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MicroRNA-34a induces apoptosis in the human glioma cell line, A172, through enhanced ROS production and NOX2 expression

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ABSTRACT

Background: MicroRNA is a type of non-coding small RNA involved in regulating genes and signaling pathways through incomplete complementation with target genes. Recent research supports key roles of miRNA in the formation and development of human glioma.
Methods: The relative quantity of miR-34a was initially determined in human glioma A172 cells and glioma tissues. Next, we analyzed the impact of miR-34a on A172 cell viability with the MTT assay. The effects of miR-34a overexpression on apoptosis were confirmed with flow cytometry and Hoechst staining experiments. We further defined the target genes of miR-34a using immunofluorescence and Western blot.
Results: MiR-34a expression was significantly reduced in human glioma A172 cells and glioma tissue, compared with normal glial cells and tissue samples. Our MTT data suggest that up-regulation of miR-34a inhibits cell viability while suppression of miR-34a enhances cell viability. Flow cytometry and Hoechst staining results revealed increased rates of apoptosis in A172 human glioma cells overexpressing miR-34a. Using immunofluorescence and Western blot analyses, we identified NOX2 as a target of miR-34a in A172 cells.
Conclusion: MiR-34a serves as a tumor suppressor in human glioma mainly by decreasing NOX2 expression.

1. Introduction

MicroRNA (miRNA) is a type of endogenous RNA about 20–24 nucleotides in length. Recent studies have disclosed various critical roles of miRNA in cell growth and apoptosis [1]. Each miRNA may have multiple target genes, and several miRNAs often regulate the same genes. Within this complex regulatory network, expression of multiple genes may be controlled either through a single miRNA or a combination of several miRNAs. Collective research to date has suggested that miRNAs regulate approximately one-third of the human genome. Recent studies indicate that about 70% of the mammalian miRNA genes are located in transcription micro RNA units most of which are present in the intron region. Some miRNA genes encoded within introns are highly conserved in different species [1]. MiRNAs are not only conserved in terms of position, but also show high sequence homology. The highly conserved characteristics of miRNA are closely related to their corresponding functions. MiRNAs are closely linked with target gene evolution. Thus, elucidation of their evolutionary history would be helpful in understanding the underlying mechanisms and functions.

In normal tissues, after transcription and processing, miRNAs bind to the complementary sites of target genes and consequently repress gene expression by inhibiting protein translation or affecting mRNA stability [2]. The ultimate effects include maintenance of cell growth, proliferation, differentiation and death at a physiological level. As a tumor suppressor gene, aberrant decrease or loss of miRNA usually leads to tumor formation. Decreased levels of mature miRNA may be attributed to defects in the biosynthesis step, which results in abnormal expression of miRNA target genes [3]. Eventually, tumors are induced due to abnormal cell proliferation, invasion, apoptosis, and differentiation. Overexpression of miRNA may additionally lead to tumor formation. In this case, miRNAs are inappropriately increased in abnormal tissue or at the developmental stage, which could lead to decreased target gene expression, thereby causing tumor formation. High-throughput detection technology has revealed that human tumor tissues display a significantly different miRNA expression

