Research Article

MicroRNA-29a-3p Regulates SH-SY5Y Cell Proliferation and Neurite Growth through Interaction with PTEN-PI3K/ AKT/mTOR Signaling Pathway

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The effects of microRNA-29a-3p in the proliferation process of nerve cells are unclear. The purpose of this study is to delve into the regulatory role of microRNA-29a-3p, via interaction with phosphatase and tension homolog (PTEN), in the SH-SY5Y cell proliferation process. Different expressions of microRNA-29a-3p in the SH-SY5Y cells were constructed by transfected miRNA-29a-3p mimic and inhibitor. The effects of cell transfection and the mRNA expressions of PTEN, Akt, and mTOR were detected by qPCR. The expressions of PTEN, Akt, and mTOR protein and the phosphorylation levels of Akt and mTOR were examined using Western blotting. Nerve cell proliferation activity and neurite length of each group were measured and examined by the use of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2Htetrazolium bromide (MTT), and morphological examination. We observed that the levels of PTEN mRNA and protein were distinctly decreased in the microRNA-29a-3p mimic group, but the expressions of the phosphorylated Akt and mTOR mRNA and protein were distinctly upregulated. In the transfected miRNA-29a-3p inhibitor SH-SY5Y cells, the expressions of miRNA-29a-3p were significantly suppressed; however, the expressions of PTEN gene and protein were significantly enhanced. The expressions of phosphorylated Akt and mTOR in the downregulated microRNA-29a-3p group distinctly were suppressed. The SH-SY5Y cell proliferation activity and neurite length in the upregulated microRNA-29a-3p group increased significantly. Our findings revealed that microRNA-29a-3p could enhance the proliferation activity of SH-SY5Y cells and promote neurite growth by inhibiting the expression of PTEN and regulating PI3K/Akt/mTOR signaling pathway.

1. Introduction

MicroRNA (miRNA) is a type of endogenous single-stranded noncoding RNA with regulatory functions found in eukaryotes. It is approximately 20–25 nucleotides in length and has been found to play a key role in many cellular development processes at the posttranscriptional level [1–3]. Growing studies have demonstrated that miRNA has an antiapoptotic effect, and current research on miRNA was mainly focused on the field of tumors [4–6]. Growing evidence has found that miRNAs were distinctly associated with the diseases and normal functions of the nervous system and also exhibited an important regulatory role in the repair and regeneration of nerve cells after injury [7–9]. Unlike the central nervous system, peripheral nerves have a certain extent of regeneration ability after injury. It has been reported that miRNA-192 could promote nerve repair in rats with peripheral nerve injury [10] which suggested that the miRNA was closely involved in the repair and regeneration processes of peripheral nerve. These results implied that miRNAs may have a potential important value for treatments of peripheral nerve injury and recovering the function of target organ. Being a liquid and protein phosphate, the phosphate and tension homolog (PTEN) acts as...
a key anticancer gene widely involved in the regulation of cell proliferation and apoptosis [11, 12]. Our research was aimed at elucidating the great significant molecular mechanisms of regulating nerve cells growth process by miRNAs through interaction with PTEN.

We have found that the expressions of miRNA-29a were the highest via biological information analysis in the regenerated nerve tissue after peripheral neurorrhaphy. Therefore, miRNA-29a may play an important regulatory role in the nerve regeneration process. There were two members in the miRNA-29a family: miR-29a-3p and miR-29a-5p according to the different processing methods of its precursors. It has been reported that the biological functions of miRNA-29a were mainly realized through the form of miRNA-29a-3p [13]. The current studies about miRNA-29a-3p were focused on tumor cell proliferation and myocardial hypertrophy. The relevant study of biological function about miRNA-29a in the nerve system was lacking. Moreover, how the miRNA-29a-3p regulated the expression of genes in the processes of nerve regeneration and its effect on nerve regeneration is still unclear.

