Oncology and Translational Medicine
DOI 10.1007/s10330-020-0438-8
February 2021, Vol. 7, No. 1, P25–P30

ORIGINAL ARTICLE

Expression of mir-34c-5p and mir-150-5p in nasopharyngeal carcinoma and up-regulated expression after invasion and apoptosis of nasopharyngeal carcinoma cells*

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Abstract

Objective MiRNAs are closely related to tumors, and we hypothesized there is specific miR expression in nasopharyngeal carcinoma (NPC). We intended to investigate the expression of mir-34c-5p and mir-150-5p in NPC and to investigate the effects of mir-34c-5p and mir-150-5p on apoptosis and invasion following up-regulated expression in HNE1 NPC cells.

Methods MiR-34c-5p and miR-150-5p expression levels in 30 individual cases of NPC and nasopharyngitis were detected with gene chip and qRT-PCR techniques. miR-34c-5p and miR-150-5p were transfected into the NPC cell line HNE1 via liposomes. Their expression levels were detected with qRT-PCR, apoptosis was evaluated by flow cytometry, and invasion ability was assessed via Transwell migration assay.

Results MiR-150-5p expression levels in NPC and nasopharyngitis were 0.165 ± 0.092 and 1.062 ± 0.280 respectively, and miR-34c-5p expression levels in NPC and nasopharyngitis were 0.417 ± 0.220 and 1.385 ± 0.739, respectively, which indicated miR-34c-5p and miR-150-5p were weakly expressed in NPC. Apoptosis rates in HNE1 cells transfected by miR-34c-5p and miR-150-5p were increased, by 12.7% and 7.6%, respectively, which were significantly higher compared to blank control (3.9%). The Transwell assay demonstrated that invasive HNE1 cell counts were 32.00 ± 2.00 and 28.33 ± 2.08, respectively, compared to 60.66 ± 8.50 in the blank control ($P < 0.001$).

Conclusion MiR-34c-5p and miR-150-5p are lowly expressed in NPC, and their down-regulation may be associated with NPC.

Received: 18 June 2020
Revised: 10 July 2020
Accepted: 25 July 2020

Key words: miR-150-5p; miR-34c-5p; nasopharyngeal carcinoma; apoptosis; invasion

Nasopharyngeal carcinoma (NPC) is a common malignancy mostly seen in southern China. Many years of research have found that although the factors of heredity, region, and EB virus exposure are implicated in its etiology, the most distinguishing feature is that its occurrence has a particular regionalism; it is also called "canton tumor," the only malignant carcinoma named after a region. This phenomenon indicates that genetic and environmental regulations together are closely associated with its occurrence. Regarding regulation, the relationship between micro RNA and tumor has received increased attention. Related studies report that miR-34c-5p and miR-150-5p are lowly expressed in many types of tumors and are closely associated with tumor progression and invasive activities, representing potential targets for tumor therapy [1–2]. The expression of miR-34c-5p and miR-150-5p in NPC and related cytobiological activities after reverse-regulation in the cultured NPC cell line

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* Supported by grants from the National Natural Science foundation of China (No. 81373698), Joint Special Scientific Research Project of the Department of Science and Technology of Guangdong Province-Guangdong Provincial Academy of Chinese Medical Sciences (No. 2017A020213020); Zhongshan Key Scientific and Technological Project (No. 2015B1003, 2019B1096).

HNE1 are yet to be studied, which provided the basis for the present study.

Materials and methods

Clinical materials

Thirty cases of NPC from outpatients of the Zhongshan Hospital, affiliated with Guangzhou University of Chinese Medicine, from between May 2014 and October 2016 were sampled, comprising 18 men and 12 women, ages from 22 to 64 years, with an average age of 46.67 ± 11.17 years. The clinical stages of NPC included 10 cases of stage II, 13 cases of stage III, and 7 cases of stage IV. There were 20 cases of differentiated nonkeratinizing carcinoma and 10 cases of undifferentiated carcinoma in pathological categorization. Thirty cases of nasopharyngitis were used as a control group, including 17 men and 13 women, ages from 20 to 55 years, with an average age of 34.17 ± 11.25 years.

