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MicroRNA-34a Inhibits Human Brain Glioma Cell Growth by Down-regulation of Notch1*

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Summary: The effects of microRNA-34a (miR-34a)-regulated Notch1 gene on the proliferation and apoptosis of the human glioma cell line U87 were investigated in this study. The U87 cells were divided into miR-34a mimics, negative control, mock transfection and blank control groups in terms of different treatments. In miR-34a mimics group, human U87 glioma cells were transfected with miR-34a mimics by using lipofectamine 2000. The cells transfected with nonsense microRNA were set up as negative control group. Those treated with lipofectamine 2000 only were designated to the mock tranfection group. In the blank control group, the cells were cultured routinely and no treatment was given. The expression of miR-34a and Notch1 was detected by using real-time RT-PCR. Western blotting was employed to monitor the change in Notch1 protein. Cell proliferation and apoptosis were measured by CCK-8 and flow cytometry. The results showed that the proliferative ability of U87 cells was significantly reduced and the apoptotic cells increased in miR-34a mimics group relative to control groups. The expression of miR-34a was significantly up-regulated in mimics group as compared with control groups (P<0.05). Furthermore, Notch1 protein levels were significantly decreased in miR-34a mimics group when compared with control groups (P<0.05), but the mRNA expression of Notch1 showed no significant difference among these groups. It was concluded that miR-34a may suppress the proliferation and induce apoptosis of U87 cells by decreasing the expression of target gene Notch1, suggesting that miR-34a may become a promising gene therapeutic target for brain glioma.
Key words: microRNA-34a; glioma; Notch1; proliferation; apoptosis

Central nervous system (CNS) tumor is one of the most common forms of cancer in children, with an incidence second to that of childhood leukemia[1]. As the most common CNS tumor, brain glioma is characterized by high ability of proliferation and invasion. Standard therapies for gliomas, such as surgery, radiation and chemotherapy, are only effective in treating patients with high-grade condition. Many gliomas patients have developed metastasis at the onset of clinical symptoms[2]. Hence, novel therapeutic targets that can suppress the proliferation and promote the apoptosis of glioma cells have always been sought after.

Notch gene was first identified by Morgan *et al* in the Drosophila wing. Numerous studies have confirmed that the Notch signaling pathway is involved in many important biological processes by regulating cell proliferation, apoptosis and differentiation[3, 4]. A growing body of evidence suggests that this pathway is implicated in occurrence and development of many human solid tumors including endometrial, lung and pancreatic cancer[5]. Abnormal Notch signaling was found to be associated with tumorigenesis[6]. However, few studies focused on the role of Notch signaling pathway in CNS tumor.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules found in many organisms, which mediate gene expression at the post-transcriptional level. They play an important role in such biological processes through down-regulating multiple target genes, as embryonic development, fat metabolism and oncology[7, 8]. Among the identified miRNAs, microRNA-34a (miR-34a) is one of the first miRNAs identified and is widely expressed in multiple organs. It was found to be down-regulated in many human tumors like glioma, colon cancer and non-small-cell lung cancer tissues or cell lines. Functionally, it was found to affect tumor formation and development by regulating diverse target genes[9, 10]. In our previous study, we found that Notch1 might be a target gene controlled by miR-34a[11]. In order to determine the role of Notch1 in biological behaviors of glioma cells and the possible mechanism, this present study examined the effects of Notch1 on the proliferation and apoptosis of U87 cells (a glioma cell line) after transient transfection of miR-34a mimics.

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1 MATERIALS AND METHODS

1.1 Materials

U87 human glioma cells were purchased from the Cell Bank of Chinese Academy of Science, China. Opti-MEMI Reduced serum medium was purchased from Gibco (USA). The sequences of has-miR-34a mimics, negative control and FAM-labeled negative control, the primers of has-miR-34a, Notch1, U6 and GAPDH were synthesized by Shanghai Genepharma Biotechology Co. (China). Lipofectamine 2000 was from Invitrogen (USA). Trizol and real-time RT-PCR kits were products of QIAGEN (Germany). Mouse anti-human Notch1 antibody and GAPDH were obtained from Santa Cruz (USA). Enhanced chemiluminescence (EZ-ECL) was procured from Bioind Co. (Israel). CCK-8 kit was purchased from the Institute of Chemistry Colleagues (Japan). Annexin Ⅴ/PI apoptosis kit was a product of Shanghai Majorbio Biotech Co. (China).

