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Original Article

Activation of NF-κB by miR-129 and enhancement of neuroglioma growth

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Abstract: Neuroglioma is the most common intracranial tumor in China. MicroRNA (miR) has been recognized as one novel drug reagent in clinics. This study thus focused on the role of miR-129 on neuroglioma cells. Quantitative PCR (qPCR) was used to detect the expression of miR-129 in human neuroglioma cells. In neuroglioma cell line U87, miR-129 was over-expressed or silenced. Western blotting was employed to test the expression of nuclear factor (NF)-κB. Meanwhile immunofluorescent assay detected the nuclear p65 expression to confirm the correlation between miR-129 and NF-κB. Further proliferation and scratch assay were performed to detect the role of miR-129 on cellular growth of neuroglioma cells. qPCR results showed elevated expression of miR-129 in human neuroglioma cells. In glioma cell line with miR-129 overexpression, pP65 protein level was elevated. The knockdown of miR-129 led to decreased pP65 protein ($P<0.01$). Immunofluorescent assay showed the nuclear entry of pP65, indicating the activation of NF-κB signal by miR-129 over-expression. Further MTT and scratch assay showed the rapid proliferation and enhanced invasion assay of U87 neuroglioma cells under miR-129 over-expression ($P<0.01$). The silencing of miR-129 also weakened cell proliferation and mobility ($P<0.01$). MiR-129 can activate NF-κB signal in neuroglioma cells for enhancing cell proliferation.

Keywords: Neuroglioma, microRNA-129, nuclear factor-κB

Introduction

Human neuroglioma is the most common malignant intracranial tumor in neurosurgery, as it occupies more than 50% of brain cancers. Recently the population of brain glioma patients is becoming younger [1, 2]. The pathogenesis of neuroglioma involves multiple processes as affected by dozens of regulatory factors. Our knowledge about underlying factors regulating neuroglioma progression, however, is still lacked. Increasing evidences have demonstrated the critical role of microRNA (miR) in regulating the occurrence and advancement of neuroglioma [3]. As one non-coding regulatory small RNA molecule, miR can mediate the translation of target gene mRNA and the stability in cytoplasm. MiR is thus involved in a series of biological activities from cellular growth, metabolism, proliferation, differentiation, apoptosis and stem cell mediation [4, 5]. Since the first evidences showing the direct correlation between miR and tumors, more and more studies have indicated the role of miR in tumor pathogenesis and metastasis [6]. In neuroglioma, miR has a differential expression profile, with over-expression or down-regulation of different miR molecules [7].

MiR-129 is one novel member of miR family, and has been shown to have over-expression in gastric carcinoma and lung cancer, and down-regulation in breast cancer and esophageal squamous cell carcinoma, indicating its close correlation with tumor [8]. The role of miR-129 in neuroglioma, however, has not been illustrated. This study thus investigated the role of miR-129 in neuroglioma. Nuclear factor (NF)-κB is one nuclear transcription factor with pluripotent roles in multiple cells, and is commonly existed in cells as homo- or hetero-dimer. P50/p65 heterodimer is the most common binding manner. NF-κB signal pathway can regulate the expression of multiple genes for further modu-

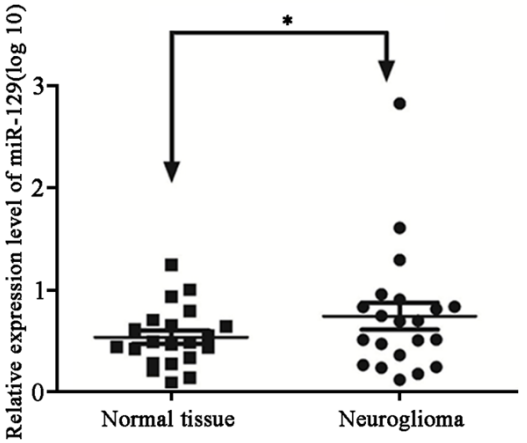

Figure 1. Expression level of miR-129 in neuroglioma tissues. *, P<0.05 compared to normal tissues.

lation of development, proliferation, differentiation, cellular growth or apoptosis and immune response [9, 10]. Hyperplasia and cell apoptosis are closely correlated with tumor occurrence. Both miR-122 and neuroglioma have been widely investigated while the role of miR-129 in neuroglioma was still unknown. This study thus aimed to investigate the role and mechanism of miR-129 in neuroglioma.