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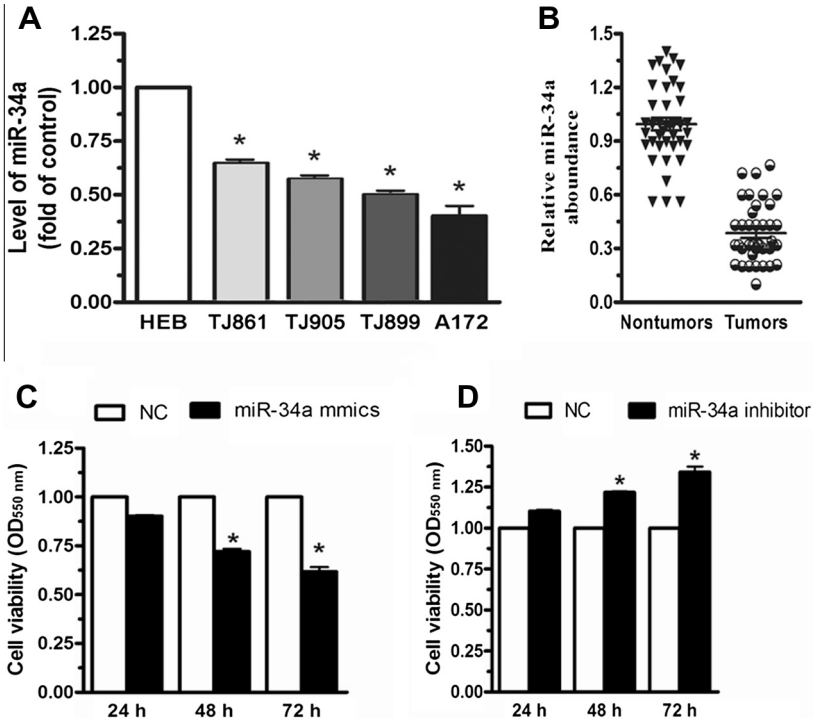

Fig. 1. Expression of miR-34a in human glioma cell lines and tissue samples. (A) qRT-PCR analysis of miR-34a expression in the human glioma cell lines, TJ861, TJ905, TJ899 and A172, as well as normal human glial cells, HEB. (B) Analysis of miR-34a expression in 60 human glioma cancer tissue and paired nontumor tissue samples. RNU48 was employed as an endogenous control. MiRNA levels were determined in individual patient samples using RT-qPCR. Fold changes relative to the control group were calculated based on the ΔCt method. Data represent means ± SEM. Statistical significance was determined with Student's *t*-test $n = 3$ independent experiments $^{*}P < 0.05$ versus control. (C) A172 cells were transfected with miR-34a mimics and inhibitors (D) or negative control for 24, 48, and 72 h, respectively. The relative expression level of miR-34 was determined using qRT-PCR. RNU48 was used an internal control. Cell viability was determined using the MTT assay. Data are presented as mean ± SEM, $n = 6$ independent experiments $^{*}P < 0.05$ versus control.

spectrum, with varying abundance of miRNAs in different cancer tissues [4]. For example, in glioma, B cell lymphoma, chronic lung cancer, liver cancer, breast cancer and colon cancer, the miRNA expression profiles are distinct from that of normal tissue [5].

Glioma is the most common primary tumor produced by the brain and spinal cord glial cells. The annual incidence rate of glioma is about 3–8 per 10 million people [6]. Similar to other tumors, glioma mainly results from genetic risk and environmental carcinogenic factors. Specific genetic diseases, such as neurofibromatosis and tuberous sclerosis disease, are predisposing factors of glioma. In these patients, the chances of glioma are significantly higher, compared to the general population [7]. Environmental carcinogenic factors may additionally be associated with the occurrence of glioma. For instance, electromagnetic radiation, such as mobile phone use, may be related to glioma [8]. However, there is no direct evidence to indicate an inevitable cause-and-effect relationship. Although most glioblastoma patients were identified with cytomegalovirus infection and the majority of glioblastoma specimens contained macrophages of viral infection in an earlier study, [9] it is currently unclear whether a causal relationship exists between them. A highly conserved gene regulatory factor, miRNA, identified in recent years, may shed light on the potential mechanism of glioma [9].

Here, we focus mainly on miRNA-34, in view of its down-regulation in various tumor types. For instance, miR-34a is significantly decreased in hepatocellular carcinoma cells and induces hepatocyte apoptosis. However, limited information is available on miR-34 expression and its significance in glioma. In the current study, we examined the effects of miR-34a on regulation of apoptosis in the human glioma cell line, A172.

2. Materials and methods

2.1. Human samples and cell lines

Human glioma cancer tissue microarrays (Lot Number: ARY-HH0118-011-02) containing 60 glioma cancer specimens and 30 normal tissues were purchased from Folio Biosciences (Columbus, OH, USA). The human glioma cell line, A172, and normal human glial cell line, HEB, were acquired from the American Type Tissue Culture Collection and cultured in DMEM/F12 with 10% fetal bovine serum.

