Int J Clin Exp Pathol 2016;9(9):8811-8816
www.ijcep.com /ISSN:1936-2625/IJCEP0027948

Review Article

Regulation of nasopharyngeal carcinoma cell proliferation by targeting Notch1 with miR-34a

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Received March 11, 2016; Accepted July 12, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Nasopharyngeal carcinoma (NPC) has the highest incidence rate of head and neck cancer and a strong attack capability. miR-34a, a member of microRNA family, has been shown to participate in the regulation of NPC proliferation through Notch1 signaling pathway. Therefore, the regulation of NPC cell proliferation by targeting Notch1 with miR-34a will be investigated in this study. qRT-PCR was used to detect the expression of miR-34a in NPC. Bioinformatics analysis was used to predict the possible targets of miR-34a which were verified by luciferase assay. Mimics-34a was designed according to miR-34a sequence and transfected into NPC cell line CNE1. Western-Blot analysis was used to detect the expression of Notch1 and flow cytometry was used to detect cell cycle distribution in order to analyze the changes in cell proliferation capability. RT-PCR analysis showed that the expression of miR-34a was significantly decreased in NPC cells compared to that in adjacent tissues ($P<0.05$). Bioinformatics analysis and luciferase reporter gene assay confirmed that Notch1 is one of miR-34a's potential targets. Overexpression of miR-34a resulted in decrease of the expression levels of Notch1, reduction of the number of cells in s phase ($P<0.05$), elongation of cell cycle, and decrease of cell proliferation. In conclusion, the expression of miR-34a is down-regulated in NPC, and miR-34a can inhibit the proliferation of NPC cells by targeting and reducing the expression of Notch1.

Keywords: miR-34a, Notch1, nasopharyngeal carcinoma, cell proliferation

Introduction

Nasopharyngeal carcinoma (NPC) has the highest incidence of head and neck cancer and a strong attack capability. The incidence of NPC is low and generally below 1/10 million worldwide. However, in China, NPC has a higher incidence and ranks the first among malignant tumors in otorhinolaryngology. The incidence of NPC can reach 30-50/100,000 in Guangdong Province of southern China [1]. It is believed that the occurrence of NPC is related to a variety of factors, including genetic factors, environmental factors, viral infections, and other factors [2]. NPC is highly malignant and the 5-year survival rate of patients with advanced diseases was only 8% to 10%. However, if treated at an early stage, the 5-year survival rate can reach 70% [3]. Therefore, in-depth study on NPC cell behavior and its regulation mechanism are of great significance for the diagnosis and treatment of NPC.

miR-34a, a family member of microRNA, can modulate the expression of its target genes to affect a variety of cellular behaviors [4]. In NPC and other malignant tumor cells, the expression level of miR-34a was significantly reduced, suggesting that miR-34a may be closely related with the development and progression of NPC [5]. Studies showed that miR-34a exerts its physiological role by affecting Notch1 signaling pathway [6]. Notch1 signaling pathway plays a role in cell differentiation, apoptosis, cell proliferation, and boundary formation. It is also closely linked with the occurrence and regulation of a variety of cancers [7-9]. This study investigates the effect of miR-34a on Notch1 signaling pathway and regulation of NPC cells proliferation.

Materials and methods

Subjects selection

Eleven patients diagnosed with NPC in the Second Affiliated Hospital of Fujian Medical University from February 2015 to December 2015 were included in this study. There were 7

Table 1. Primer sequences used qRT-PCR

Name	Sequence	Tm (°C)
miR-34a-F	5'TGTGAGTGTTTCTTTGGCAGTG3'	55.7
miR-34a-R	5'ACAACGTGCAGCACTTCTAG3'	54.2
U6-F	5'CTCGCTTCGGCAGCACA3'	55.3
U6-R	5'AACGCTTCACGAATTTGCGT3'	55.4

males and 4 females with a mean age of 42.1 ± 6.5. All patients were newly diagnosed and have not been treated with chemotherapy or surgery. Cancerous tissues and adjacent tissues were removed from patients and kept frozen in liquid nitrogen for later use. This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Fujian Medical University and informed consents have been obtained from all subjects.

qRT-PCR

qRT-PCR was used to detect the expression of miR-34a in both NPC tissue and adjacent tissue. PCR primers were designed for RT-PCR amplification according to miR-34a sequence (GeneBank accession number NR_029610) (**Table 1**). Total RNA was extracted from frozen tissue using RNAprep Pure Tissue Kit (QIAGEN). mirVanat qRT-PCR miRNA Detection kit (Ambion) was used to perform RT-PCR at 95°C/3 min, 95°C/15 sec, 60°C/30 s, 40 cycles. U6-RNA was used as the reference gene and $2^{-\Delta\Delta Ct}$ was used to analyze the data [10].

