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Original Research

MiR-122 exerts anti-proliferative and apoptotic effects on nasopharyngeal carcinoma cells via the PI3K/AKT signaling pathway

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Abstract: To investigate the effects of microRNA-122 (miR-122) on the proliferation and apoptosis of nasopharyngeal carcinoma (NPC) HONE-1 cells, and its correlation with the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. Human NPC cell line (HONE-1) was transfected with miR-122 inhibitor (anti-miR-122 group), negative controls (vector control group) via lipofectamines, and HONE-1 cell lines undergoing no transfection were selected (non-transfection group). The expression of miR-122, cell proliferation, apoptosis, and expressions of PI3K/AKT pathway and downstream target proteins in the three groups were determined using fluorescence quantitative polymerase chain reaction (qPCR), cell counting kit-8 (CCK8), immunofluorescence (IF) and Western blotting, respectively. The expression of miR-122 in the anti-miR-122 group was significantly lower than corresponding expressions in the non-transfection and vector control groups after 48h of transfection (p <0.05). The proliferation of cells in the anti-miR-122 group was significantly reduced with time after transfection (p <0.05). After 48h of transfection, the extent of apoptosis in the anti-miR-122 group (47.11 ± 1.95%) was significantly higher than that in normal control (7.37 ± 0.82%) and vector control group (8.54 ± 0.96%; p <0.05). There were no significant differences in the expressions of PI3K, AKT, mTOR protein, and the downstream signal proteins (p70S6K and 4E-BP1) in the three groups (p >0.05). However, the expressions of phosphorylated forms of these proteins were significantly lower in the anti-miR-122 group than in the non-transfection and vector control groups (p <0.05). IF results revealed that there were no significant differences in the fluorescence intensity value of PI3K and Akt among the three groups of patients (p>0.05). Inhibition of the expression of miR-122 in NPC suppresses the proliferation, and promotes their apoptosis through the PI3K/AKT signal transduction pathway.

Key words: MiR-122; Nasopharyngeal carcinoma; PI3K/AKT pathway; Proliferation; Apoptosis.

Introduction

Nasopharyngeal carcinoma (NPC), which is common in Southern China, is a squamous epithelial cell carcinoma characterized by high degree of malignancy and local invasion (1). Although little is known about the specific molecular mechanism of the occurrence and development of NPC, three main causes will lead to NPC, namely, genetic susceptibility, environmental factors and Epstein-Barr virus infection. Although NPC treatment is sensitive, the long-term survival rate of patients at the advanced stage is still very low, and the 5-year survival rate after chemotherapy is about 60%. More than 30% of patients with NPC experience metastasis within five years, and, once this happens, prognosis becomes very poor (2). In order to find a more effective treatment for NPC, its pathogenesis should be understood. Recently, research on microRNAs (miRNAs) and the pathogenesis of NPC have formed the fulcrum of major scientific discussions. MiRNAs are a class of small, non-coding RNAs that often bind to the three prime untranslated region (3'-UTR) of a target mRNA and induce mRNA degradation or translational inhibition. miRNAs are involved in the regulation of the growth and development of plants and animals and many other complex life processes. Several studies have shown that miRNAs may function as tumor suppressor genes or oncogenes (3, 4). Overexpression of miR-122 has been found in renal clear cell carcinoma (5), cancer of the liver (6) and non-small cell lung cancer (NSCLC) (7). However, the expression of miR-122 in NPC, and the signaling pathway involved, have not been reported. The aim of this study was to investigate the anti-proliferative and apoptotic effect of miR-122 on NPC, and its link with the PI3K/AKT pathway.