2.2. Transient transfection

MiR-34a mimics, miR-34a inhibitor and negative control (Genepharma, Shanghai, China) were pre-incubated with HiperFect transfection reagent (QIAGEN, Germantown, MD, US), according to the manufacturer's instructions. The final concentration of microRNA analogs was 100 nmol/L.

2.3. Cell culture

The human glioma cell line, A172, and normal human glial cell line, HEB, were maintained in DMEM/F12 (HyClone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS, HyClone), 100k U/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were cultured at approximately 70% confluency and starved in serum-free DMEM (SF-DMEM) overnight. Each experiment was performed at least three times.

2.4. Western blot analysis

Cellular proteins were extracted using RIPA buffer (SolarBio, Beijing, China, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl 1% (v/v) NP-40, 0.1% (w/v) SDS) containing 1% (v/v) PMSF (SolarBio), 0.3% (v/v) protease inhibitor (Sigma–Aldrich, St. Louis, Missouri, USA) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma–Aldrich). Lysates were centrifuged at 12,000 rpm at 4 °C for 15 min, and the supernatant collected for total protein. The BCA protein assay kit (Pierce, IL, US) was employed to determine protein concentrations. Equal amounts of protein (15 μg) were separated on an SDS gel (10% (v/v) polyacrylamide) and transferred to a PVDF membrane. Non-specific binding was blocked using 8% (w/v) milk in TBS-T for 2 h at room temperature (RT). Membranes were incubated with primary antibodies against GAPDH (Abmart, Shanghai, China), NOX2 (Cell Signaling), p-Akt or Akt (Cell Signaling) overnight at 4 °C. After several washes with TBS-T, membranes were incubated in HRP-conjugated goat anti-rabbit and anti-mouse IgG or HRP-conjugated mouse anti-goat IgG (Abmart, 1:5000 dilution) for 2 h at RT and washed. Target proteins were visualized using enhanced chemiluminescence (Millipore) according to the manufacturer's recommendations, quantified using density analysis normalized against GAPDH according to the manufacturer's recommendations, and expressed as fold change, compared to the control.

2.5. MTT assay

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, A172 cells (5×10^4 cells per well) were seeded in 96-well tissue culture plates. At 70–80% confluence, cells were incubated for 16 h in serum-free DMEM F-12 medium. After drug treatment, A172 cells were cultured in fresh medium containing 0.5 mg/ml MTT for a further 4 h. The blue formazan products were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

2.6. Hoechst 33258 staining

A172 cells (1×10^5 cells per well) were cultured in six-well tissue culture plates. At 70–80% confluence, cells were incubated for 16 h in serum-free DMEM F-12 medium. After drug treatment, medium was removed, and cells were rinsed once with cold PBS and fixed with 4% formaldehyde in PBS for 15 min at 37 °C. Cells were washed three times with PBS. Nuclei were stained with Hoechst 33258 (10 μg/mL) for 5 min, washed three times with PBS and dried.

2.7. Immunofluorescence analysis

Cells grown on chamber slides were washed with PBS for 15 min (total), fixed in 4% paraformaldehyde for 30 min at RT, and permeabilized with 0.1% TritonX-100 at RT for 5 min. After three washes with PBS for 15 min (total), non-specific binding was blocked with 3% bovine serum album (BSA) in PBS for 1 h at RT. Next, cells were incubated with human NOX2 and GAPDH primary antibodies (diluted 1:100 in PBS with 1% BSA) for 2 h at RT, washed with PBS, and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG (Zhongshan