Experimental materials

The NPC cell line HNE1 was purchased from American Type Culture Collection (ATCC).

Main reagents and apparatus

Experimental reagents

The miRCURY LNA™ microRNA Array (Exiqon, Denmark) was used. Three thousand and one hundred capture probes were employed, covering human, mouse, and rat microRNA in miRBase, as well as viral microRNA related to these species. Other reagents included the miRCURYTM Array Power Labeling kit (Cat #208032-A, Exiqon), 2X PCR master mix (Arraystar), RPMI1640 basic culture medium (Hyclone Co., cat: SH30022.03B), fetal bovine serum (FBS, GIBCO Co., cat: 16400-044), bispecific antibody (Prospec Co., cat: SV30010), Lipofectamine 2000(Invitrogen Co., cat: 11668-027), and real-time PCR kit (Thermo Co., cat: 11762-100), TRIZOL (Invitrogen Co., cat: 15596-026), and primer synthesis was done by Shanghai Sangon Biotech Co.

Experimental apparatus

An axon GenePix 4000B microarray scanner, real-time fluorescent quantification PCR cycler (Eppendorf Co., lot No.:X226488N), primer design software Primer 5.0, CO_2 incubator (Shanghai Boxun Biotech Co., cat:BC-J160S), sterile bench (Shang Boxun Biotech Co., type:SW-CJ-2FD), transwell chamber (Costar Co., cat: 3422), and fluorescence microscope (Olympus IX71) were employed in this study.

Experimental methods

Determination of miR-34c-5p and miR-150-5p expression in NPCs via gene chip technique.

RNA was extracted from 100 mg of -80°C frozen fragmented nasopharynx tissues by Trizol, including 6 cases each of NPC and nasopharyngitis.

RNA was labeled with the miRCURY™ Array Power Labeling kit after quality control by gel electrophoresis detection. Labeled RNA samples were hybridized to the microarray with the miRCURY LNA™ microRNA Array and washed with buffer kit.

The microarrays were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA), and results were saved after being transformed into data; then, the original data were analyzed with corresponding software.

Determination of miR-34c-5p and miR-150-5p expression in NPC by RT-PCR.

RNA was extracted from tissues of 30 patients with NPC and 30 patients with nasopharyngitis, and quality was controlled via gel electrophoresis detection. For cDNA sample synthesis, a compound RT mixed reaction solution, which included 5 μL of 2x Master Mix, 0.5 μL of 10 uM PCR specific primer F, 0.5 μL of 10 uM PCR specific primer R, and water to a total volume of 8 μL. cDNA samples of 2 μL were amplified by mixing with the reaction solution, then running 40 PCR cycles (95°C for 10 s, 60°C for 60 s). PCR primer sequences were listed below, Table 1.

miRNA transfection into NPC cell line HNE1

Cells were digested and seeded one day before transfection. Into each of two centrifuge tubes (No.1 and No.2), 100 μL of RPMI-1640 was added. With the pipette tip well below the liquid level, 3 μL of lipofectamine 2000 was added into tube 1 and 1 μg of DNA (100 pmol siRNA) into tube 2. Both were homogenized by flicking, and placed at 25°C for 5 min. The liposome dilution in tube 1 was transferred into tube 2 with the pipette tip well below the liquid level; then, the mixture was homogenized by flicking and placed at room temperature for 20 min. RPMI-1640 (0.8 mL/well) was added to HNE1 cells for transfection. Blank and negative controls were made for siRNA transfection. Next, 0.2 mL of DNA-liposome (siRNA-liposome) mixture was dripped into culture plates. They were then homogenized and incubated in an incubator at 37°C with 5% CO_2. Five hours later, the culture solution was changed to 10% FBS-RPMI-1640, and the culture continued for 48 h. After 48 h of transfection, RNA was routinely extracted using Trizol reagent. RNA reverse transcription (37°C for 60 min, 65°C for 20 min) and RT-PCR was performed (hU6 was used as the internal standard, the primer design software was Primer5, and primer sequences of all target genes were listed below, Table 1).