Prediction of miR-34a function

Bioinformatics software TargetScan Release 5.1 (www.targetscan.org) was used to predict miR-34a function. Luciferase reporter gene assay was used to verify miR-34a targets. The region of 155-206 of 3'UTR, which might contain possible targets of miR-34a, was chosen to synthesize oligonucleotides of Notch1-3'UTR, according to Notch1 mRNA sequence (Genebank accession number NM_017617). Notch1-3'UTR: 5'-GTCACTAGTATGTACTTTTATTT-TACACAG-AAACACTGCC TTTTTATTTATATGTACT-GAAGCTTCTC-3'. The Notch1-3'UTR sequence was inserted into downstream of the coding region of firefly luciferase gene within pmirGLO vector to construct pmirGLO-notch1 vector. NPC cells were transfected with either pmirGLO-notch1 or pmirGLO first, followed by transferring mimics-34a to enhance the activity of miR-34a. Successfully transfected cells were cultured 48h and then analyzed for fluorescence intensity using a dual luciferase reporter assay system (Promega) and MicroLumat Plus LB96V photometer (Berthold). PmirGLO vector transfected cells were used as a control to calculate the relative fluorescence intensity.

Overexpression of miR-34a

Mimics-34a was designed according to miR-34a sequence and synthesized by Shanghai Biological Engineering Co. Liposome transfection kit (Polyplus transfection) and Interfer in TM (50 nM) were used to perform cell transfection experiments. NPC cell line CNE1 was purchased from the cell bank of Chinese Academy. Frozen cells were cultured until reaching logarithmic growth phase, trypsinized, counted, diluted with fresh medium, and inoculated to 96 well plates. Transfection experiments were performed 24 h after inoculation according to manufacturers' instructions [11].

Western blot

CNE1 cells were collected, homogenized with 100 uL cell lysis buffer in a glass homogenizer, and centrifuged at 13000 g for 10 min. Supernatant was used for Western-Blot analysis. 15% gel was used for SDS-PAGE electrophoresis. Protein was transferred to PVDF membrane. Membranes were blocked with 5% skim milk for 1 h, washed with TBST, and incubated with mouse anti-human Notch1, GAPDH antibodies (1:2000 dilution) at 4°C overnight. Membranes were washed with TBST, incubated with horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (1:1000 dilution) at room temperature for 1 h, washed with TBST, incubated with freshly prepared DAB at dark for 10 min, washed with distilled water to stop color development [12]. Gel imaging analysis system was used to analyze the Western-Blot image. GAPDH was used as an internal reference.

Flow cytometry analysis

Flow cytometry was used to detect the effect of transfection of pcDNA3-miR-34a on the proliferation of CNE1 cells. Cells at logarithmic phase were pelleted, washed with PBS, fixed