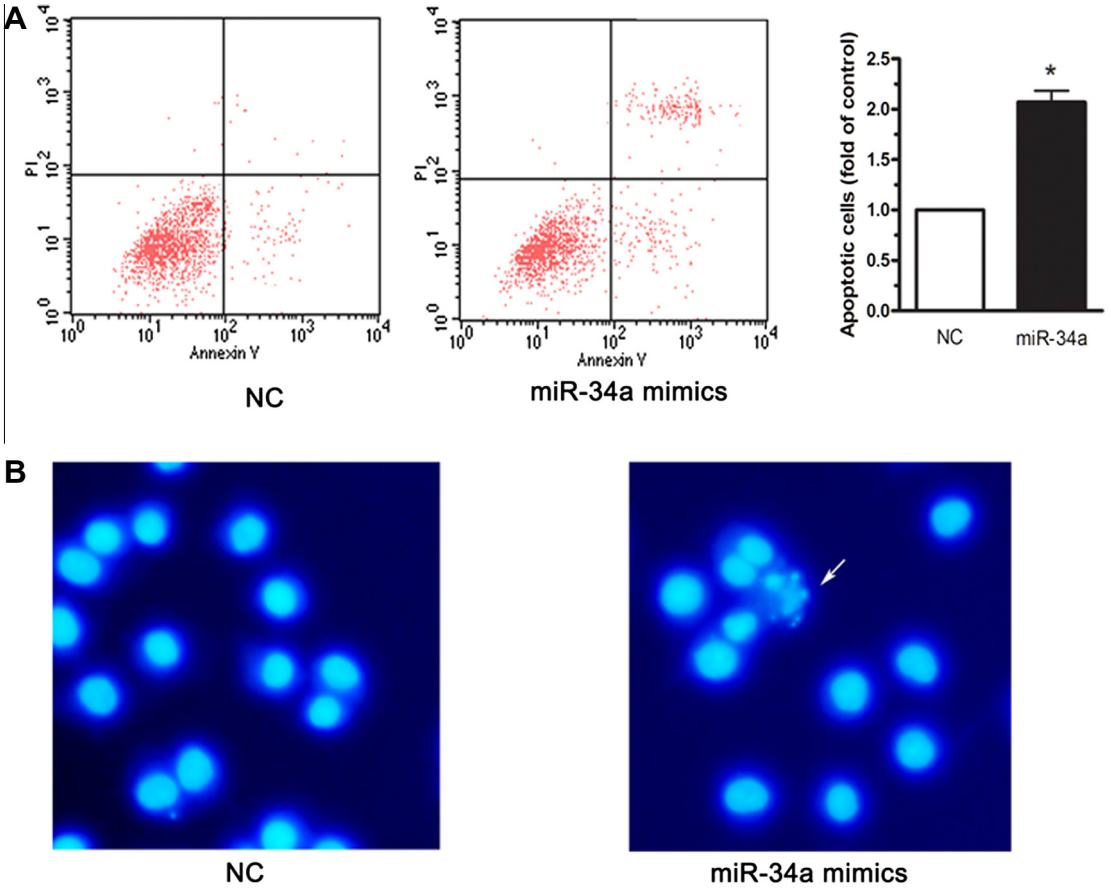

Fig. 2. Apoptosis of A172 cells is induced upon overexpression of miR-34a. (A) Flow cytometry using the Annexin V and PI kit was applied to determine the rate of cell apoptosis. (B) Morphological analysis of apoptosis using Hoechst 33258 staining.