Materials and methods

Tissue collection

A total of 22 neuroglioma tissue samples were collected from surgical resection in the department of neurosurgery in our hospital from 2014 to 2015 as the experimental group, in parallel with 22 samples of normal human brain tissues collected from intracranial decompression. This study has been pre-approved by the ethical committee of Union Hospital of Tongji Medical College. Tissue samples were rinsed twice in TBS, and kept in liquid nitrogen for further use. All neuroglioma patients have been diagnosed by pathological examination.

Cell stable transfection for over-expressing or silencing miR-129

Neuroglioma cell line U87 was purchased from ATCC (US) and was cultured in DMEM medium (Sigma, US) containing 10% FBS (Hyclone, US) in a humidified 37 °C chamber (Thermo, US) with 5% CO_2. Other equipment includes: cold high-speed centrifugation (Thermo, US) and cell culture workstation (Thermo, US).

U87 cells at log-phase were seeded into 6-well plate at 5 × 10^5 per well. Lipo2000 reagent (2 μL) was mixed with 0.1 mL serum-free medium and was incubated for 5 min. Plasmids with over-expression or silencing of miR-129 (Ruibo, China) was mixed in serum-free medium for 5 min. Both transfection mixture and plasmids mixture were mixed and incubated for 20 min. 1.8 mL serum-free medium was then added into each well. After 48-hour incubation, RNA was extracted for detecting miR-129 expression level. Meanwhile empty vector was also employed in transfection as the negative control.

qPCR

Using reverse transcription test kit (TaKaRa), equal volume of tissues were mixed with Trizol reagents, and were centrifuged at 12000 g for 10 min at 4°C. Supernatants were saved and incubated for 5 min to lyse cells, followed by the addition of 0.2 mL chloroform. The mixture was vibrated for 15 sec, and was incubated for 3 min. After centrifugation for 15 min at 12000 g under 4°C, the upper phase was saved and mixed with equal volume of isopropanol. The mixture was incubated at room temperature for 10 min, and was centrifuged for 10 min at 12000 g under 4°C. The pellet was washed in 75% ethanol (in DEPC-treated H_2O) by centrifugation at 7500 g for 5 min under 4°C. RNA pellet was re-suspended in 20 μL DEPC-treated H_2O. Real-time fluorescent quantitative PCR was employed to detect the expression of miR-129 in neuroglioma using specific primers (miR-129-F, 5'-GCCAG CTACA TTGTC TGCTG GGTT-3'; miR-129-R, 5'-GTCGA GGATC CGACG TATTCC G-3'; U6-F, 5'-CGGCG GTAGC TTATC AGAAT GATG-3'; U6-R, 5'-GCAGT CGAGG TAGTA TT-3'. The reaction parameters were: 95°C perdenature for 10 min, followed by 40 cycles each containing 95°C denature for 30 sec, 55°C annealing for 30 sec, and 72°C elongation for 30 sec. The melting curve analysis was employed to detect the relative expression level.

Western blotting

Total proteins were extracted by SDS-lysis buffer on iced incubation for 10 min, and were scratched by knives. The lysate was heated for 10 min, and was centrifuged at 12000 g for 10 min. The supernatants were saved and quanti-