Table 1 Primer sequences for all target genes

miRNA mimics	Sequence
hsa-miR-150-5p mimics-Sense	5'-UCUCCCAACCCUUGUACCAGUG-3'
hsa-miR-150-5p mimics-anti sense	5'-CUGGUACAAGGGUUGGGAGAUU-3'
hsa-miR-34c-5p mimics- Sense	5'-AGGCAGUGUAGUUAGCUGAUUGC-3'
hsa-miR-34c-5p mimics-anti sense	5'-AAUCAGCUAACUACACUGCCUUU-3'
Mimics-sense	5'-UCACAACCUCCUAGAAAGAGUAGA-3'
Mimics-anti sense	5'-UACUCUUUCUAGGAGGUUGUGAUU-3'
RT primer	
hsa-miR-150-5p	TGCTCCAACCCTTGTACCAGTG
hsa-miR-34c-5p	TGCTAGTGTAGTTAGCTGATTGC
hU6	CGCAAGGATGACACGCAAATTC

Determination of apoptosis by flow cytometry

HNE1 cells were classified into the following groups: hsa-miR-150-5p mimics transfection group, hsa-miR-34c-5p mimics transfection group, negative control transfection group, and normal control group. At 48 h after transfection, the supernatant was discarded. The cell pellet was washed twice with PBS solution and digested with trypsin. Then, 500 μL of AnnexinV binding buffer was added to prepare the cell suspension, 5 μL of AnnexinV-FITC was added, and the mixture homogenized. Next, 5 μL of propidium iodide was added and the mixture homogenized and placed at room temperature in darkness for 10 min. Apoptosis was examined by flow cytometry (Ex = 488 nm; Em = 530 nm).

Determination of cell invasion ability by Transwell assay

HNE1 cells were classified into the following groups: hsa-miR-150-5p mimics transfection group, hsa-miR-34c-5p mimics transfection group, negative control transfection group, and normal control group. They were digested and passaged with routine methods. Cell suspension was prepared, cell counting was performed, and cell concentration was adjusted to 2×10^5/mL. Next, 100 μL of diluted Matrigel was perpendicularly added to the bottom center of the upper chamber and incubated at 37°C for 4–5 h to form a gel. Afterwards, 100 μL of cell suspension was pipetted into the chamber and 600-800 μL of 10% serum culture medium was added into the lower chamber (the bottom of 24-well plate), and the culture continued for 48 h. Then the chamber was taken out, washed twice with PBS solution, and transferred into 100% methanol to fix at room temperature for 20 min. Then it was transferred into 800 μL of Giemsa solution to stain at room temperature for 15–30 min, and washed with PBS. With a wet cotton swab, cells were carefully removed from Matrigel and the surface of the bottom membrane in the upper chamber. After the chamber was completely dry, counting and photography were performed under a fluorescent inverted microscope.

Statistical analysis

Statistical analyses and plotting were performed with GraphPad prism 5.0. Data were processed with MS EXCEL. Relative quantification was used to analyze RT-PCR results, and relative expression levels were calculated with $RQ=2^{-\Delta\Delta CT}$. Expression of mir-34c-5p and mir-150-5p are expressed as mean ± standard deviation, and Student's *t*-test was performed for two-group comparisons. The data on apoptosis ratio were analyzed by non-parametric statistical methods, while the numbers on cell invasion were analyzed with a variance analysis test. $P < 0.05$ indicated statistical significance.

Results

Expression of miR-150-5p and miR-34c-5p in NPC

The original data were preprocessed and homogenized, and the results of microRNA Array chip detection indicated certain characteristic microRNA expression profiles in NPC. miR-34c-5p expression levels in nasopharyngitis and NPC were 1.099 ± 1.015 and 0.361 ± 0.659, respectively, and miR-150-5p expression levels in nasopharyngitis and NPC were 5.884 ± 4.374 and 2.236 ± 1.708, respectively, which indicated miR-34c-5p and miR-150-5p were weakly expressed in NPC.