Neurite growth is astonishingly sensitive to shallow concentration gradients, but a widely observed feature of both growth and guidance regulation, with important consequences for development and regeneration [14]. The important genes regulating the process of nerve cell proliferation and neurite growth included ATF3, mTOR, and PTEN [15, 16]. It was found that PTEN is widely expressed in the nervous system and regulated nerve cell proliferation, differentiation, migration, and apoptosis through different signal pathways. It not only participated in the regulation of the normal morphology and functions of the nervous system but also played a crucial role in the process of repair and regeneration after nerve injury [17]. Various studies had found that the inhibition of PTEN gene could promote the regeneration of damaged nerves in the central and peripheral nervous systems [18, 19]. There was a negative regulatory relationship between PTEN gene and mTOR protein. mTOR was a downstream protein of the PI3K/Akt signaling pathway in which a classic signaling pathway played a key biological activity in regulating cell proliferation and regeneration [20]. It has been reported that miRNA-26a could inhibit PTEN expression and promote axonal growth [21]; therefore, we speculated that miRNA-29a-3p could affect the PI3K/Akt/mTOR pathway through PTEN and then intervene the repair and regeneration process of the impaired nerves. This research was aimed at delving into the biological effects of miRNA-29a-3p on nerve cells and clarifying the probable specific mechanism of its action through interaction with PTEN in the signaling pathway.

2. Material and Methods

The cell bank of the Shanghai Branch of the Chinese Academy of Science provided SH-SY5Y cells. The cells were cultured in a MEM/F12 medium containing 10% FBS in a 37°C, 5% CO₂ incubator. The cells were transfected with IMAX reagent when the density was about 40%, and the transfection concentration was 40 nM. According to different transfection methods, the SH-SY5Y cells were divided into 5 groups: control, mimic-NC, mimic miRNA-29a-3p, inhibitor-NC, and inhibitor miRNA-29a-3p.

2.1. Detection of the Expressions of miRNA-29a-3p, PTEN, Akt, and mTOR by qPCR. The cells were lysed in TRIzol for three minutes at room temperature 24 hours after transfection, and then, they were centrifuged at 12000 g for fifteen minutes at 4 degrees Celsius. After combining the topmost aqueous phase with isopropanol and incubating it for four minutes at room temperature, the mixture was separated by centrifugation at a speed of 12000 g for ten minutes, while the temperature was set to four degrees Celsius. When the supernatant had been removed, the RNA-containing pellet had been washed with 75% ethanol and retrieved by centrifugation for 5 minutes at 4°C. Drying at room temperature for 10 minutes, solubilization in RNase-free water and a nucleic acid microspectrophotometer were used to determine the RNA concentration in the sample. Primer 6 and Oligo 7 were used to build primers for amplification of the miRNA sequence acquired from the National Center for Biotechnology Information (NCBI) database. Instrumentation for fluorescence quantitative PCR amplification was utilized. The two-step method of PCR amplification was applied to examine the expressions of miR-29a-3p in the reaction mixture. 48 h after transfection, the same method was applied to examine the expressions of PTEN, Akt, and mTOR. The primers are shown in Table 1.

2.2. Detection of the Expressions of PTEN, Akt, p-Akt, mTOR, and p-mTOR by Western Blotting. The SH-SY5Y cells were collected after transfection for protein extraction and quantification using a BCA kit (Solarbio Science & Technology, China). SDS-PAGE was used to separate 30 g of protein from each sample before the protein was transferred to PVDF membranes. They were blocked with 5% skimmed milk for 1 h and were probed with anti-rabbit PTEN

| Name       | Primer sequences (5’–3’) |
|------------|-------------------------|
| PTEN: forward | TGGATTCGACTTATGACCTTGACCT |
| PTEN: reverse | GGTGGGTTATGCTTCAAAAGG |
| Akt: forward | TCACCTCTGAGACCGAACCC |
| Akt: reverse | ACTGGCTGAGTACGAAGACTGG |
| mTOR: forward | ATGCTTGGAACCAGCCTG |
| mTOR: reverse | TCTCTGACACATCTCCTCAGGAGTT |
| microRNA-29a-3p: forward | CCCTAGACCATCATCTCA |
| microRNA-29a-3p: reverse | AGTGCAAGGGTCCAGGATT |
| GAPDH: forward | CTGGGCCTACATCTGAGCACC |
| GAPDH: reverse | AAGTGTCGCTTGGAGGCAAT |
| U6: forward | GCCGCGTCTGAAAGGTTGG |
| U6: reverse | GTGCGAGGTCCAGAGGT |
Measurement of Neurite Length. The SH-SY5Y cells were incubated with goat anti-rabbit secondary antibody IgG (1:10000, ab66721, Cambridge, MA, USA). ECL kit chromogenic substrate was produced and applied to the membrane for 5 minutes at room temperature. By the use of Image-Pro Plus 6.0 (version 6.0; Media Cybernetics, Rockville, MD, USA), target protein concentrations were computed and evaluated. The expressions of p-Akt/Akt and p-mTOR/mTOR were compared between different groups in the SH-SY5Y cells.