Materials and Methods

Materials

Human NPC cell line (HONE-1) was a product of Shanghai Xuran Biotechnology Co., Ltd; RPMI-1640 cell culture medium was purchased from Shanghai Guandao Bioengineering Co., Ltd, while Gibco Australia fetal cattle serum was purchased from Beijing Zhijie Fang Yuan Technology Co., Ltd. Kits for CCK8 kit were purchased from Shanghai Xuan Ling Biotechnology

Co., Ltd; MiR-122 inhibitor. The negative control vector was product of Biomarker Biotechnology Co., Ltd, while lipofectamine 2000 kit was obtained from Shanghai Weijin Biotechnology Co., Ltd. Annexin V-FITC/PI cell apoptosis detection kit was product of Shanghai Yisheng Biotechnology Co., Ltd; Opti-MEM culture medium was purchased from Shanghai Tuo Yang Biotechnology Co., Ltd, and Trizol total RNA extraction kit was from Shanghai Chaoyan Biotechnology Co., Ltd. The qPCR primers were products of Shanghai Xingyuan Ruimin Biological Engineering Co., Ltd, while the first and second antibodies were purchased from BD Inc.

Culture of HONE-1 cells

This was carried out in 10,000-grade purification room to ensure an aseptic operation. HONE-1 cells were evenly dispersed in 10% fetal bovine serum- supplemented RMPI-1640 culture medium and incubated at 37˚C in an incubator. The culture medium was replaced with fresh medium every two days, until the cells grew to a density of 80%.

Transfection and grouping

Culture plates were inoculated with HONE-1 cells, followed by the addition of RMPI-1640 cell culture medium. After the cells attained a density of 60%, the observation and negative groups were transfected with miR-122 inhibitor and mimic, respectively. Cells that were not transfected served as control. After 24h of transfection, the plasmid vector lipofectamine 2000 diluted in Opti-MEM medium was added and incubated for another 6h. Thereafter, it was replaced with complete culture medium.

Determination of the expression of miR-122

After 48h of transfection, Trizol reagent was used to extract the total RNAs of cells in the three groups. The purity and concentrations of the extracted total RNAs were also determined. Complementary DNA (cDNA) was synthesized using reverse transcription and qPCR amplification at temperatures of 94°C for 20 sec, 84°C for 30 sec, 60°C for 45 sec, and 40 cycles. Primer sequences: miR-122: 5'-AGGGGCGCCTGGAGTGTGACAATG-3' and β-actin: forward: 5'-ACACTGTGCCCATCTACGAGGGG-3', and reverse: 5'-ATGATGGAGTTGAAGGTAGTTTCGTGGAT-3'. The levels of expressions of miR-122 in the three groups were calculated using $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control.

Determination of cell proliferation

Transfected and control cells (5 x 10^3/wells) were seeded into 96-well plates. Each well was incubated with 10μl CCK8 reagent and further incubated in the dark for 2h. The absorbance of each well was measured at 492nm using Sunrise enzyme labeling instrument, and cell proliferation was calculated thus:

Cell proliferation (%) = $(A_{EW} - A_{BW}) / (A_C - A_{BW}) \times 100$

where A_{EW} = absorbance of experimental well; A_{BW} = absorbance of blank well; and A_C = absorbance of control well.

Apoptosis assay

Transfected and control cells were digested with trypsin, and the cells (1×10^6/ml) were collected and re-suspended in 100μl binding buffer containing 10μl of Annexin V-FITC and 5μl PI. After incubation in the dark at room temperature for 20 min, the extent of apoptosis was determined using a flow cytometer.

Western blotting

Transfected and control cells were washed with phosphate-buffered saline (PBS) and lysed with ice-cold radio-immunoprecipitation assay buffer (RIPA). Determination of protein content was done using BCA protein assay kit and stored at ultra-low temperature for later use. Protein separation was achieved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the bands transferred to PVDF membranes within 35 min. Membranes with target proteins were blocked with 5% skim milk powder at 37°C, and incubated with primary antibodies (PI3K, p-AKT and caspase-3) within 90 min, washed, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 1h. The primary and secondary antibodies were diluted at a ratio of 1 to 200 overnight at 4°C.The densities of the bands were estimated using Chemi-doc XRS imaging system with β-actin as the internal control.

Statistical analysis

Data are expressed as mean ± SEM, and statistical analysis was performed using SPSS (19.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Inhibitory effect of miR-122 detected by qPCR

Total RNA was extracted from each group after transfection for 48h. qPCR results showed that there was no significant difference in the relative expression level of miR-122 between the vector control group and the non-transfection group ($p>0.05$). However, the expression of miR-122 in the anti-miR-122 group was significantly lower than corresponding expressions in normal and vector control groups after 48h of transfection ($p<0.05$). However, there was no significant difference in the expressions of miR-122 between the non-transfection and vector control groups ($p>0.05$), as shown in Figure 1.