RT-PCR detection of microRNA expression levels are displayed in Table 2. Statistical testing indicated miR-150-5p and miR-34c-5p were weakly expressed in NPC, and the expression levels were significantly lower compared to nasopharyngitis, with $t = 16.673$, $P < 0.01$ and $t = 6.874$, $P < 0.01$, respectively. Both gene chip detection and RT-PCR amplification results indicated miR-150-5p and miR-34c-5p were weakly expressed in NPC.

Transfection and amplification of NPC cell line HNE1

Expression levels were elevated in HNE1 cells

Table 2 Expression of miR-150-5p and miR-34c-5p in NPC by RT-PCR

Groups	*n*	miR-150-5p	miR-150-5p
NPC	30	0.165 ± 0.092	0.417 ± 0.220
nasopharyngitis	30	1.062 ± 0.280	1.385 ± 0.739

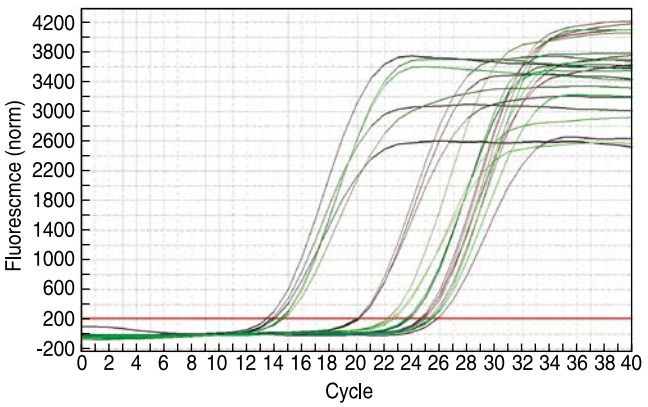

Fig. 1 Amplification curves of the NPC cell line HNE1 when transfected with hsa-miR-150-5p and hsa-miR-34c-5p

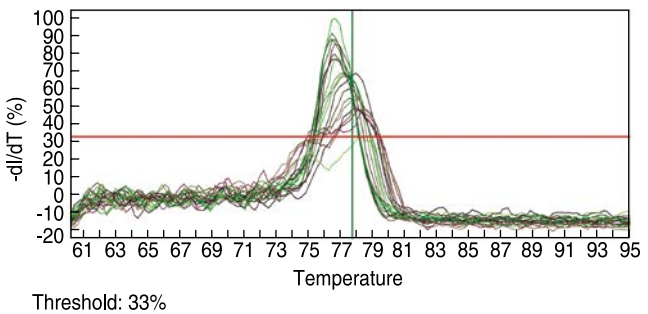

Fig. 2 Solubility curves of the NPC cell line HNE1 when transfected with hsa-miR-150-5p and hsa-miR-34c-5p

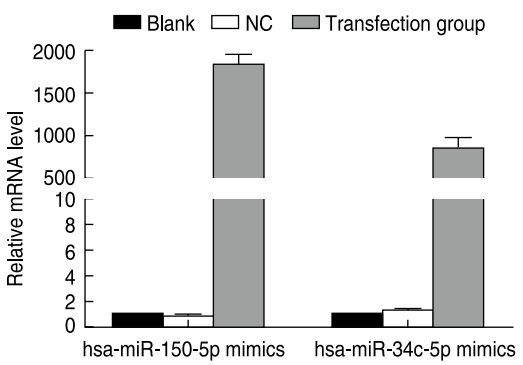

Fig. 3 Expression levels of the NPC cell line HNE1 when transfected with hsa-miR-150-5p and hsa-miR-34c-5p

transfected with hsa-miR-150-5p mimics and hsa-miR-34c-5p mimics (Fig. 1–3).

Apoptosis analysis by flow cytometry

After transfection, the average apoptosis rates in HNE1 cells of the-miR-34c-5p mimics (12.7%) and hsa-miR-150-5p mimics (7.6%) groups were significantly higher compared to the blank control (3.9%) and negative control (5.7%) groups (Fig. 4).