2.3. Detection of Proliferation Activity by 3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyl-2Htetrazolium Bromide (MTT) and Measurement of Neurite Length. The SH-SY5Y cells were cultured in vitro to prepare single-cell suspension at a density of 1 x 10⁷/ml, and 100 μl was added to each well in the 96-well plate. 72 h after transfection, 20 μl of MTT solution was added to each well until the incubator for 4 hours. The medium was removed, and 150 μl of dimethyl sulfoxide was added to each well. Then, the 96-well plate was shaken at low speed for 10 minutes, and the absorbance at 490 nM was measured in the microplate reader. An inverted microscope was utilized to examine and capture images 72 hours after transfection. The longest neurite in each cell was manually traced to obtain the neurite length. Each separate experiment was conducted with a total of six replicate wells, each of which included 10 cells.

2.4. Statistical Analysis. SPSS21.0 and GraphPad Prism7 statistical software were used for statistical analysis. The data were presented as mean ± standard deviation. The least significant difference (LSD) method was used to analyze the data, and comparisons between the groups were drawn. P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Detection of the Expressions of miRNA-29a-3p, PTEN, Akt, and mTOR by qPCR. The results of detecting the levels of miRNA-29a-3p in different SH-SY5Y cells of each group were exhibited in Figure 1(a). The levels of miRNA-29a-3p in the mimic miRNA-29a-3p group were the highest, and the inhibitor miRNA-29a-3p group was the lowest. There was no distinct difference among the other three groups. In the PTEN gene detection results (Figure 1(b)), the mRNA levels of PTEN in the mimic miRNA-29a-3p group were the lowest, while in the inhibitor miRNA-29a-3p group, the levels of PTEN gene were the highest, which was opposite to the results of mRNA levels of miRNA-29a-3p. The mRNA expressions of PTEN and miRNA-29a-3p in the two NC groups were similar to those in the control groups. In the detection results of the mRNA expression levels of Akt (Figure 1(c)) and mTOR (Figure 1(d)), there was no significant statistical difference in the different groups.

3.2. Detection of the Expressions of p-mTOR, mTOR, p-Akt, and PTEN. The different protein expressions of p-mTOR, mTOR, p-Akt, and PTEN in different groups were examined by the use of Western blotting assays. The PTEN protein levels were distinctly reduced in the mimic miRNA-29a-3p group and significantly increased in the inhibitor miRNA-29a-3p group, which was consistent with the qPCR results. There were statistical differences in the expression level of PTEN protein in the two different groups compared to the control group. There were no distinct differences in the levels of PTEN protein among the other three groups (Figure 2).

The expressions of Akt protein in each group were examined by the use of Western blot, and there was no distinct difference between each group (Figures 3(a) and 3(b)). The phosphorylation level of Akt was further detected. The levels of phosphorylated Akt (p-Akt) protein in the mimic miRNA-29a-3p group were the highest, and in the inhibitor miRNA-29a-3p group, the levels of phosphorylated Akt protein were the lowest, which were significant statistical differences compared to control group. There was no significant difference in the expressions of phosphorylated Akt between control group and NC group (Figure 3(c)). The ratio of phosphorylated Akt/total Akt was also detected in the different groups, and the whole tendency in the ratio of p-Akt/Akt in the different groups was consistent with the results of p-Akt expression level in the different groups (Figure 3(d)). The ratio of p-Akt/Akt in the mimic miRNA-29a-3p group was the highest, followed by the control group, and the ratio of p-Akt/Akt in the inhibitor miRNA-29a-3p group was the lowest. There was a statistical difference in the ratio of p-Akt/Akt between mimic miRNA-29a-3p group and inhibitor miRNA-29a-3p group, which also was a statistical difference when compared to NC group and control group (P < 0.05).

