The mechanism of miR-410-3p and miR-34c in nasopharyngeal carcinoma development and progression

Li Zhu, Zhigang Ni, Kuai Liang, Yan Tang, Min Zhao, Chun Chen, Xiaolin Yuan*

Department of Otorhinolaryngology and Head and Neck Surgery, Chengdu Ren Pin Otorhinolaryngology Hospital, Chengdu, China.

*Correspondence to: yuanxiaolin0322@163.com

Abstract: To investigate the relationship between miR-410-3p, miR-34c and nasopharyngeal carcinoma development, we detected the expression of miR-410-3p and miR-34c in nasopharyngeal carcinoma tissues and evaluate its clinical value as a molecular marker for predicting the prognosis in patients with nasopharyngeal carcinoma through clinical case study. To identify the role and mechanism of miR-410-3p and miR-34c in nasopharyngeal carcinoma development and progression. The expression of miR-410-3p and miR-34c in 300 cases of nasopharyngeal carcinoma tissues and 30 cases of paired adjacent normal breast tissues was detected by RT-qPCR. The paired t-test was used to compare the differences of miR-410-3p and miR-34c levels between the nasopharyngeal carcinoma and normal groups. The Chi-square test was used to compare the differences between miR-410-3p and miR-34c expression and clinicopathological factors. The Kaplan-Meier survival curve was used to analyze the relationship between miR-410-3p and miR-34c expression and 5-year overall survival (OS). The Cox proportional hazards regression model was used to evaluate the prognostic value. The results were validated by TCGA database. The expression of miR-410-3p was down-regulated in nasopharyngeal carcinoma tissues compared with that of paired normal tissues (P<0.001). The patients with lower miR-410-3p expression have a higher ratio of positive lymph node status (P=0.039) and a poorer 5-year disease-free survival (P=0.001) and 5-year overall survival (P = 0.002). The expression of mi R-34c was down-regulated in nasopharyngeal carcinoma tissues compared with that of paired normal tissues (P<0.001). Up-regulation of the miR-34c inhibited the viability of paired normal tissues (P<0.01), but there was no significant change in migration of the paired normal tissues. Downregulation of mi R-34c promoted the viability and migration of paired normal tissues (P<0.05). The expression of miR-410-3p and miR-34c levels are predictors for the OS in patients with nasopharyngeal carcinoma. The expression of miR-410-3p and miR-34c are molecular markers of early nasopharyngeal carcinoma metastasis and an independent prognostic biomarker for patients with nasopharyngeal carcinoma.

Key words: Nasopharyngeal carcinoma; Prognosis; miR-410-3p; miR-34c.

Introduction

Nasopharyngeal carcinoma (Nasopharyngeal carcinoma NPC) is a common malignant tumor(1). The incidence of nasopharyngeal carcinoma in China accounts for about 80% of the whole world, and it is a serious threat to people's life and health in southern China, especially in the vicinity of Guangdong Province. At present, the combination of radiotherapy and chemotherapy is the main treatment method (2). Although early-stage NASopharyngeal cancer can be treated with radiotherapy (RT), the 5-year survival rate has been as high as 95%. However, in clinical practice, most patients have been in the middle and late stage of nasopharyngeal carcinoma due to the hidden onset site and various clinical symptoms. In addition, due to drug resistance and other conditions of the tumor, 19%-29% of patients were accompanied by cervical lymph node and/or distant metastasis after treatment, with significantly increased risk of metastasis and recurrence, and sharply increased mortality(3-7). Due to the limitation of research progress, there is currently a lack of highly effective and low-toxicity targeted drugs and new therapeutic methods, and there are still some patients with nasopharyngeal carcinoma who cannot be cured due to distant metastasis. Therefore, improving the early diagnosis and prognosis of nasopharyngeal carcinoma is an effective way to improve the overall treatment level of nasopharyngeal carcinoma.

