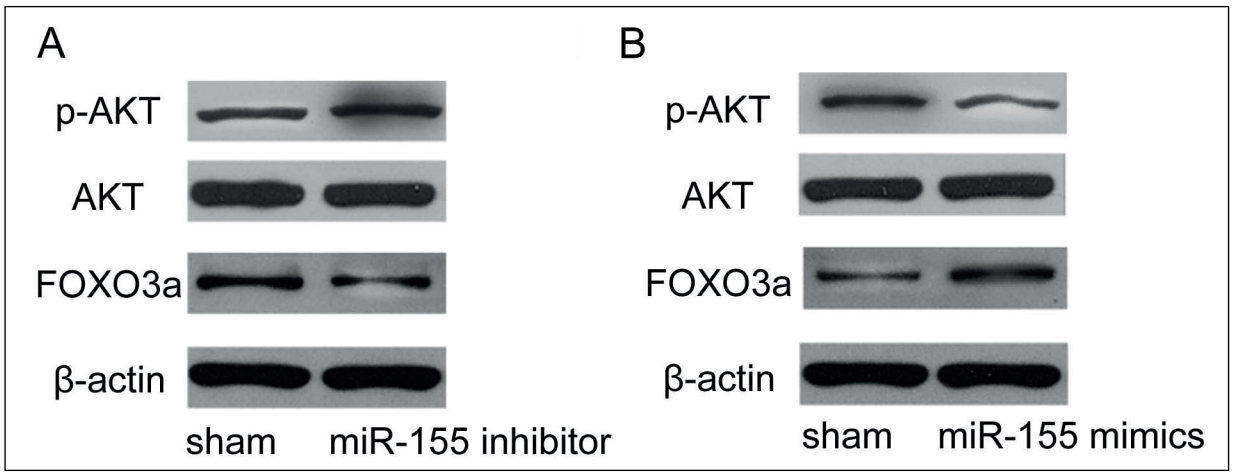

Figure 4. Effect of miR-155 on PI3K/AKT-FOXO3a signaling pathway. ***A,*** Treatment with miR-155 inhibitor. ***B,*** Treatment with miR-155 mimics.

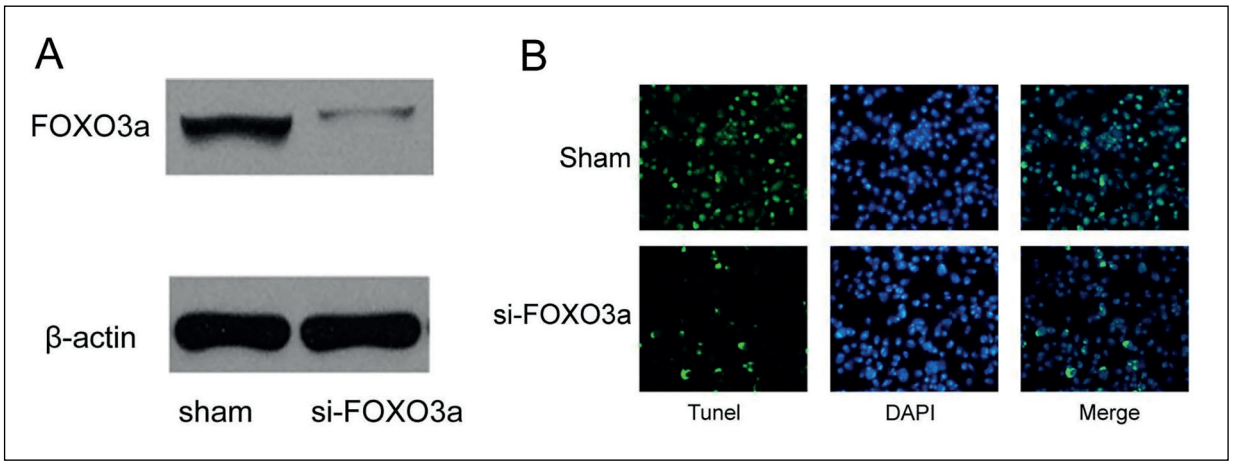

Figure 5. Effect of FOXO3 knock down on cell apoptosis. ***A,*** Knock down FOXO3a. ***B,*** TUNEL staining (× 40).

cancer, pancreatic cancer, and thyroid cancer[14]. Currently, there are several studies on miR-155, and the oncogene-like effect of miR-155 was firstly discovered in hematopoietic malignancies[15,16]. In tumor-related reports, miR-155 was identified as overexpressed in chronic lymphocytic leukemia. Despite this, there are few studies on its transcriptional regulation or its biological function. However, how miR-155 is expressed in nasopharyngeal carcinoma cell lines and what role it plays has not been reported. In this study, high expression of miR-155 in nasopharyngeal carcinoma was confirmed for the first time, and the inhibitory effect of miR-155 on the proliferation of nasopharyngeal carcinoma cells and the promotion of cell apoptosis were observed.

In this study, we used cell transfection to knock down or overexpress the level of miR-155, while immunoblotting was performed to detect changes in the protein expression levels. The MTT assay was used to detect changes in the cell proliferation rate, and the level of apoptosis was observed by AV-PI staining. In addition, in apoptotic cells, endogenous endonucleases are activated, the cell's own chromosome or DNA is cleaved, the single-stranded or double-stranded gaps appear, the same number of 3'-OH ends, and the number of DNA breaks are produced. According to this feature, we also directly observed the apoptotic cells before morphological changes using the TUNEL staining method.

The phosphatidylinositol trikinase (PI3K)/protein kinase B (AKT) signaling pathway is a classical signaling pathway that regulates cell proliferation, apoptosis, metastasis, and gene transcription[17,18]. PI3K/AKT is closely related to the development of various malignant tumors. It has been reported[19,20] that miR-155 may act as an upstream regulator of PI3K to activate PI3K/AKT signaling. FOXO3a is a transcription factor with a fork-like structure that plays a key role in cell apoptosis and is considered to be an important tumor suppressor[21]. In this study, it was found that the up-regulation of miR-155 level in the nasopharyngeal carcinoma CNE-1 cell line inhibits the PI3K/AKT signaling pathway and down-regulates FOXO3a expression, decreases cell apoptosis, and was accompanied by accelerated cell proliferation. By contrast, after transfection of the miR-155 inhibitor, the PI3K/AKT signaling pathway was activated, and the expression of FOXO3a was up-regulated, the proliferation of nasopharyngeal carcinoma cells was inhibited, and cell apoptosis was increased. Thus, we concluded that miR-155 might play an important role in the development of nasopharyngeal carcinoma. Therefore, miR-155 may be an effective target for the treatment of nasopharyngeal carcinoma, and its regulatory pathway may be achieved through the regulation of PI3K/AKT signaling pathway, causing an abnormal expression of FOXO3a.

Conclusions

In the nasopharyngeal carcinoma CNE-1 cell line, miR-155 can inhibit the proliferation and promote apoptosis of nasopharyngeal carcinoma cells by targeting PI3K/AKT-FOXO3a signaling. MiR-155 may be a novel target for the treatment of nasopharyngeal carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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