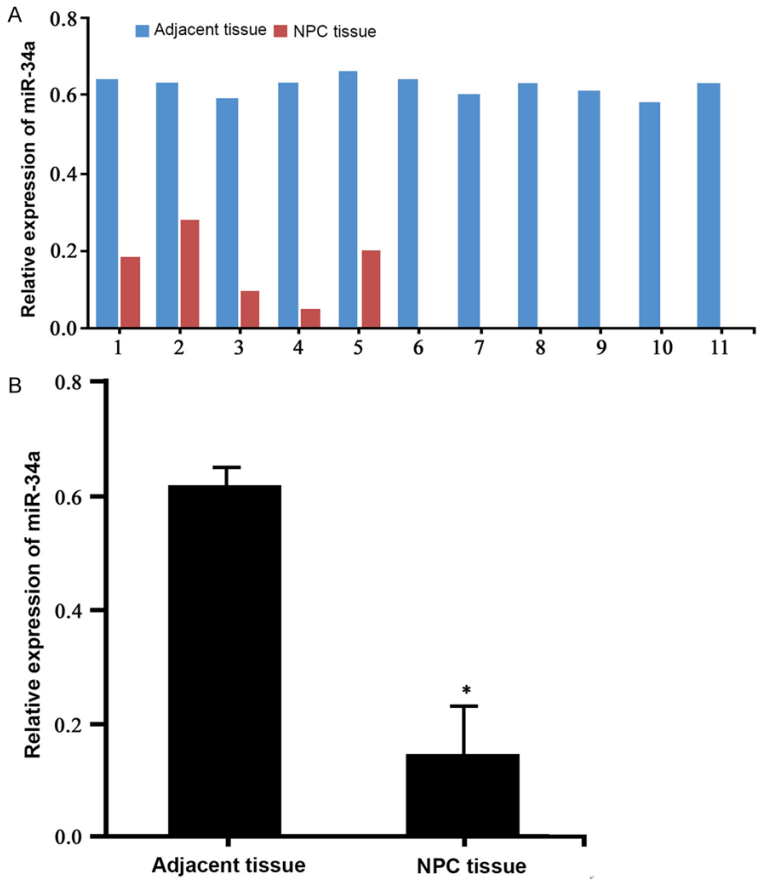

Figure 1. A. Expression of miR-34a in different NPC tissues. RNA was extracted from NPC tissue or adjacent tissue followed by analysis of the expression of miR-34a by qRT-PCR. B. Average expression level of miR-34a in both adjacent tissue and NPC tissue. RNA was extracted from NPC tissue or adjacent tissue followed by analysis of the expression of miR-34a by qRT-PCR. Note: *P<0.05 compared to adjacent tissues.

Figure 2. Sequence homology analysis of Notch1 3'UTR and miR-34a.

with 90% ethanol at 4°C overnight. Untreated CNE1 was used as control. After removal of ethanol, RNase was added and incubated at 37°C for 30 min. Propidium iodide (PI) was used to stain cells. Flow cytometer (Becton Dickinson) with argon ion excitation was used to detect fluorescence. Excitation wavelength was 488 nm, and emission wavelength was 630 nm. 10000 fluorescence signals were collected for each sample. Modifit software was used to analyze the FL-2 area and DNA histograms. All experiments were repeated three times [13].

Statistical analysis

The results were expressed as mean ± standard deviation. T test was performed using SPSS 20.0. P<0.05 indicates significant difference.

Results

RT-PCR results

RT-PCR was used to detect the expression of miR-34a in NPC tissues and adjacent tissues. The relative expression of miR-34a was shown in **Figure 1**. Compared with the corresponding adjacent tissues, the expression of miR-34a from different NPC tissues was reduced. By calculating the average expression of miR-34a in adjacent tissue and NPC tissue, we found that the relative expression of miR-34a in NPC tissue (0.16 ± 0.09) was decreased by 76.6 ± 11.5% as compared to that in adjacent tissues (0.62 ± 0.03) (P<0.05).

Prediction of miR-34a targets

Target Scan Release 5.1 software (www.targetscan.org) was used to predict miR-34a function. Notch1 was found to be a potential target for miR-34a. Sequence homology analysis was shown in **Figure 2** and certain homology was found between 3'UTR region of Notch1 mRNA and miR-34a sequence.

Cell transfection and overexpression of miR-34a

Mimics-34a was transfected into CNE1 cells. Transfected cells were cultured until reaching logarithmic phase, and Western-Blot assay was used to measure the relative expression of Notch1 protein (**Figure 3**). The result showed that cells transfected with mimics-34a have reduced Notch1 protein expression (0.28 ± 0.06) compared to blank control (0.91 ± 0.16)