In view of the high incidence and poor prognosis of nasopharyngeal carcinoma, recent studies have found that microRNA (miRNA) is involved in the whole regulation of tumor genesis and development (8-11). The miRNA is a class of endogenous, non-coding small RNA consisting of 18~25 nucleotides in length (12). Bruce et al. detected the expression level of miRNA in tumor tissues and found that it could predict the risk of distant metastasis of nasopharyngeal carcinoma (13). MiR-34 is a class of non-conserved miRNAs in progression (14). The human family of miR-34 (miR-34s) includes three homologous genes: miR-34a, miR-34b and miR-34c (15). In normal cells, miR-34a can promote cell senescence, arrest cell cycle in G1 phase and induce cell apoptosis. MiR-34a is also involved in the occurrence of nasopharyngeal cancer, prostate cancer, colon cancer, malignant glioma, pancreatic cancer, lung cancer and other diseases (16-18). We found that miR-34c is involved in the occurrence of common cancers, mainly by promoting tumor cell apoptosis. Inhibit the proliferation and differentiation of tumor cells; Biologi-
eral effects such as inhibition of invasion and migration of tumor cells play a role in tumor inhibition (19-22).

However, there is no report on the function of miR-34c in nasopharyngeal carcinoma at present. Through miRNA sequencing, the results showed that miR-34c was specifically expressed in nasal mucosal carcinoma tissue, suggesting that miR-34c has high clinical value in the treatment of nasopharyngeal carcinoma.

MiR-410-3p was localized in human chromosome 14q32.31 (23). Many studies have confirmed that the expression level of miR-410-3p is significantly different between tumor tissues and paracancerous tissues (24). Through the study of cancer cells in different parts, it was found that their expression levels were significantly different, suggesting that miR-410-3p plays different roles in different types of tumors (25).

To investigate the mechanism, on the one hand, miR-410-3p can directly bind to FHL1 mRNA 3′-UTR to inhibit its expression. On the other hand, miR-410-3p can increase the binding ability of DNMT3a (DNA methyltransferase 3 Alpha) to the FHL1 promoter, leading to DNA methylation in the promoter region and inhibiting the expression of FHL1. In NSCLC, multiple studies have shown that miR-410-3p can affect the Wnt/β-catenin signaling pathway by targeting GSK3β, SLC34A2 and BRD7, and promote the proliferation and invasion of NSCLC and induce stem cell characterization (26,27). However, the role of miR-410-3p in the development and progression of nasopharyngeal carcinoma is still unclear, and its molecular mechanism needs to be further elucidated.

Materials and Methods

All the 30 cases of NASopharyngeal carcinoma tissues and matched para-carcinoma normal tissues were collected from 30 patients with NASopharyngeal carcinoma admitted to the Affiliated Oncology Department of Guangzhou Medical University from January 2018 to June 2021. Paraffin samples were collected from 300 patients with nasopharyngeal carcinoma in the Affiliated Cancer Hospital of Guangzhou Medical University, and the personal information and clinical data were collected. The staging standards of the American Joint Commission for Cancer (AJCC) and the Union for International Cancer Control (UICC) were followed. Tissue samples are stored in liquid nitrogen. All samples were collected and used with the consent of nasopharyngeal carcinoma patients, and the informed consent was signed by the Medical Ethics Committee of Oncology affiliated with Guangzhou Medical University.

Inclusion criteria included 1/ All patients were diagnosed with NPC examination and surgery interval < 1 week; 2/ No preoperative treatment was performed; 3/ Immunohistochemical determination of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 by immunohistochemistry HER2; 4/ The combined treatment of radiotherapy and chemotherapy was performed and specimens were collected. (5) The bed material is complete; (5) Patients or their family members sign informed consent.

Exclusion criteria included 1/ Multiple tumors; 2/ Patients without comprehensive treatment; 3/ Pregnant or lactating women with cognitive dysfunction or mental illness; (4) Patients who fell off, dropped out and lost follow-up in clinical trials.

The Reagent included TaqMan miRNA Assay Kit Applied Biosystems inc, TaqMan miRNA Reverse transcription Kit Applied Biosystems Inc, 10 mirVana miRNA Isolation kit Thermo Scientific Inc and HONE1 cell of nasopharyngeal carcinoma Shanghai Institute of Biological Chemistry and Cell Biology.

The experimental instruments included Axygen, Micropipettes from Gilson, Real-time Fluorescent quantitative PCR instrument CFX96 Touch Bio-rad inc, High-speed cryogenic centrifuge Eppendorf Inc, -80 °C ultra-low temperature refrigerator -20°C low-temperature refrigerator company and Mili-q Ultra pure water Manufacturing system by Millipore Inc

Experimental methods

The extraction of microRNAs

miRNAs were isolated from tissues using the mirVana miRNA Isolation Kit.