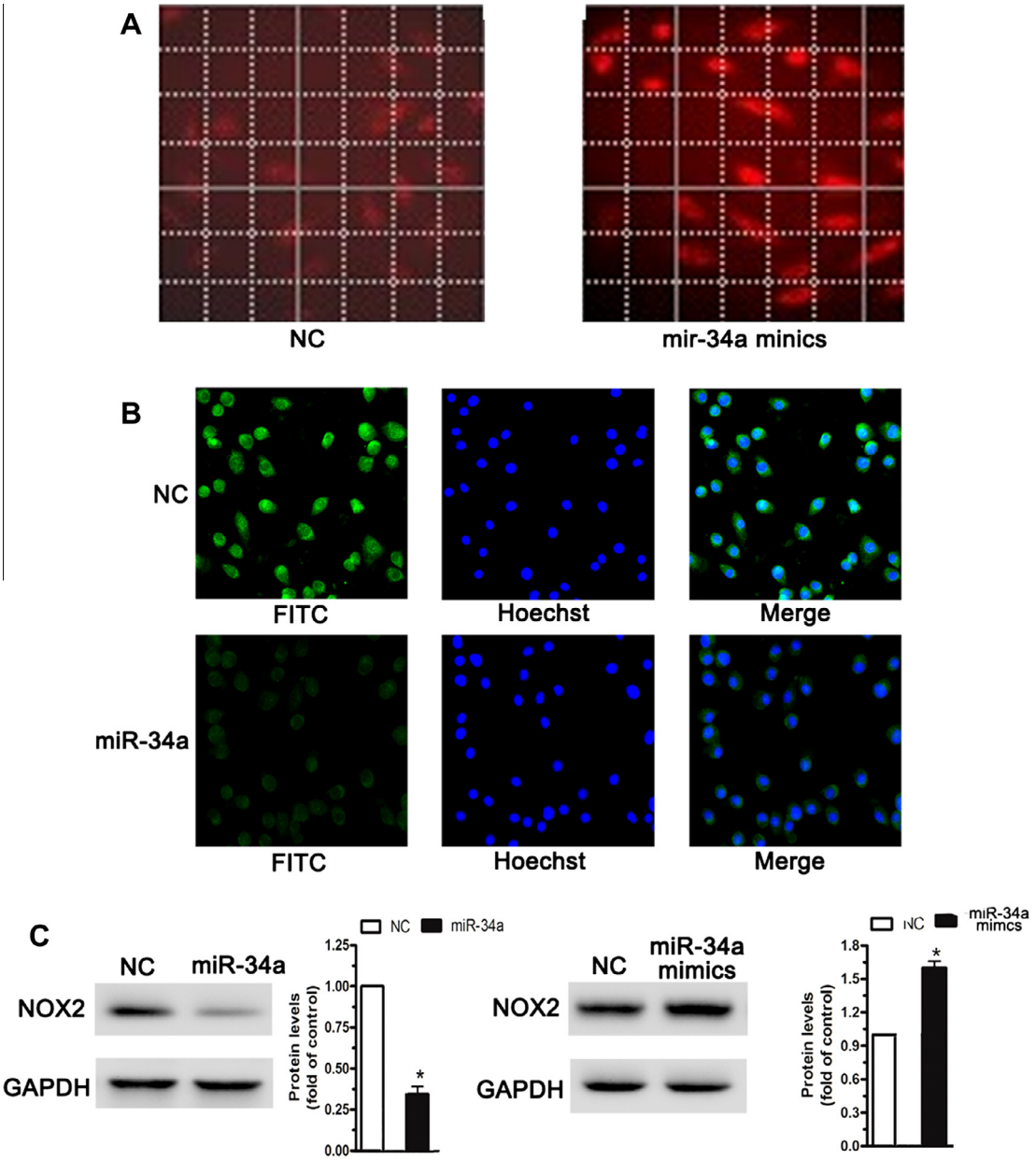

Fig. 3. MiR-34a enhances ROS production and NOX2 expression in the human glioma cell line, A172. (A) ROS levels were explored in A172 cells transfected with miR-34a mimics or negative control. (B) Immunofluorescence analysis of NOX2 expression in A172 cells transfected with miR-34a inhibitors or negative control. (C) Western blot analysis of NOX2 expression in A172 cells transfected with miR-34a mimics and inhibitors (C) or negative control. Data represent mean values ± SEM, $n = 3$ independent experiments $^{*}P < 0.05$ versus control.

Biotechnology; 1:50 dilution in PBS with 1% BSA) for 1 h at RT. After several washes for 15 min (total) with PBS, cell nuclei were visualized with Hoechst 33258 staining (10 μg/mL) for 10 min at RT. Slides were washed again, dried, mounted, and examined under a fluorescence microscope.

2.8. Determination of ROS

Cells cultured on six-well chamber slides were washed with PBS three times for 5 min each. Slides were incubated with ROS Fluorescent Probe-DHE (Vigorous Biotechnology Beijing Co., Ltd) in serum-free DMEM F-12 for 30 min at 37 °C in the dark, fixed in 4% paraformaldehyde for 30 min at RT, re-washed and mounted. Immunofluorescence images were captured using fluorescence microscopy.