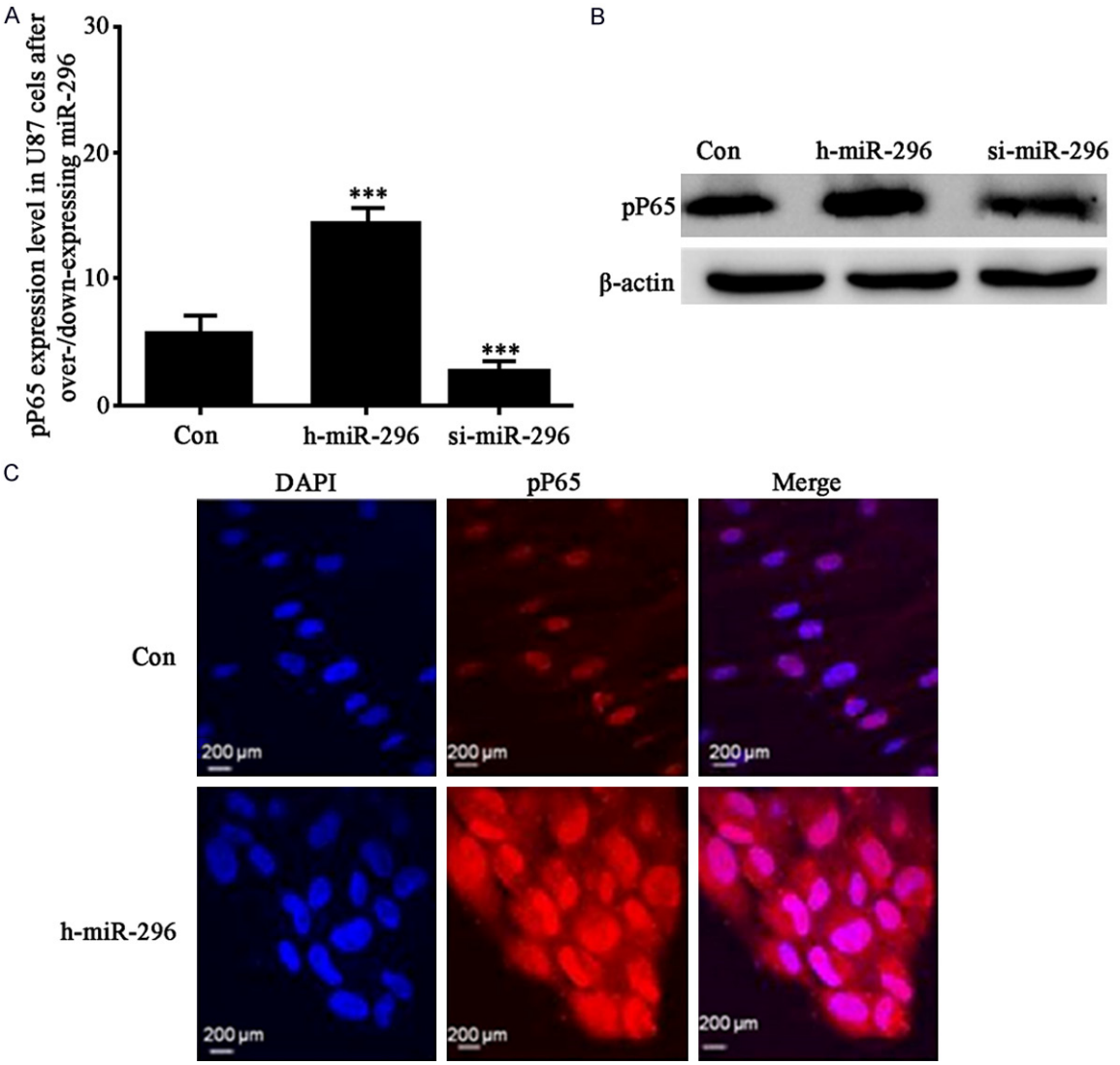

Figure 2. pP65 protein expressions in U87 cells. A. Relative expression level of pP65 in U87 cells after over-expressing miR-129 (middle) or down-regulating miR-129 (right). B. Western blotting bands showing the level of pP65 protein in U87 cells. C. Immunofluorescent assay showing the nuclear localization of pP65. ***, P<0.001 compared to control group.

fied by BCA assay. 2 μL protein sample was mixed with 18 μL PBS, and in 200 μL AB mixture. All protein samples were adjusted to equal concentration using loading buffer, which contains 5 × bromophenol blue. Protein samples were adjusted to equal concentrations, along with 6 μL protein marker. The electrophoresis started at 80 V, and continued at 120 V for separation. The PVDF membrane was rinsed in methanol for 5 min, and was applied in transfer at 250 mA field for 90 min. The membrane was then rinsed in TBST, and was blocked in 5% defatted milk powder. With gentle rinsing in TBST, primary antibody was added for overnight incubation. On the next day, the membrane was rinsed for three times in TBST (5 min each), and was mixed with secondary antibody for 1-hour incubation. The membrane was then rinsed in TBST (5 min × 3 times), and was developed in ECL chromogenic substrate (1:1 for A and B reagent).