[1] For paraffin-embedded specimens, the tissue was removed and put into the centrifuge tube, and an appropriate volume of paraffin-removing solution was added. After the instantaneous centrifugation, the specimens were violently rotated for 10 s, incubated at 56°C for 3 min and cooled for later use.

[2] For fresh tissue specimens or tissue blocks stored at -80 °C, take the appropriate amount of tissue and quickly freeze it in liquid nitrogen, then grind it into powder in a mortar;

[3] The organizations were put into THE EP tube and the 500 µL Lysis/Binding Buffer was added.

[4] A 1/10 volume of miRNA Homogenate Additive was added and incubated on ice for 10 min.

[5] Phenolic chloroform and Lysis/Binding Buffer were added and mixed with scroll and centrifuged at 12000 g for 20 min.


[7] Transfer the liquid to the separation column, centrifuge it at 10000 g for 15 s, and discard the waste liquid;

[8] Add 700 µL miRNA Wash Solution 1 and discard the waste liquid after instantaneous centrifugation;

[9] Add 500 µL miRNA Wash Solution in 2/3, centrifuge the waste liquid, centrifuge at 10000 g for 1 min;

[10] Add 80 µL preheated DEPC water, centrifuge at 10000 g for 30 s, and store at -80°C.

Reverse transcription of miRNA

Reverse transcription was performed using Taqman miRNA Reverse transcription Kit

[1] The corresponding reagent and template RNA were thawed on ice, reversed and centrifuged before use, and mixed;

[2] The corresponding reagent was added to eight rows of tubes in the following volume, and the dosage per tube was as follows (Table 1), and centrifuged for 5 min on ice;

[3] Reverse transcription was performed under the following conditions: 16 °C for 30 min; 42 °C for 30 min. 85 °C for 5 min. 4 °C forever.
miRNA amplification by qPCR

TaqMan miRNA assay kit was used to detect the expression level.

[1] Add the corresponding reagent to the eight-row tube in the following volume (Table 2).

[2] Reverse transcription was performed at 95°C for 10 min. (95°C 15s + 60°C 60s) a total of 40 cycles; 4°C forever.

Detection of cell migration ability

HONE1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/µL penicillin and 100 µg/mL streptomycin in a cell incubator at 37°C and 5% CO2.

The cells were inoculated in 6-well plates, and the transfection agent was added after the cells were attached to the wall. When the cell growth density reached over 90%, the 6-well plates were scratched with a "1" shape with 200 µL sterile gun head, and then the suspended cells were rinsed with PBS immediately, and cultured in RPMI 1640 medium. The cell healing was observed under an inverted fluorescence microscope at 0 h, 24 h, 48 h and 72 h after the scratch, respectively. The images were processed by ImagePro Plus 6.0 and the relative migration distance was recorded.

Detection of cell proliferation

Mir-34c and Mir-410-3pmimics were transfected into HONE1 cells with endogenous low expression of Mir-410-3p and Mir-34c, and the expression of Mir-410-3p and Mir-34c was detected by RT-QPCR. It was determined that the expression of Mir-410-3p and Mir-34c was significantly down-regulated (> 25%), and there was a statistical difference between them (P < 0.001) (Figure 1).

Statistical Analysis

SPSS 20.0 was used as statistical analysis software, and the measurement data conforming to the normal distribution were expressed in the form of mean ± standard deviation, while those not conforming to the normal distribution were expressed in the form of mean (value range). If the variance of the data is uniform, the t-test ratio of paired samples is used to compare the apparent difference of Mir-410-3p and Mir-34c between the normal nasopharyngeal tissues and the paired para-carcinoma normal tissues. If the variance is not uniform, mann-Whitney U test is used for comparison. The subjects worked on the receiver operating characteristic curve (ROC), and the value that maximization detection sensitivity and specificity at the same time was selected as the threshold value for the expression levels of Mir-410-3p and Mir-34c. Nasopharyngeal carcinoma patients were divided into the high expression group of Mir-410-3p and the low expression group of Mir-34c.