2.9. RNA isolation and real-time RT-PCR

Total RNA was isolated with RNAiso Plus (Takara Bio) following the manufacturer's instructions. For quantitation of miRNA, 10 ng total RNA was reverse-transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific primers for miR-34a and RNU48, and PCR amplification performed in reaction volumes of 20 μl containing 10 μl TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 1 μl 20× TaqMan MicroRNA Assay mix (Applied Biosystems) and 1.33 μl template cDNA in the same system used for mRNA quantitation. Thermal cycling conditions were as follows: a hot-start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative expression of miR-34a was normalized against the endogenous control, RNU48, using the comparative delta-delta

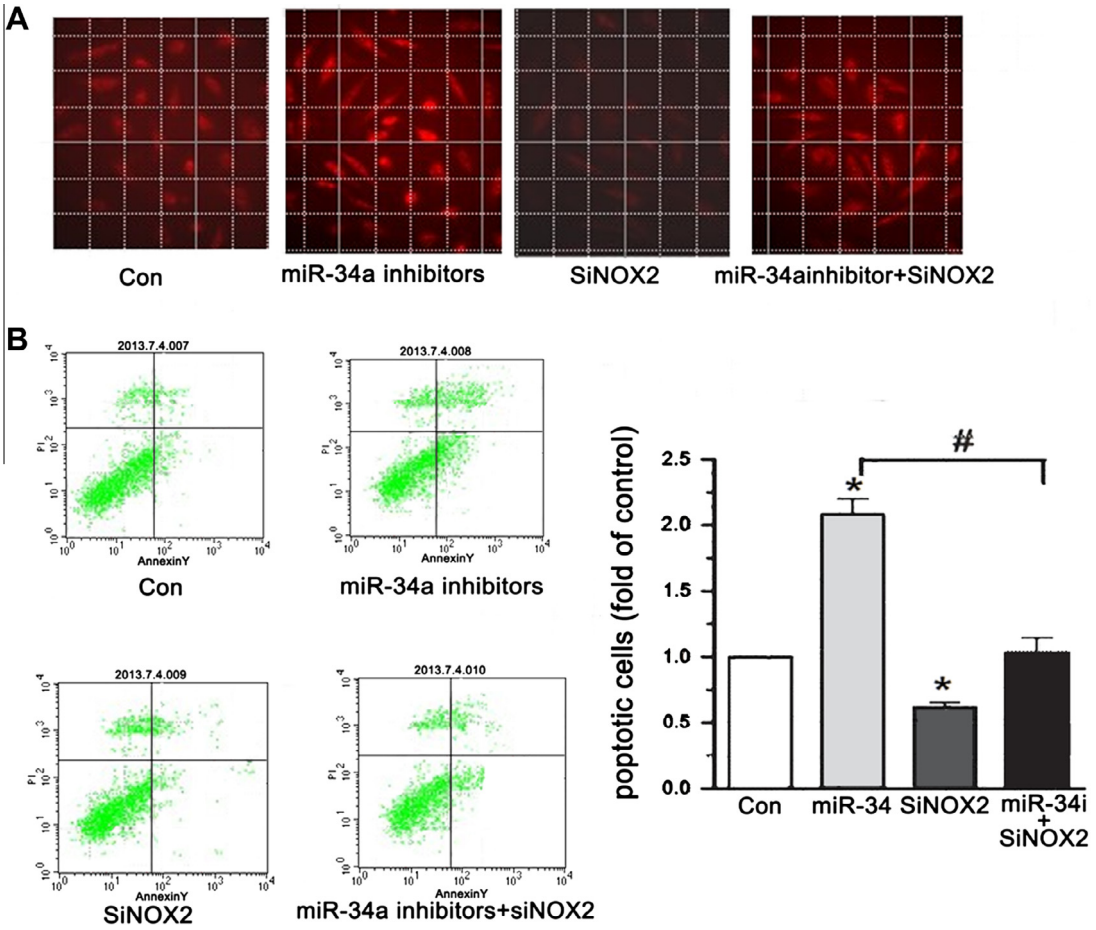

Fig. 4. Overexpression of miR-34a induces an increase in ROS. (A) A172 cells were transfected with miR-34a mimics, siNOX2 or negative control, and the ROS levels determined. (B) Apoptosis was analyzed using Annexin V and PI kit under similar conditions. Data represent means ± SEM, $n = 3$ independent experiments $^{*}P < 0.05$ versus control.