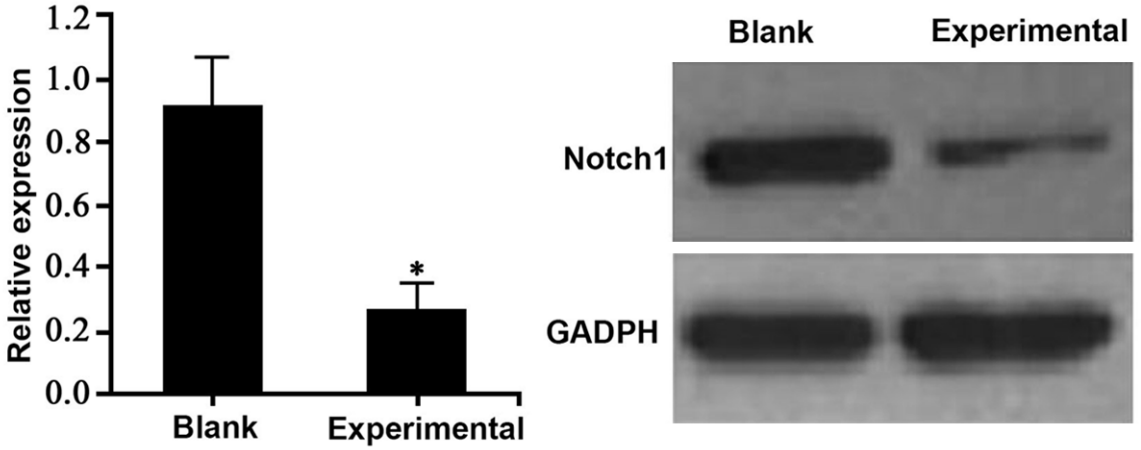

Figure 3. Effect of over-expression of miR-34a on expression levels of Notch1. NPC cell line CNE1 was transfected with Mimics-34a using Liposome transfection kit. Then cells were collected to extract RNA and protein for analysis of mRNA and protein expression of Notch1 by qRT-PCR and western blot.

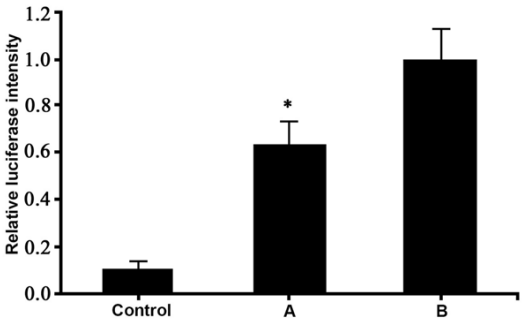

Figure 4. Luciferase assay. Note: A: CNE1 cells transfected with pmirGLO-notch1 + mimics-34a; B: CNE1 cells transfected with pmirGLO + mimics-34a; *P<0.05 compared with group B.

(P<0.05), indicating that overexpression of miR-34a in NPC cell line CNE1 decreased the expression levels of Notch1 protein.

Luciferase assay

Luciferase reporter gene assay was designed according to bioinformatics analysis results (**Figure 4**). The **Figure 4** showed that, compared to CNE1 cell transfected with pmirGLO + mimics-34a (1.03 ± 0.15), CNE1 cells transfected with pmirGLO-notch1 + mimics-34a had decreased fluorescence intensity (0.62 ± 0.11) (P<0.05), indicating that CNE1 cell transfected with pmirGLO-notch1 + mimics-34a had lower levels of luciferase, which suggested that miR-34a could bind to 3'UTR region of Notch1 gene.

Flow cytometry measurement of cell proliferation

Flow cytometry was used to measure the proliferation of CNE1 cells transfected with mimics-34a. Modifit software was used to analyze sample's FL-2 area and the DNA histograms (**Figure 5A**). Cell numbers of different cell cycles were recorded (**Figure 5B**). The result showed that, compared with the control group (35.6 ± 7.2%), CNE1 cells transfected with mimics-34a had less cells in S phase (17.1 ± 4.5%) (P<0.05), however, cells in the G0/G1 and G2/M phase did not change significantly (P>0.05), indicating that CNE1 cells transfected with mimics-34a had prolonged cell cycle and lower proliferation capability.

Discussion

NPC has the highest incidence rate of head and neck cancer and a strong attack capability. Studies confirmed that there is a big difference between the expression profiles of cellular microRNA in normal cells and NPC cells, including miR-26a, miR-144, miR-218 and miR-34a, etc. [14-16]. In this study, the expression level of miR-34a was found to be significantly decreased in NPC tissues by qRT-PCR assay, suggesting that miR-34a may play an important role in the development of NPC. Recent studies showed that miR-34a is the target of tumor suppressor gene P53. It can arrest cells in G1 phase and induce apoptosis [17]. Long et al.