Chi-square test was used to investigate the relationship between the expression levels of Mir-410-3p and Mir-34c in nasopharyngeal carcinoma tissues and clinicopathological factors. Kaplan-meier analysis and Log-Rank test were used to compare 5-year disease-free survival and overall survival in nasopharyngeal carcinoma patients with high and low expression of Mir-410-3p and Mir-34c. Univariate and multivariate analyses of Cox proportional risk regression models were used to evaluate mir-410-3p and Mir-34c levels as prognostic factors for nasopharyngeal carcinoma. Test level α=0.05. P < 0.05 was considered statistically significant.

Results

RT - qPCR detection

Both Mir-410-3p and Mir-34c are down-regulated in nasopharyngeal carcinoma tissues. In this study, rT-QPCR was used to detect the expression levels of Mir-410-3p and Mir-34c in 30 primary nasopharyngeal carcinoma tissues and their matching para-carcinoma normal tissues, and it was found that 63.3% of the patients, The expression of Mir-410-3p and Mir-34c was significantly down-regulated (> 25%), and there was a statistical difference between them (P < 0.001) (Figure 1).

The expression levels of Mir-410-3p and Mir-34c in 300 primary nasopharyngeal carcinoma tissues were detected by RT-QPCR. It was determined that the expression of Mir-410-3p and Mir-34c in nasopharyngeal carcinoma tissues was correlated with lymph node status (P = 0.040, P (Mir-34c) = 0.032), and lymph node metastasis was more common in nasopharyngeal carcinoma patients with low expression of Mir-410-3p and Mir-34c. Mir-410-3p and Mir-34c were close to clinical stage with statistical significance (P = 0.056).

Relationship between the expression levels of Mir-410-3p and Mir-34c and prognosis of nasopharyngeal carcinoma patients

Kaplan-meier survival analysis showed the relationship between the expression levels of Mir-410-3p and Mir-34c and the prognosis of patients. As shown in Figure 2 (A and B), the 5-year OS of patients with low

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<tr>
<td>dNTPs</td>
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<td>MultiScribe Reverse Transcriptase (50 U/µl)</td>
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<td>RNase Inhibitor (20 U/µl)</td>
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<td>Nuclease-free water</td>
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<td>TaqMan® Universal Master Mix II</td>
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<td>No AmpErase® UNG (2X)</td>
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Figure 1. Expression levels of Mir-410 and Mir-34c detected by RT-QPCR in 30 primary nasopharyngeal carcinoma tissues and normal adjacent tissues.
expression of Mir-410-3p and Mir-34c was lower than that of patients with high expression of Mir-410-3p (OS Mir-34cP = 0.001; Osmir-410-3p P = 0.002). Through univariate and multivariate analysis, clinical staging (HR = 3.072; 95% CI = 1.671-5.651; P < 0.001), mir-410-3p expression level (HR = 0.461; 95% CI = 0.256-0.829; P = 0.010) expression level of Mir-34c (HR = 0.543; 95% CI = 1.026-3.857; P = 0.032) as an independent prognostic factor.

Liposome transfection regulates the expression of Mir-34c and Mir-410-3p in NASopharyngeal carcinoma

HONE1 cells of nasopharyngeal carcinoma were selected and inoculated into the culture plate. When the degree of cell fusion reached 60% ~ 80%, liposome transfection was carried out according to the specifications of Lipofectamine RNAiMAX Reagent. The experiment was divided into two groups: Mir-34C mimics group (final concentration: 50 nmol/L) and Mir-34c inhibitor group (Mir-34c inhibitor, Mir-410-3p mimics group (Mir-410-3pmimics, final concentration of 50 nmol/L), Mir-410-3p inhibitor group (Mir-410-3pinhibitor, the final concentration was 50 nmol/L) and the control group (CONTROL).

Hsa - mir - 34 c mimics sequence was: AAUACUAACACAGGGCACAG, hsa - mir - R - 34 c inhibitor sequence is: CCUGGCGUUGGUUAGGAUU.

The sequence of hSA-Mir-410-3pmimics was: AAUAUAACACAGGGCACAG, hsa - Mir-410-3 P inhibitor was ACAGGCGCUAGAGUAAUU.

Its expression was measured as follows (Figure 3).