CT method. Bio-Rad CFX Manager Software was employed for quantitation of both mRNA and miRNA.

2.10. Apoptosis assay

Cells (50–60% confluent) were treated with or without miR-34a (40 μM, 30 min) and washed twice with 1 × PBS. Apoptosis was assessed using an Annexin-V FITC/PI Apoptosis Kit (Invitrogen, Carlsbad, CA, USA). This assay employs fluorescein-labeled Annexin-V in concert with propidium iodide (PI) to detect cells undergoing apoptosis. Briefly, cells were washed twice with 1 × PBS and suspended at 2–3 × 10^6 cells/mL in 1 × Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V FITC and Propidium Iodide buffer were added to cells, which were subsequently incubated at RT for 15 min in the dark. Cells were analyzed using flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 h of staining using the FL1 (FITC) and FL3 (PI) lines.

2.11. Statistical Analysis

Data are expressed as mean values ± SEM. The number of independent experiments is represented by "*n*". Multiple comparisons were performed using one-way ANOVA followed by Tukey's multiple-comparison test, with $P < 0.05$ considered statistically significant.

3. Results

3.1. MiR-34a is down-regulated in human glioma cells and tissue samples

We examined miR-34a expression in human glioma cells and tissue samples. The relative quantity of miR-34a was determined using real-time quantitative RT-PCR (qRT-PCR). Our data showed that miR-34a is decreased by nearly 64% in the human glioma cell line, A172, when expression is normalized to RNU48 (Fig. 1A; $P < 0.01$). MiR-34a expression patterns were further determined in 60 pancreatic cancer specimens along with 30 normal tissues. Compared with normal tissues, the average expression of miR-34a was reduced by 68% (Fig. 1B; $P < 0.01$). Clearly, miR-34a is significantly decreased in both human glioma cells and tumor tissues.

We further investigated whether miR-34 affects A172 cell viability, in view of its evident suppression in human glioma cells and tissues. Using the transient transfection method, the human glioma cell line, A172, was transfected with miR-34a mimics, inhibitors or negative control for 24, 48 and 72 h, respectively. In our study, mimics are analogs that enhance the miR-34a level while inhibitors are analogs that suppress miR-34a expression. As expected, upon transfection of miR-34a mimics into the human glioma cell line, A172, viability was significantly decreased by 25% and 34% at 48 and 72 h, respectively (Fig. 1C). Conversely, when miR-34a expression was inhibited, cell viability was enhanced by 15% and 30% at 48 h and 72 h, respectively (Fig. 1D). Our results

suggest a negative correlation between miR-34a expression and A172 cell viability.

3.2. MiR-34a mimics induce A172 apoptosis

To determine the mechanism by which miR-34a affects A172 cell viability, we analyzed whether its expression is correlated with apoptosis. Flow cytometry analysis using the Annexin V and PI kit was performed. Upon overexpression of miR-34a, the apoptosis rate was significantly increased. As shown in Fig. 2, compared with the negative control, cell apoptosis was increased by 4% (Fig. 2A). Hoechst staining was applied to detect cellular apoptosis, and apoptotic cells observed under a microscope (Fig. 2B). The cell phenotype data suggest that miR-34a overexpression induces apoptosis in the human glioma cell line, A172.

3.3. NOX2 is the target gene of miR-34a

In view of the finding that cellular apoptosis is positively correlated with reactive oxygen species (ROS), [10] we performed DHE staining to determine the relative levels of ROS in A172 cells. ROS production was significantly increased upon transfection of miR-34a mimics into A172 cells, as shown in Fig. 3A, strongly supporting a key role of ROS in glioma cell apoptosis. In recent years, NOX2 has been identified as a leading candidate for reactive oxygen species (ROS) production in various cell types [11]. Accordingly, we investigated the correlation between miR-34a and NOX2. To establish whether miR-34a regulates NOX2, immunofluorescence was used to detect NOX2 expression. Upon transfection of the human glioma cell line, A172, with miR-34a inhibitors for 48 h, NOX2 density was significantly decreased, compared with the negative control (Fig. 3B). Furthermore, at 48 h after transfection with miR-34a mimics, NOX2 expression was increased by 120%. Conversely, NOX2 levels were reduced when miR-34a was inhibited in A172 cells (Fig. 3C). Based on these findings, we conclude that miR-34a regulates NOX2 in human glioma cells and tissues.