Influence of miR-122 on the proliferation of NPC cells

The difference in the cell proliferation among the three groups of cells was measured using the cell counting assay. As shown in Figure 2, the number of cells in the non-transfection group and the vector control group was increased with time, and there was no significant difference at each detection time point ($p>0.05$). The number of cells in the anti-miR-122 group began to be decreased at 12h after transfection. Compared with that in the vector control group, the number of viable cells in the anti-miR-122 group was significantly decreased ($p<0.05$), indicating the knockout of miR-122 remarkably inhibits the proliferation of NPC cells.

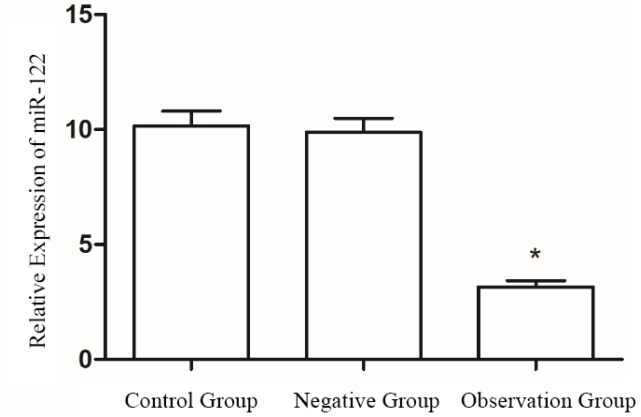

Figure 1. Expressions of miR-122 in the three groups of cells. *p <0.05, compared with the vector control.

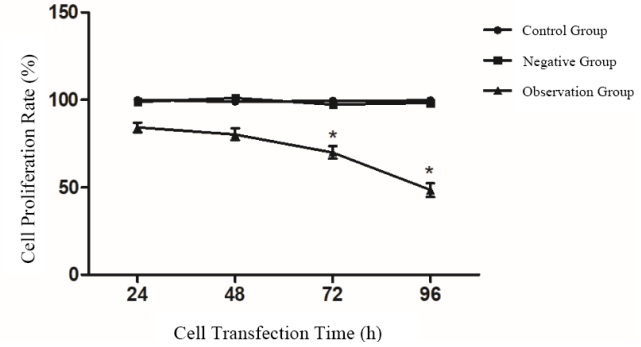

Figure 2. Influence of miR-122 on the proliferation of NPC cells. Note: *p<0.0.1 vs. vector control group.

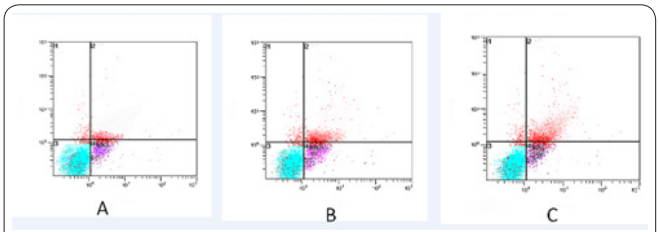

Figure 3. Flow cytometogram of apoptosis in the three groups of cells.

Difference in the apoptosis among the three groups of cells detected via flow cytometry

After 48h of transfection, flow cytometry results revealed that the apoptosis rate was (4.37±1.82)% in the vector control group, (3.54±1.56)% in the non-transfection group, and (32.11±5.95)% in the anti-miR-122 group. The apoptosis rate in the anti-miR-122 group was significantly higher than that in the vector control group (p <0.05). However, there was no significant difference in the apoptosis rate between the vector control and the non-transfection group (p >0.05; Figures 3 and 4).

Expressions of PI3K/AKT signaling pathway-related proteins

Western blotting was further adopted to detect the levels of PI3K, AKT and the downstream target molecule mTOR as well as its phosphorylated proteins. The results manifested that the total protein content of PI3K, AKT and downstream signal mTOR did not change significantly in the three groups (p>0.05). However, the relative expression levels of phosphorylated-PI3K (p-PI3K), p-AKT and p-mTOR in the anti-miR-122 group were evidently lower than those in the vector control group (p<0.05) (Figure 5 and Figure 6).