1.2 Cell Culture

The cells were cultured in DMEM media supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2, and the media were replaced routinely.

1.3 Transfection

To eliminate the interference of transfection reagent, four groups were set up: (1) miR-34a mimics group, in which the cells were transfected with miR-34a mimics at a final concentration of 100 nmol/L; (2) negative control group, in which the cells were subjected to the transfection with unrelated sequence and lipofectamine; (3) mock transfection group, in which the cells were treated only with lipofectamine; (4) blank control group, in which the U87 cells were cultured routinely and no treatment was given.

1.3.1 Detection of Transfection Efficiency The transfection efficiency was determined by using the FAM labeled NC-miRNA. Approximately 24 hours before transfection, U87 cells were inoculated in 96-well plates. FAM labeled NC-miRNA were diluted with Opti-MEMI to give different concentrations (final concentration: 50, 100 and 200 nmol/L), and then mixed with lipofectamine 2000 and incubated at room temperature for 20 min. Afterwards, the mixture was added to the cultured U87 cells when the cells reached 80%–90% confluence. Forty-eight hours after the transfection, the miRNA transfection efficiency was measured by fluorescence microscopy to determine the optimal final concentration for transfection.

1.3.2 Transfection of miR-34a Mimics The sequences were as follows: miR-34a mimics, sense, 5'-UGGCAGUGUCUUAGCUGGUUGU-3', antisense, 5'-AACCAGCUAAGACACUGCCAUU-3'; negative control (NC, non-homologous to any human genome sequence), sense, 5'-CAGUACUUUUGUGUAGUACAA-3', antisense, 5'-UUGUACUACACAAAAGUAC UG-3'.

Cells were cultured to 80% to 90% confluence after seeded into 6-well plates. They were transfected with Lipofectamine 2000 according to the manufacturer's instructions. miR-34a mimics (10 μL) in 250 μL of Opti-MEM Ⅰ reduced serum medium were mixed with 5 μL of Lipofectamine 2000 transfection reagent dissolved in 250 μL of the same medium and were allowed to stand at room temperature for 20 min. The resultant 500 μL transfection solutions were then added to each well. After 6 hours, the cultures were replaced with 2 mL fresh medium supplemented with 10% FBS and antibiotics.

1.4 Assessment of Cell Proliferation

The proliferative ability of cells was measured by using the Cell Counting Kit-8 24, 48 and 72 hours after transfection, according to the manufacturer's protocol. Briefly, 10 μL of CCK-8 solution was added to each well. After incubation at 37°C for 4 h in 5% CO_2, the absorbance (A) of each well at a wavelength of 490 nm was detected by using the microplate reader.

1.5 Assessment of Cell Apoptosis

The cells were harvested, washed and re-suspended in binding buffer 48 hours after transfection. Apoptotic cells were determined with an FITC Annexin V Apoptosis Detection kit according to the instructions of the manufacturer. Briefly, the cells were washed twice in PBS and subsequently incubated for 15 min at room temperature in the dark in 100 μL of 1× binding buffer containing 5 μL of AnnexinⅤ-FITC and 5 μL of propidium iodide (PI). Afterward, cell apoptosis was analyzed by flow cytometry within 1 h.

1.6 Quantitative Real-time RT-PCR (qRT-PCR)

qRT-PCR was performed to determine the expression levels of miR-34a and its potential target gene Notch1. Forty-eight hours after transfection, total RNA was extracted from the transfected U87 cells by using Trizol according to the manufacturer's instructions. Then it was reversely transcripted into cDNA with a TaqMan Reverse Transcription kit. The amplified primers were designed as follows: miR-34a, sense, 5'-CGTCATC AAACCGTTACCATTAC-3', antisense, 5'-AAAGGTTGTTCTCCACTCTCTCTC-3'; U6 (internal standard of has-miR-34a), sense, 5'-ATTGGAACGATACAGAGAAGATT-3', antisense, 5'-GGAACGCTTCACGAATTTG-3'; Notch1, sense, 5'-CAATGAGTTCCAGTGCGAGTGC-3', antisense, 5'-AGGTGTAAGTGTTGGGTCCGTCC-3'; GAPDH (internal standard of Notch1), sense, 5'-CATGAGAAGTATGACAACAGCCT-3', antisense, 5'-AGTCCTTCCACGATACCAAAGT-3'. The reaction conditions were 40 cycles of 95°C for 3 min, 95°C for 12 s, and 62°C for 40 s. All samples were measured in triplicate. The analysis was performed in ABI Prism 7500 Sequence Detection system. The relative gene expression was calculated by employing the $2^{-\Delta\Delta Ct}$ method[12].