The influence of Mir-34c and Mir-410-3p expression levels on the migration ability of HONE1 cells

The scratch experiment showed that the migration distance of each group increased gradually as time went on. Compared with the 72 h migration and expansion distance of the control group, there was no significant difference in the migration ability of the Mir-34c and Mir-410-3p simulated group and the control group, but the migration ability of the Mir-34c and Mir-410-3p inhibited group was significantly enhanced, indicating that the down-regulation of Mir-34c expression can promote cell migration. The results are shown in Tables 3 and 4 and Figure 4.

Overexpression of Mir-34c and Mir-410-3p inhibited proliferation and invasion of NASopharyngeal carcinoma HONE1 cells.

Mir-34c and Mir-410-3pmimics were transfected into HONE1 cells with endogenous low expression of Mir-34c and Mir-410-3p, and the expression of Mir-34c and Mir-410-3p was detected by RT-QPCR. It was found that the expression of Mir-34c and Mir-410-3p in the experimental group were up-regulated about 11.3 times and 14.2 times respectively. EdU experiment also confirmed that the proportion of positive cells in the overexpression group of Mir-34c and Mir-410-3p was significantly lower than that in the control group. The effects of Mir-34c and Mir-410-3p on the invasion ability of NPC cell HONE1 were further evaluated by Transwell, and the results showed that the number of HONE1 cells overexpressing Mir-34c and Mir-410-3p was significantly reduced through the ma-

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<th>miR-34c Simulation</th>
<th>miR-34c suppression</th>
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<td>38.669±3.118</td>
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<td>72h Migration extension distance</td>
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<td>40.611±3.540</td>
<td>50.662±3.018*</td>
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Table 3. Effect of miR-34c on the migration of nasopharyngeal carcinoma cell line HONE1 cells.

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<th>miR-410-3p suppression</th>
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<td>48h Migration extension distance</td>
<td>26.917±2.132</td>
<td>35.198±2.836</td>
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<tr>
<td>72h Migration extension distance</td>
<td>35.646±3.866</td>
<td>39.611±3.661</td>
<td>51.482±4.127*</td>
</tr>
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</table>

Table 4. Effect of miR-410-3p on the migration of nasopharyngeal carcinoma cell lineHONE1 cells.

Figure 2. Kaplan-Meier survival analysis showed the relationship between the expression levels of Mir-34c and Mir-410-3p and the survival of patients with nasopharyngeal carcinoma. A: the relationship between the expression level of Mir-34c and the 5-year disease-free survival of NPC patients. B: the relationship between the expression level of Mir-410-3p and the 5-year disease-free survival of NPC patients.

Figure 3. Expression of Mir-34c in CHONE1 cell (left) and expression of Mir-410-3p in DHONE1 cell (right) in different times.
miR-410-3p and miR-34c in nasopharyngeal carcinoma.

Figure 5. Overexpression of Mir-410-3p inhibits proliferation and invasion of NASopharyngeal carcinoma cell HONE1. A: Expression levels of Mir-410-3p in NASopharyngeal carcinoma cell line HONE1 were detected by RT-QPCR in mimics transfection group and control group; B: The effects of Mir-410-3p on the proliferation ability of HONE1 cells were detected by EdU assay; C) Transwell was used to detect the effect of Mir-410-3p overexpression on HONE1 invasion ability of NASopharyngeal carcinoma cells. (P < 0.01).

Figure 6. miR-34C overexpression inhibits the proliferation and invasion of NASopharyngeal carcinoma cell HONE1. A: Expression levels of Mir-34c in NASopharyngeal carcinoma cell line HONE1 were detected by RT-QPCR in mimics transfection group and control group; B) The effects of Mir-34c on the proliferation ability of HONE1 cells were detected by EdU assay; C) Transwell was used to detect the effect of mir-34c overexpression on HONE1 invasion ability of NASopharyngeal carcinoma cells. (P < 0.01).

Discussion

In recent years, there has been an explosion of research on miRNAs and oncogenesis and development. Studies have found that miRNAs are involved in the regulation of proliferation, apoptosis, differentiation, invasion, metastasis, angiogenesis and tumor microenvironment of cancer cells, which are closely related to the occurrence, development and drug resistance of cancer cells (28-30). Multiple tests have proved that the abnormal expression of miRNA is closely related to the occurrence and development of nasopharyngeal carcinoma, the degree of malignity, and the sensitivity of treatment, and has the potential as a tumor-related marker (31,32). Tumor angiogenesis is a key process leading to the formation of new blood vessels and plays an important role in tumor growth, invasion and metastasis (1,33-36). Typically, solid tumors larger than 1-2 cubic millimeters in diameter require the formation of new blood vessels to remove metabolic waste and transport nutrients and oxygen, which can allow tiny tumors to develop into an uncontrolled state. Ways to control this complex process have become the focus of innovative therapies.