Similarly, the expressions of mTOR protein and p-mTOR were detected through Western blotting assay. The mTOR and p-mTOR protein stripe images in the different groups are shown in Figure 4(a). There was no distinct difference in the levels of mTOR protein in the different groups. The relative expressions of p-mTOR in the mimic miRNA-29a-3p group were the highest, and the relative expressions of p-mTOR in the inhibitor miRNA-29a-3p group were the lowest, which were distinct statistical differences compared to control group (Figure 4(b)). The phosphorylated mTOR protein level significantly decreased when the expression of miRNA-29a-3p had been inhibited (Figure 4(c)). The expressions of p-mTOR/mTOR in these different groups were in line with the expressions of p-mTOR in these different groups (Figure 4(d)).

3.3. Detection of SH-SY5Y Cell Proliferation Activity by MTT and Neurite Length Measurement. The results of neurite length and proliferation activity in different groups are exhibited in Figures 5(a) and 5(b). The microscopic images of nerve cell proliferation process and morphology in the different groups are exhibited in Figure 5(a). The cellular proliferation activity of each group was examined by MTT assays. The proliferation activity of SH-SY5Y cells in the mimic miRNA-29a-3p group was the highest, which was
Figure 1: RT-PCR for the mRNA levels for miRNA-29a-3p, PTEN, Akt, and mTOR. (a) The expression of miRNA-29a-3p increased in the mimic miRNA-29a-3p group and decreased in the inhibitor miRNA-29a-3p group (***: p < 0.001). (b) The expression of PTEN was the lowest in the mimic miRNA-29a-3p group and in the inhibitor miRNA-29a-3p group; the expressions of PTEN gene were the highest (***: p < 0.001). (c and d) There was no distinct difference in the mRNA expression of Akt and mTOR between the groups (p > 0.05).

Figure 2: Relative expressions of PTEN. The expression of PTEN protein was decreased in the mimic miRNA-29a-3p group, while in the inhibitor miRNA-29a-3p group, the expression of PTEN protein was increased (***: p < 0.001). For each different group, the SH-SY5Y cells were separately repeatedly measured 6 times.
higher than the proliferation activity of SH-SY5Y cells in the control group and NC group. The proliferation activity of SH-SY5Y cells in the inhibitor miRNA-29a-3p group was the lowest, and there was a statistical difference in the nerve cell proliferation activity compared to control group and NC group (Figure 5(b)). The neurite length of distinct SH-SY5Y cells was found to be compatible with the results of cell proliferation activity in various groups. The neurite length in the mimic miRNA-29a-3p group was the longest, while the neurite length in the inhibitor miRNA-29a-3p group was the shortest. The relevant results of neurite length in the above two groups were both have significant statistical difference compared to NC group and control group (\(P < 0.001\)) (Figure 5(c)).

4. Discussion

miRNA was a class of endogenous small single noncoding RNA with important regulatory functions in eukaryote. miRNA regulated about one-third of human gene expression and a series of important life process including cell proliferation, differentiation, development, and apoptosis, involved in various pathophysiological processes in humans [3]. At present, the research about miRNA was mainly concentrated in the field of tumor. More and more studies have found that miRNA was closely related to the normal function of nervous system and the occurrence of nerve diseases with an important regulatory function in the repair and regeneration after nerve damage. Liu et al. found different expressions of miRNA after 4 h of T10 spinal cord injury rat model through miRNA chip [22]. Nakanishi et al. found differences in the miRNA expression after 6 h and 12 h in the compression of the spinal cord in mice T11-12 through gene chip [23]. Unlike the central nervous system, peripheral nerve had a certain degree of regenerative ability after injury. After peripheral nerve injury, the expression of miRNA in the denervated muscles would change accordingly and participate in the process of nerve repair. Taylor et al. found...
that the expression of miRNA-206 increased significantly after sciatic nerve injury, and the increased miRNA-206 targeted regulation of the key protein expression in nerve regeneration signaling pathways and promoted the regeneration of neuromuscular synapses [24]. Zhou et al. injected miRNA-9 agonists into the rat models of sciatic nerve injury, and in vivo experiments found that highly expressed miRNA-9 inhibited the migration of Schwann cell and the regeneration of sciatic nerve [25]. Therefore, exploring the molecular mechanism of miRNA regulation nerve regeneration is of great significance for repairing nerve damage and promoting nerve regeneration.