Discussion

MicroRNAs are relatively stable small RNAs in cells. They can combine with mRNA sequence of a target gene through the principle of complementary base pairing to inhibit the translation of the mRNA, and downregulate expressions of the target genes, thereby silencing them (8, 9). In recent years, considerable attention has been focused on the role of miRNAs in tumorigenesis, and several reports have been published on the topic. Studies have shown that miRNAs are closely related to the development of tumors. In one of such studies, it was found that about 50% of human miRNAs are localized in cancer-related gene regions, where they function as tumor suppressor genes or oncogenes (10, 11). Bioinformatics have revealed that each miRNA in a human cell can effectively regulate the transcription of hundreds of genes, an indication that they have potential regulatory effects on most biological signaling

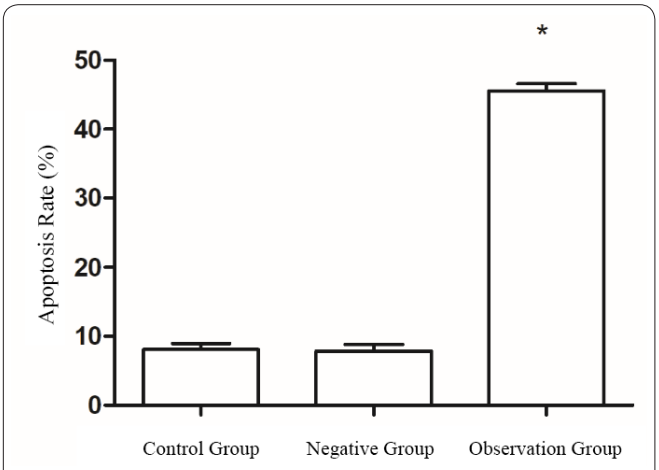

Figure 4. Extent of cell apoptosis. *p <0.05, compared to vector control and non-transfection control.

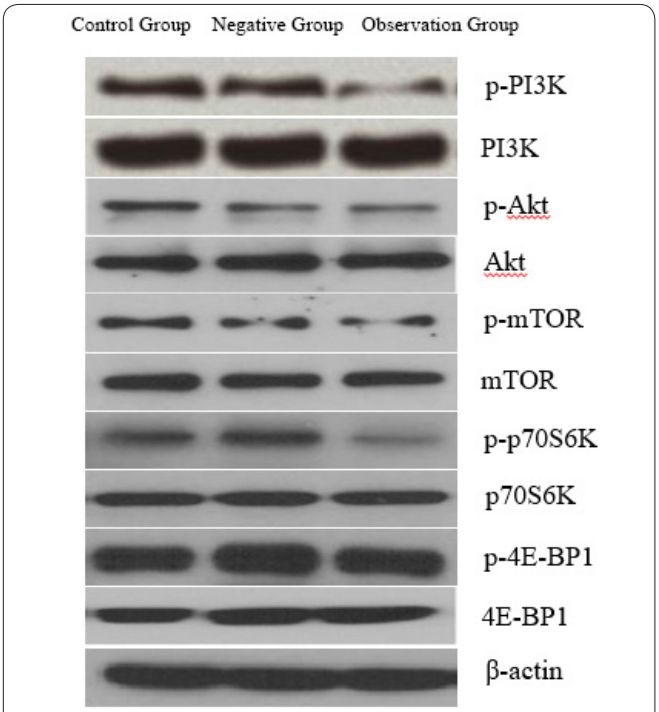

Figure 5. Western blotting images of the expressions of the PI3K/Akt signaling pathway-related proteins in the three groups of cells.