MTT assay

Neuroglioma cell line U87 was seeded in 96-well plate at 5 × 10^3 per well and was divided into two groups (5 wells each), which were transfected with miR-129 plasmid or si-miR-129 plasmids, along with blank control group. After 12-hour post-transfection, 0.1 mL fresh medium was added. At 24, 48 and 72 hours post-transfection, 10 μL MTT reagent (5 mg/

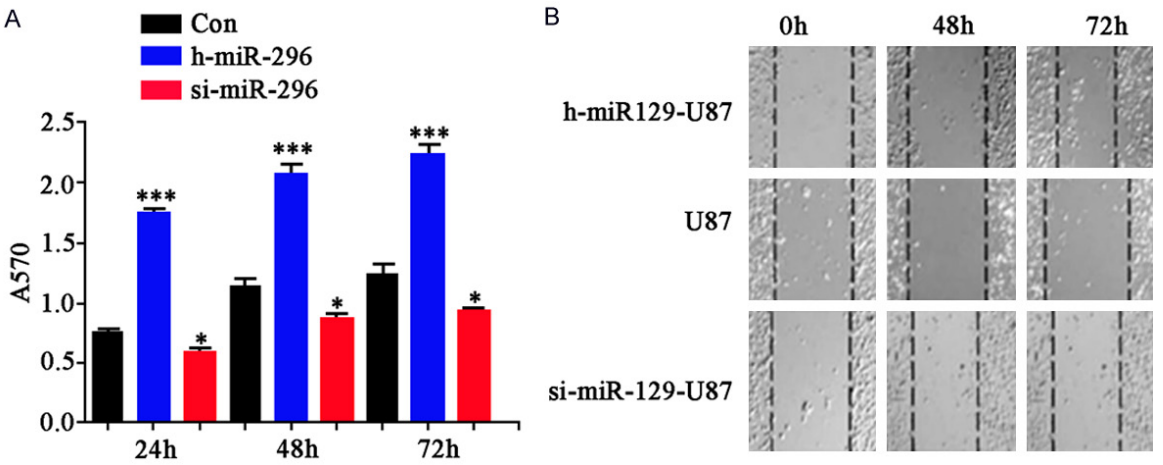

Figure 3. Effect of miR-129 expression on cell proliferation and invasive mobility of U87 neuroglioma cells. A. MTT assay showed cell proliferation ability when miR-129 was up-regulated or down-regulated. B. Scratch assay detected cell mobility under miR-129 upregulation or downregulation. **, P<0.01 compared to control group; ***, P<0.001 compared to control group.

mL in PBS, pH=7.4) were added into each well for 4-hour continuous incubation. Supernatants were discarded, and 0.15 mL DMSO was added into each well, followed by 37°C incubation for 10 min until complete resolving. Absorbance values at 490 nm were recorded from a microplate reader. The growth curve was plotted using time and absorbance values.

Scratch assay

Neuroglioma cell line U87 was transfected with miR-129 plasmid or si-miR-129 plasmids, along with blank control group. After reaching a confluence over 90%, a "+" shape scratch was made using pipette tips on the culture dish, which was further incubated at 37°C incubation. At 0, 12 and 24 hour, images were taken to calculate the healing rate of scratch, which was equal to (scratch distance at 0 h-scratch distance at 24 h)/scratch distance at 0 h × 100%.

Statistical analysis

Experimental data were expressed in mean ± standard error of means (SEM). SPSS 13.0 software was employed to compare sample means by student t-test. A statistical significance was defined when P<0.05.