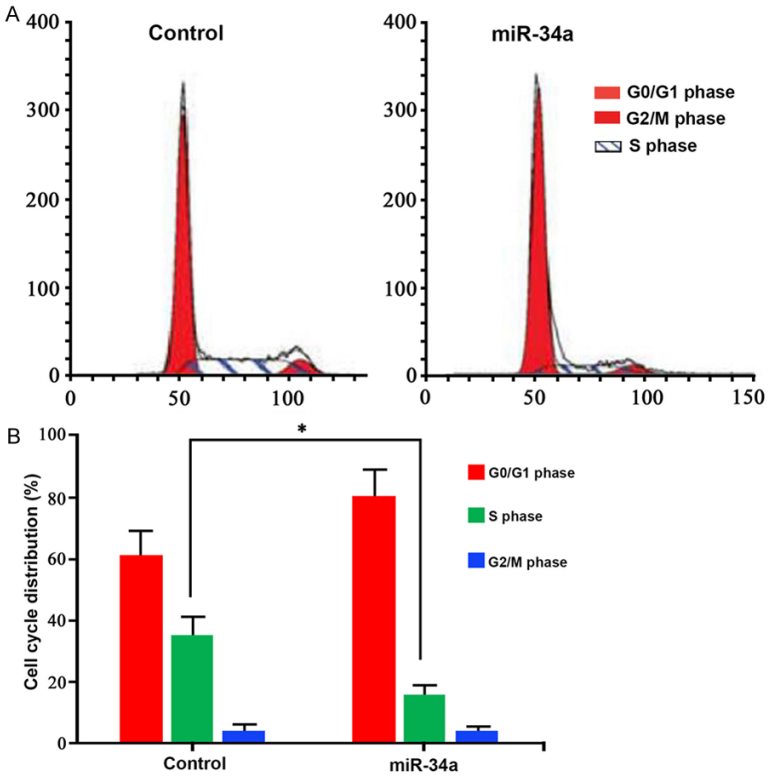

Figure 5. A. Cell cycle of CNE1. Cells at logarithmic phase were pelleted, washed with PBS, fixed with 90% ethanol at 4°C overnight. Untreated CNE1 was used as control. After removal of ethanol, RNase was added and incubated at 37°C for 30 min. Propidium iodide (PI) was used to stain cells followed by flow cytometer analysis of cell cycle. B. Cell cycle changes after overexpression of miR-34a. Cells with miR-34a overexpression at logarithmic phase were pelleted, washed with PBS, fixed with 90% ethanol at 4°C overnight. Untreated CNE1 was used as control. After removal of ethanol, RNase was added and incubated at 37°C for 30 min. Propidium iodide (PI) was used to stain cells followed by flow cytometer analysis of cell cycle. Note: *P<0.05 compared to control group.

found that miR-34a expression level was significantly reduced in NPC compared with that in normal tissue [18]. It is debatable how did the expression of miR-34a decrease in NPC. Some scholars believe that the aberrant methylation of CpG island in the promoter region of miR-34a resulted in the down-regulation of miR-34a level [19], but this conclusion needs more experimental data to confirm.

miR-34a belongs to microRNA family. It can bind to the mRNA of its target gene to regulate gene expression at the transcriptional level [6]. We have found through bioinformatics and luciferase reporter gene assay that Notch1 may be a potential target of miR-34a. We also found that overexpression of miR-34a in CNE1 resulted in the reduction of Notch1 expression, elongation of CNE1 cell cycle, decrease of cell proliferation capability. In short, miR-34a could inhibit Notch1 expression to inhibit cell proliferation and cell division in NPC cells.

Li *et al.* showed by bioinformatics analysis and luciferase reporter gene assay that miR-34a binds to the 3'UTR region of Notch1 mRNA to inhibit Notch1 expression at the transcriptional level [20]. Notch1 signaling pathway involves in evolutionary conserved cell-cell interactions and plays an important role in cell differentiation, development, and apoptosis. Adjacent cells transfer Notch signaling by binding Notch1 receptor and ligand, thereby propagate the signaling, determine cell differentiation fate, organ formation, and morphogenesis [6]. Studies have also showed that Notch1 was closely associated with the pathogenesis and development of a number of malignancies including breast cancer, prostate cancer [7, 8]. Notch1 exerts its function mainly through influence of the cell cycle. A recent study found that Notch1 has dual roles in cancers and acts either as a proto-oncogene, or a tumor suppressor gene [21, 22].

NPC, number one of ear, nose and throat cancer, seriously threats human health. Therefore, early diagnosis and treatment of NPC has become a common concern of doctors and patients [2]. This study showed the relationship and interaction between Notch1 and miR-34a in NPC, providing a theoretical basis for clinical diagnosis and future application of RNA interference on the treatment of NPC.

Acknowledgements

This project supported by the Fujian provincial natural science foundation (No. 2016J01441).

Disclosure of conflict of interest

None.

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