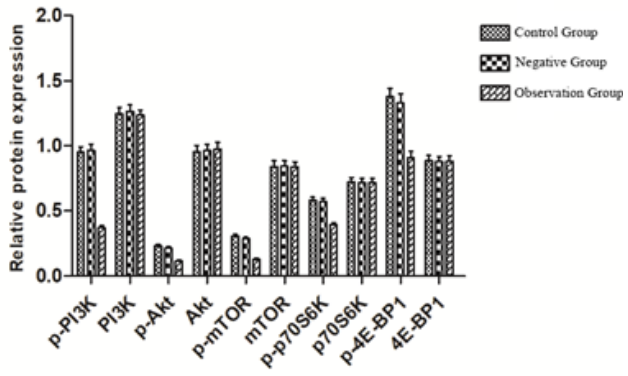

Figure 6. Expressions of PI3K/AKT signaling pathway and downstream target proteins in the three groups of cells.

pathways (12). It has also been reported that abnormal expressions of miRNAs associated with Epstein-Barr virus, such as miR-100, miR-29, and miR-141 promote the development of NPC (13). As a member of miRNAs family, miR-122 has been shown to be overexpressed in renal clear cell carcinoma, cancer of the liver, and NSCLC (5-7). Yang *et al*. (14) reported that the overexpression of miR-122 in renal clear cell carcinoma enhances the proliferation activity of cancer cells (14). Valentina *et al*. reported that miR-122 overexpression reduced the sensitivity to chemotherapy-induced apoptosis by activating the AKT signaling pathway and inhibiting the p53 signaling pathway (15). However, the mechanism by which miR-122 exerts its effect in NPC is unclear.

In the present study, the expression of miR-122 in the anti-miR-122 group was significantly lower than corresponding expressions in the non-transfection and vector control groups after 48h of transfection. However, there was no significant difference in the expressions of miR-122 between the non-transfection and vector control groups. The proliferation of cells in the anti-miR-122 group was significantly reduced with time after transfection. There were no significant differences in cell proliferation between the three groups at 24 and 48h of transfection, but, at 72 and 96h, proliferation of cells in the anti-miR-122 group was significantly lower when compared to the other two groups. After 48h of transfection, the extent of apoptosis in the anti-miR-122 group was significantly higher than apoptosis in the two control groups. There were no significant differences in the expressions of PI3K, AKT, mTOR protein and the downstream signal proteins (p70S6K and 4E-BP1) in the three groups. However, the expressions of phosphorylated forms of these proteins (p-PI3K, p-AKT, p-mTOR, p-p70S6K and p-4E-BP1) were significantly lower in the anti-miR-122 group than in the non-transfection and vector control groups. These results are in agreement with those of Gong *et al.* (15). Inhibition of the expression of miR-122 in primary cancer cells of the liver inhibits proliferation of hepatocellular carcinoma (HCC), while promoting apoptosis. However, overexpression of miR-122 in HCC enhances proliferation of the cells, while inhibiting apoptosis. It has been reported that NSCLC miR-122 exhibits oncogene characteristics by effectively promoting proliferation and distant metastasis of cancer cells, suggesting that miR-122 might be a target in clinical treatment for NSCLC (16).

The PI3K/AKT pathway is one of the most classical signal transduction pathways known. Studies have shown that PI3K/AKT pathway is closely related to cell proliferation, apoptosis, cancer metastasis and invasion. The phosphorylated form of AKT is often the active form *in vivo*, and it has been shown that p-AKT is overexpressed in a number of malignant tumors, such as colorectal cancer (17), breast cancer (18) and prostate cancer (19), and has an effect on downstream target proteins. However, the role of PI3K/AKT pathway in the overexpression of miR-122 in NPC has remained unclear until now. Lin *et al.* found that paclitaxel could promote the degradation of PI3K protein in CNE2 cells of NPC, while inhibiting p-AKT protein, but had no effect on the expression of AKT (20). The results obtained from this study suggest that miR-122 acts as a cancer gene and exerts its effect through the PI3K/AKT signaling pathway.

This study has demonstrated that inhibition of the expression of miR-122 in NPC cells suppresses proliferation, and promotes apoptosis of the cells through the PI3K/AKT signal transduction pathway.

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Interest conflict
There is no conflict of interest to be declared by the author.

Author's contribution
All work was done by the author(s) named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. Chen Cheng analyzed the data and wrote the text; Wang Xiaohua, Jiang Ning collected the samples; Ji Hong performed the cell and animal experiments; He Xia read and approved the text prior to publication.

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