Results

miR-129 was significantly up-regulated in neuroglioma cells

We employed the expression level of miR-129 in both neuroglioma and normal brain tissues by fluorescent qPCR. Results showed that after GAPDH normalization, miR-129 expression level was significantly elevated (**Figure 1**).

pP65 protein expression level of U87 cells

We further used qPCR to detect the transfection efficiency of miR-129 plasmids. As shown in **Figure 2**, expression level of miR-129 was effectively elevated by transfecting h-miR-129 plasmid, which was miR-129 overexpressing vectors (P<0.01). The stable transfection of si-miR-129 significantly depressed miR-129 expression in U87 cells (P<0.01). In those cells with successful transfection, Western blotting was further replenished to detect the level of pP65 protein, which showed significantly elevated expression of pP65 protein when miR-129 was overexpressed (P<0.01, **Figure 2B**) and depressed pP65 protein level when miR-129 expression was silenced (P<0.01, **Figure 2B**). Immunofluorescent assay also showed the nuclear translocation of pP65 in U87 cells with elevated expression of miR-129 (**Figure 2C**).

Effect of miR-129 on cell proliferation and invasive mobility of neuroglioma cells

MTT assay showed that, when miR-129 was over-expressed, cell proliferation ability was significantly enhanced (P<0.01, **Figure 3A**). The opposite effect occurred when miR-129 was down-regulated (P<0.01). Scratch assay showed significant retard of healing speed of U87 cells with miR-129 down-regulation compared to miR-129 over-expressing cells (P<

0.01, **Figure 3B**), suggesting miR-129 can stimulate the mobility of neuroglioma cells.

Discussion

Increasing number of studies have suggested the important role of miR in regulating pathogenesis and progression of neuroglioma via targeting tumor related genes [11]. Other scholars have reported the facilitation of miR on invasive ability of neuroglioma, along with the inhibitory role on tumor migration and invasion by miR [12]. For example, miR-146b has been shown to inhibit migration and invasion of neuroglioma [13]. Previous study has shown inhibitor or facilitator roles on tumors by various miR. Neuroglioma is the most common intracranial tumor. Our study has revealed the high expression of miR-129 in human neuroglioma cells. Some reports suggested the down-regulation of Cdk6 by miR-129 in mouse pulmonary cancer cell line for regulating tumor cell proliferation [14]. The expression of miR-129 in non-tumor tissues was found to be related with the lymph node metastasis of colorectal cancer [15]. The role of miR-129 in neuroglioma progression, however, is still unknown. This study thus aimed to investigate the function and possible mechanism of miR-129 in neuroglioma cells.

NF-κB is one nuclear transcription factor existed in multiple cells with pluripotent regulatory functions, and is usually formed in homo-/hetero-dimer in cytoplasm. P50/p65 heterodimer is the most common binding way for its complex. Multiple researches have shown the involvement of NF-κB in regulating proliferation and apoptosis related gene's transcription, while both apoptosis and proliferation are closely correlated with tumor pathogenesis [16-18]. NF-κB exerts its function mainly via activating IKB kinase (IKK) to phosphorylate IkB, which is one inhibitor of NF-κB to activate downstream pathway releasing p50 and p65, which were translocated into the nucleus for initiating downstream cascade reaction [19, 20]. Immunohistochemistry study has shown the nuclear location of NF-κB p65, whose activation was featured with the nuclear translocation of its dimers. As immunofluorescent assay showed nuclear localization of p65, it is suggested that miR-129 can facilitate neuroglioma growth via activating NF-κB signaling transduction pathway. We then confirmed the potency of miR-129 to facilitate growth and invasion motility on cellular level by MTT and scratch assay.

In summary, miR-129 can facilitate neuroglioma cell growth via activating NF-κB signaling transduction pathway. It has been demonstrated on cellular level that miR-129 could facilitate growth and invasion motility of neuroglioma cells, thus providing new insights for studying and treating neuroglioma. To further confirm the function of miR-129 in glioma, we need further confirmation in animal model, along with illustration on mechanisms.

Disclosure of conflict of interest

None.

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