1.7 Western Blot Analysis

Forty-eight hours after transfection, cells were harvested and centrifuged, and total protein was extracted. Protein concentrations were determined using the BCA protein assay. After heated for 10 min at 100°C, 20 μg of denatured protein was subjected to 10% SDS-PAGE. Then proteins were transferred electrophoretically for 1 h at 200 mA at 4°C onto PVDF membranes. Membranes were blocked for 1 h at room temperature in TBS containing 5% non-fat dry milk. Blots were washed 3 times for 10 min each with 0.1% TBS-T and subsequently treated overnight at 4°C with primary antibodies against Notch1 (1:1000) or GAPDH (1:500). After washing 3 times for 10 min each with 0.1% TBS-T, the blots were incubated with anti-mouse antibody (1:5000) conjugated with horseradish peroxidase for 1 h at room temperature. Bands were visualized by using EZ-ECL detection re-

agents. The scanned images were quantified using Quantity One software. GAPDH was used as an endogenous protein for normalization. The ratio of Notch1/GAPDH was used for semi-quantification and comparison between different groups.

1.8 Statistical Analysis

Results were expressed as $\bar{x}\pm s_{\bar{x}}$. The difference among groups was assessed by AVONA and Student's *t*-test. Differences were considered significant when the *P* value was less than 0.05.

2 RESULTS

2.1 Transfection Efficiency

The transfection efficiency was evaluated by determining the percentage of cells transfected with FAM-labeled NC-miRNA. The results showed that the number of apoptotic cells was increased along with the concentration of transfection agents. Fluorescence microscopy showed cell growth remained good after tranfection with FAM-labeled NC-miRNA at a lower concentration, and the transfection efficiency could reach 70% (fig. 1). So the optimal final concentration of miRNA for transfection was 100 nmol/L.

2.2 Effect of miR-34a Mimics on Cell Proliferation

As shown in fig. 2, the cell proliferation rate was obviously decreased in the miR-34a mimics group relative to other control groups 24, 48 and 72 hours after transfection (P<0.05 for each), indicating that miR-34a could significantly inhibit the proliferation of glioma cells.

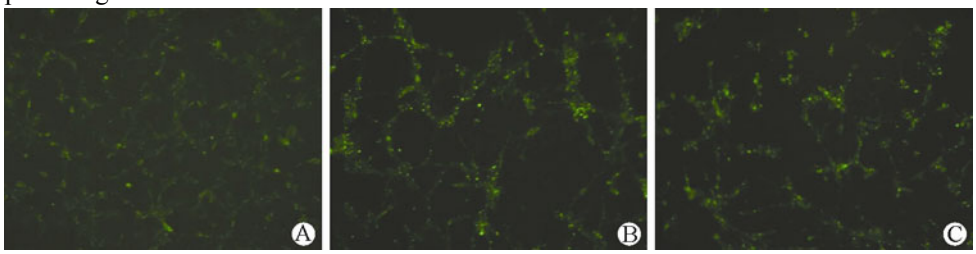

Fig. 1 FAM expression in U87 cells transfected with different concentrations of FAM-labeled NC-miRNA 48 h after transfection by fluorescence microscopy (×200)
A: 50 nmol/L FAM-labeled NC-miRNA; B: 100 nmol/L FAM-labeled NC-miRNA; C: 200 nmol/L FAM-labeled NC-miRNA

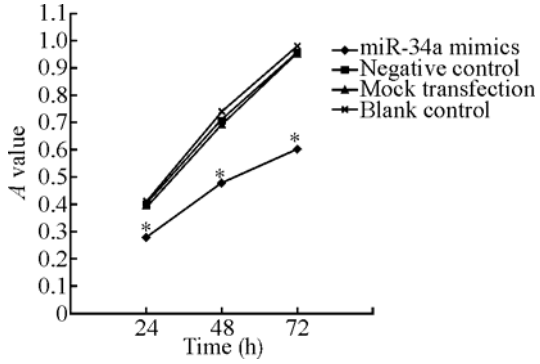

Fig. 2 miR-34a significantly inhibits the proliferation of U87 glioma cells at different time points.
*P<0.05 *vs.* negative control, mock tranfection and blank control groups