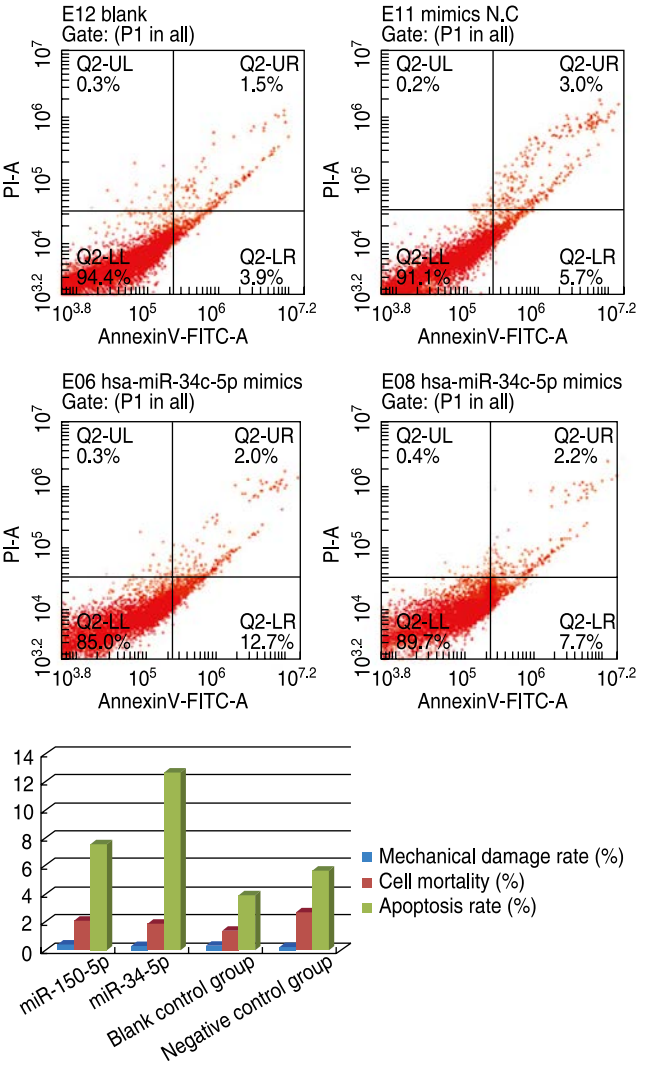

Fig. 4 Photographs of HNE1 cell apoptosis after up-regulation of hsa-miR-34c-5p and hsa-miR-150-5p

Transwell cell invasion test

A Transwell cell invasion test showed that migration (invasion) counts of HNE1 cells in the miR-150-5p mimics and miR-34c-5p mimics groups were 32.00 ± 2.00 and 28.33 ± 2.08, respectively, while they were 60.66 ± 8.50 and 56.6 ± 12.09 in the blank control and negative control groups, respectively ($F = 14.572$, $P < 0.001$). There was a significant difference when the miR-150-5p mimics and miR-34c-5p mimics groups were compared with the blank control and negative control groups ($P < 0.01$). There was no significant difference between either the miR-150-5p mimics and miR-34c-5p mimics groups or between the blank control and negative control groups (Fig. 5).