Recent studies have shown that Mir-410-3p can affect the occurrence and development of multiple tumors by regulating multiple target genes. In neuroblastoma and osteosarcoma, Mir-410-3p can target to inhibit the expression of VEGF and regulate tumor growth and angiogenesis (37). Studies have confirmed that Mir-410-3p is up-regulated in NSCLC, which promotes proliferation and invasion of NSCLC and induces stem cell characteristics through targeted inhibition of GSK3 β, SLC34A2 and BRD7 target genes and activation of Wnt/ β-catenin signaling pathway (27). In addition, Mir-410-3p is the target of LINC 00680, and its main role is to secrete Mir-410-3p in the cytoplasm. In addition, as a downstream target of Mir-410-3p, HMG1 was up-regulated by Mir-410-3p, while LINC 00680 enhanced the expression of HMGB 1 in A 549 and H 1299 cells (38).

The human Mir-34 family includes Mir-34a, Mir-34b and Mir-34c. In normal cells, Mir-34a plays a role in promoting cell senescence, arresting the cell cycle in G1 phase and inducing cell apoptosis (15). Mir-34a is also involved in breast cancer, prostate cancer, colon cancer, glioblastoma, pancreatic cancer and lung cancer and other diseases (19). Mir-34a also has potential application value in prognosis and gene therapy in patients with TSCC lymph node metastasis. Mir-34b and Mir-34c also have potential anti-cancer effects (20). Mir-34c-3p mimics and Mir-34c-5PMimics were transferred to glioma cells, and the results showed that Mir-34c could inhibit the proliferation, migration and invasion of glioma cells, halt the cell cycle in the S phase and G2/M phase, and induce cell apoptosis (39).

In this study, miRNAs and nasopharyngeal carcinoma tissues were studied, and the expression levels of Mir-34c and Mir-410-3p in nasopharyngeal carcinoma patients were detected by RT-QPCR. Chi-square test, Kaplan-Meier survival curve and Log-rank were used
to investigate the survival rate of patients after treatment, and data analysis was conducted. Cox proportional risk regression model was used to analyze the prognostic value of Mir-410-3p and Mir-34c as molecular markers, and finally, transfection test was performed. In this study, the effects of Mir-34c and Mir-410-3p on the biological behavior of nasopharyngeal carcinoma cell HONE1 were investigated by in vitro transfection of Mir-34c and Mir-410-3p. The experimental results of this study showed that the number of HONE1 cells that overexpressed Mir-34c and Mir-410-3p crossed the matrix glue was significantly reduced, which proved that Mir-34c and Mir-410-3p could inhibit the proliferation and migration of HONE1 cells in nasopharyngeal carcinoma. The high expression of Mir-34c and Mir-410-3p can inhibit cell proliferation and migration, and promote cell apoptosis. Apoptosis is an important physiological process to maintain cell proliferation homeostasis and prevent unrestricted cell division. However, mir-34c and Mir-410-3p were found to be down-regulated in a variety of tumors, suggesting that Mir-34c has a potential anti-cancer effect.

Due to the characteristics of miRNA, it is a new strategy for tumor therapy to inhibit tumors by regulating miRNA expression. MiRNA-based gene therapy has entered a new stage and has great prospects for application (40). When the expression of miR is inhibited or down-regulated, the expression of miR can be increased through the synthesis of miR analogues. Or when abnormal expression of miR leads to pathological changes, antisense oligonucleotides can be used to directly bind to miR and block its activity, thus playing a therapeutic role (41).

Limited by the experimental level, the target of Mir-34c and Mir-410-3p on nasopharyngeal carcinoma cells has not yet been explored (42-47). Therefore, further exploration and verification will be conducted in the future for the clinical use of Mir-34c and Mir-410-3p as markers of nasopharyngeal carcinoma.

Acknowledgements
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References