2.3 Effect of miR-34a Mimics on Cell Apoptosis

Flow cytometry showed that the apoptosis rate was (6.13±0.40)% in the negative control group, (6.28±0.20)% in mock transfection group and (5.74±0.50)% in blank control group, with no significant difference found. The cell apoptosis rate in miR-34a mimics group was (12.17±1.40)%, significantly higher than that in control groups (P<0.05 for each).

2.4 qRT-PCR Results of miR-34a and Notch1

No significant differences were noted in miR-34a and Notch1 mRNA levels among control groups 48 h after transfection (P>0.05). The miR-34a mRNA expression level was markedly increased in the miR-34a mimics group as compared with control groups (P<0.05 for each) (fig. 3). However, although the expression level of Notch1 mRNA was slightly higher in the miR-34a mimics group than in the control groups, the difference was not statistically significant (P>0.05, fig. 4).

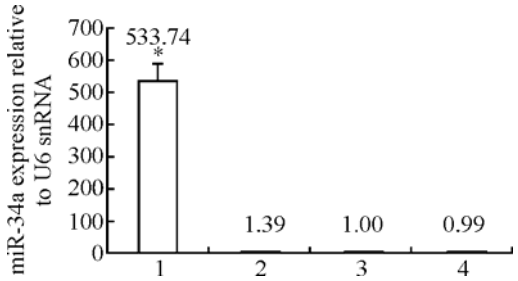

Fig. 3 The relative expression of miR-34a mRNA in U87 cells after transfection
1: miR-34a mimics; 2: Negative control group; 3: Mock transfection group; 4: Blank control group
*P<0.05 *vs.* negative control, mock tranfection and blank control groups

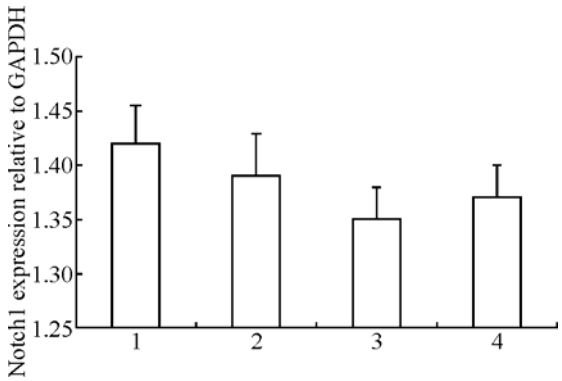

Fig. 4 The relative expression of Notch1 mRNA in U87 cells after transfection
1: miR-34a mimics; 2: Negative control group; 3: Mock transfection group; 4: Blank control group

2.5 Western Blotting Result of Notch1

Western blot analysis showed that the expression of Notch1 protein in mimics group was much lower than in control groups (P<0.05 for each). There was no significant difference in the expression of Notch1 protein among the negative control, mock transfection and blank control groups (P>0.05, fig. 5).

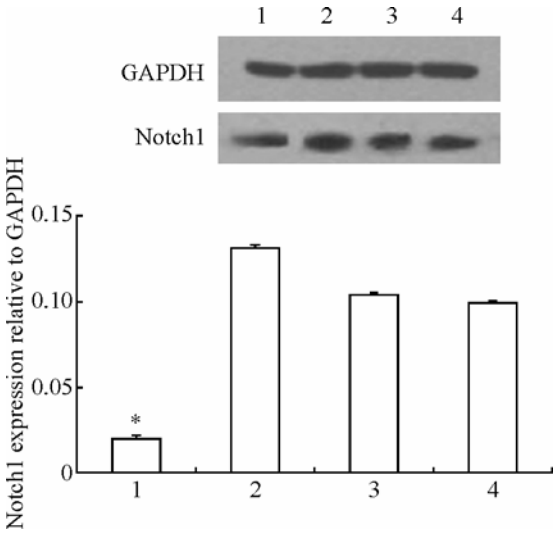

Fig. 5 Western blot analysis of Notch1 protein 48 h after transfection

1: miR-34a mimics; 2: Negative control group; 3: Mock transfection group; 4: Blank control group