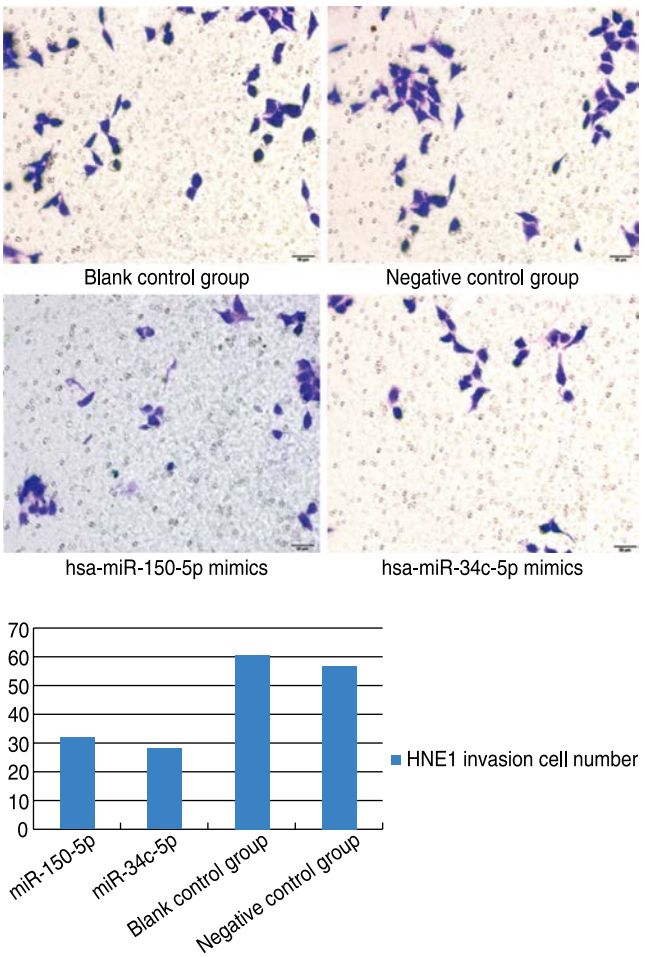

Fig. 5 Photographs of HNE1 cell invasion after up-regulation of hsa-miR-34c-5p and hsa-miR-150-5p

Discussion

MicroRNAs (miRNAs) are a group of endogenous and highly conserved non-encoding RNAs that regulate the expression of related target genes by complementary combination with the 3′ non-translated region of the genes. Because miRNA regulation is closely associated with cancer, it has become a hotspot in cancer research. Among the many miRNAs associated with cancer, down-regulation of miR-150-5p and miR-34c-5p has been associated with various types of tumors. For example, over-expression of miR-34c-5p may block the cell cycle at the G1 phase and inhibit the proliferation and metastasis of cervical carcinoma [3], inhibit MAPT gene expression in gastric cancer tissues and promote apoptosis of cancer cells [4], and regulate the KITLG gene to inhibit the proliferation and metastasis of colon cancer cells [5], while miR-150-5p can inhibit the proliferation and invasion of liver cancer cells [6] and Mir-150-5p inhibits the proliferation and migration of glioma cells by targeting matrix metalloproteinases [7]; these studies all show that Mir-34C-5p and Mir-150-5p are closely related to tumors. Thus, further study is warranted to uncover whether miR-34c-5p and miR-150-5p are related to NPC.

Our study on gene chip detection indicated certain characteristic microRNA expression profiles in NPC, among which miR-34c-5p and miR-150-5p were significantly downregulated in NPC. Thus, we selected miR-34c-5p and miR-150-5p for further study in NPC. First, we started with enlarging the number of clinical samples to 30 cases of NPC and nasopharyngitis each, and RT-PCR amplification result for those samples indicated miR-150-5p and miR-34c-5p were down-regulated in NPC, and the difference was statistically significant. Then, interference experiments on miR-150-5p and miR-34c-5p were performed using the NPC cell line HNE1, and the results indicated that after up-regulation of miR-150-5p and miR-34c-5p in the HNE1 cell line by transfection, apoptosis rates of HNE1 cells as examined by flow cytometry were significantly increased. A Transwell invasion test indicted that the invasion ability of NPC HNE1 cells was significantly decreased, demonstrating that decreased expression of miR-34c-5p and miR-150-5p was associated with malignant changes in NPC cells. Other studies [8] have indicated similar cytobiological activities of miR-34c-5p in NPC HONE1 cells, which provides corroborative evidence to our conclusions.

The present study reveals the relationship between down-regulation of miR-150-5p and miR-34c-5p and NPC, but additional research is needed to clarify the related mechanism and its relevance in the molecular diagnosis of NPC.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-020-0438-8
Cite this article as: Zhou XJ, Lin BR, Wang Y. Expression of mir-34c-5p and mir-150-5p in nasopharyngeal carcinoma and up-regulated expression after invasion and apoptosis of nasopharyngeal carcinoma cells. Oncol Transl Med, 2021, 7: 25–30.