3.4. Suppression of NOX2 leads to reduced ROS production

To determine the levels of ROS, DHE staining was performed in A172 cells transfected with miR-34a mimics, siNOX2 or negative control. As shown in Fig. 4A, ROS levels were significantly enhanced in A172 cells transfected with miR-34a mimics. Experiments with siRNA targeting NOX2 were performed to explore the effects of NOX2 on ROS production. Upon knockdown of NOX2, cellular ROS was significantly reduced (Fig. 4A). Notably, siNOX2 suppressed ROS production, even in A172 cells transfected with miR-34a inhibitors (Fig. 4A). The data suggest that ROS production is mainly enhanced through increased expression of NOX2. Under similar conditions, the apoptosis rate was detected using an Annexin V and PI kit. As shown in Fig. 4B, siNOX2 induced a significant reduction in apoptosis while upregulation of miR-34a enhanced apoptosis over onefold. In comparison, knockdown of NOX2 reversed apoptosis when A172 cells were transfected with miR-34a inhibitors (Fig. 4B). The results collectively suggest that miR-34a mainly induces human glioma cell apoptosis by regulating NOX2, which is positively correlated with cellular ROS production.

4. Discussion

MiRNAs are non-coding small RNAs that mainly repress mRNA translation in cells. Through incomplete binding to partially complementary sequences of several mRNAs, miRNAs perform important functions in various oncogenic signaling pathways, such as cell proliferation and migration [12]. Based on their functions, miRNAs are divided into oncogenes and tumor suppressor genes. Glioma is the most common malignant tumor of the central nervous system, accounting for about 40–50% of all intracranial tumors [13]. Modern microsurgery, radiotherapy, chemotherapy and other comprehensive treatment measures are not ideal for prognosis of glioma patients. Therefore, elucidation of the molecular mechanisms underlying glioma occurrence and development, as well as the key signaling pathways and regulatory factors constitute the focus of brain glioma research [14].

In human tumors, miR-34 is reduced due to selective loss of pressure [14]. Here, we examined the miR-34a expression patterns in human glioma cells and tissues. qRT-PCR findings revealed that the relative quantity of miR-34a is significantly down-regulated, compared with that in normal cells and tissues. Ectopic expression of miR-34a inhibited A172 cell growth and enhanced apoptosis. Similarly, reduction of miR-34a in A172 cells enhanced cell viability and growth *in vitro*. Our results further indicate that NOX2 is a direct target of miR-34a.

ROS plays a vital role in the process of apoptosis [15]. NOX2 is widely reported to increase cellular ROS production in various cell types [16]. NOX2 is a positive regulatory factor that induces apoptosis upon external stimulation in many cell types [15–16]. In the current study, overexpression of miR-34a enhanced cellular ROS and NOX2 levels in the human glioma cell line, A172. Additionally, knockdown of NOX2 using targeted siRNA led to a significant reduction in cellular ROS production. Our results indicate that miR-34a exerts pro-apoptotic activity mainly by enhancing NOX2 expression and ROS production in human glioma cells. However, further research is required to determine the factors underlying the positive regulation of NOX2 by miR-34a.

In conclusion, we have clearly demonstrated a decrease in miR-34a expression in both human glioma cells and tissues for the first time. Additionally, miR-34a appears to inhibit A172 cell viability and induce apoptosis. NOX2 was further identified as a regulatory target of miR-34a in A172 cells. We propose that the decrease in miR-34a expression in the glioma cell line, A172, leads to lower expression of NOX2, resulting in inhibition of apoptosis and infinitely increased cell proliferation.

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