*P<0.05 *vs.* negative control, mock tranfection and blank control groups

3 DISCUSSION

miRNAs are a class of small, non-coding RNAs (approximately 23 nt in length) and have profound impact on a wide array of biological processes[13]. miRNAs mediate posttranscriptional gene silencing by binding to the 3'-untranslated region (3'-UTR) of target messenger RNA (mRNAs) through fully complementary or imperfect base-pairing repressing the translation or promoting RNA degradation[14–16]. They participate in regulation of diverse cellular processes including cell proliferation, apoptosis, differentiation and carcinogenesis[17]. Increasing evidence shows that miRNAs are involved in tumor development and progression by functioning as oncogenes or tumor suppressor genes[18]. Among these miRNAs, miR-34a is one of the potential tumor suppressors[19]. It is commonly repressed in multiple tumor cell lines, such as human glioma cells[11] . As a tumor suppressor, overexpression of miR-34a was found to be able to affect cell proliferation, apoptosis and invasion by regulating target mRNAs and related signal pathway[20]. In the present study, by up-regulating miR-34a expression level, the proliferation rate of U87 cells was remarkably decreased at different time points, while the cell apoptosis rate increased.

The miRNAs in the human genome are estimated to regulate up to one-third of all the protein-coding genes, and a complicated regulation network is formed among them[21]. Fortunately, bioinformatics and related computer software facilitate investigation of this complex network[22]. To predict target genes of miR-34a and investigate related mechanisms, we screened target genes of miR-34a using miRanda (http://www.microrna.org) software. It was found that miR-34a may target multiple genes, including Notch1, Bcl2 and SIRT1. Among these target genes, Notch1 has been the focus of tumor-associated research in the last few years[23, 24]. The Notch gene encodes a 2753-amino-acid transmembrane receptor, which is composed of an extra-cellular domain, a transmembrane domain and an intra-cellular domain[25, 26]. When the Notch ligand expressed on an adjacent cell binds to the Notch receptor, the receptor is exposed to proteolytic activation. Then the Notch intracellular domain (NICD) dissociates from the receptor, translocates into the nucleus and binds to the members of the CSL transcription factor family (CBF-1/Suppressor-of-hairless/Lag-1) which becomes a transcriptional activator and regulates differentiation and cell survival[27]. Notch pathway appears to play diverse roles in a variety of pediatric malignancies, affecting cell differentiation, metastasis and angiogenesis. For example, approximately 50%–70% of patients with T-cell acute lymphoblastic leukemia (ALL) have mutations of Notch1[28]. The role of Notch1 and related pathway in CNS tumor, however, remains unclear. Aberrant activation of this pathway was revealed to be implicated in glioma, medulloblastoma and meningeoma[29–31].

To determine if the effects of miR-34a on biological characteristics of glioma cells are mediated by Notch1, miR-34a mimics was transfected into the U87 cells with lipofectamine 2000 and then the expression level of Notch1 was detected. Chemically-synthetic miRNAs mimics were designed to mimic endogenous mature miRNA molecules and enhance the regulatory role of endogenous miRNA by up-regulating its expression level. In order to carry out the study successfully, we tested transfection efficiency by using the FAM labeled NC-miRNA at different concentrations. The result showed that cell viability was better when miRNA was given at a lower concentration and the transfection efficiency reached about 70% when the concentration of FAM labeled NC-miRNA was 100 nmol/L. Therefore, 100 nmol/L miRNA was used for transfection.

We detected the expression level of Notch1 and miR-34a in U87 cells 48 hours after transfection by using Western blotting and real-time RT-PCR. The results showed that the miR-34a mRNA level was remarkably elevated and the Notch1 protein level but not mRNA level significantly decreased in miR-34a mimics group relative to control groups. It was suggested that miR-34a regulated cell proliferation and apoptosis by inhibiting Notch1 at the protein level but not at the mRNA level. This result was consistent with previous studies which found that miRNA was mainly involved in posttranscriptional gene regulation[32]. Besides, it was proven that Notch1 was a target gene regulated by miR-34a negatively[33].

In summary, our studies demonstrated that miR-34a mimics inhibited proliferation and induced apoptosis of glioma U87 cells by down-regulation of Notch1 protein. miR-34a may play an important role in the development of glioma, and it may serve as a therapeutic target for brain glioma.

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