We detected the expressions of different miRNAs in the regenerative nerve tissue after peripheral nerve anastomosis in the rat model through biological information analysis using TargetScan software and found that the expressions of miRNA-29a in regenerated nerve tissue were the highest. It was reported that the expressions of miRNA-29a were positive correlation to nerve growth factor (NGF) in vitro cultured PC12 cells with different concentrations of NGF. Therefore, we inferred that miRNA-29a may have an important regulatory role in the process of nerve regeneration. miRNA-29a was divided into miRNA-29a-3p and miRNA-29a-5p, according to the different processing methods of its precursors. The expressions of miRNA-29a-3p in cells were higher than miRNA-29a-5p, and miRNA-29a-3p was widely studied in the tumor proliferation and cardiomyocyte remodeling [13]. The relative research about miRNA-29a-3p in the nervous system and nerve regeneration was little [26, 27]. In vitro, the expressions of miRNA-29a-3p were, respectively, evaluated in the miRNA-29a-3p mimic transfected SH-SY5Y cells and miRNA-29a-3p inhibitor transfected SH-SY5Y cells using qPCR. The qPCR confirmed that the expressions of miRNA-29a-3p in the mimic group was distinctly higher than that in the control group, while the levels of miRNA-29a-3p in the inhibitor group was distinctly decreased, less than that in the control group.
It was found that PTEN was a direct target of miRNA-26a through luciferase reporter assay. Ma et al. reported that miRNA-29a-3p was involved in the progression of liver cancer through negative regulation of PTEN [28]. PTEN, being an important anticancer gene, had a lipid and protein dual phosphatase activity as a distinctly negative regulation factor in PI3K/Akt pathway [29]. PTEN was widely expressed in the nervous system and was involved in the repair and regeneration for nerve injury [17]. Park et al. found that nerve cell regeneration activity was significantly enhanced in the optic nerve impaired mice when the PTEN gene was knocked out [30]. Gallaher and Steward reported that reducing the expressions of the PTEN gene could enhance nerve regeneration after crush injury of the sciatic nerve in mice [31]. PTEN had been determined to be a direct target of miRNA-26a in vascular smooth muscle cell (VSMC) injury of patients involved in the PI3K/Akt pathway. Therefore, we inferred miRNA-29a-3p regulation nerve cell repair and regeneration after nerve injury by interaction with PTEN.

Bioinformatics analysis using TargetScan software 7.2 showed that the 676-683 specific zone in 3′-UTR of PTEN could be matched with the sequence of miRNA-29a-3p. The overexpression of miRNA-29a-3p in the miRNA-29a-3p mimic transfected SH-SY5Y cells decreased the expression PTEN protein. The downregulated PTEN increased phosphorylation and activation of PI3K, an important protein in the proliferative and antiapoptotic pathways. When the miRNA-29a-3p inhibitor was transfected in the SH-SY5Y cells, targeting miRNA-29-a-3p in the nerve cells, it could notably increase the expression of PTEN. The upregulated PTEN decreased the phosphorylation and activation of PI3K to suppress proliferative impact and promote nerve regeneration after nerve injury by interaction with PTEN.

Figure 5: Quantitative analysis of neurite length and the proliferation activity in different groups. (a) Morphological observation for micrographs of SH-SY5Y cells and neurites. (b and c) The histogram revealed the attenuation 570 values and the neurite length of SH-SY5Y cells in different groups. After transfection with mimic miRNA-29a-3p, the cell proliferation activity was significantly increased, and the neurites became significantly longer. While in the inhibitor miRNA-29a-3p group, the SH-SY5Y cell proliferation activity and neurite length were minimum (***: p < 0.001; **: p < 0.01; *: p < 0.05). For each different group, the SH-SY5Y cells was separately repeatedly measured 6 times.
cells apoptosis. mTOR is a downstream protein of the PI3K/Akt signaling pathway. As a major signaling pathway, PI3K/Akt/mTOR plays a key biological activity in regulating cell proliferation and apoptosis [32, 33]. Therefore, miRNA-29a-3p, being an important positive regulator, could activate PI3K/Akt/mTOR pathway by reducing the expression of PTEN, promoting nerve cell proliferation and neurites growth in the process of nerve repair.

Our findings indicated that the overexpression of miRNA-29a-3p exerted a protective effect by reactivating the PI3K/Akt/mTOR signaling pathway. The expressions of p-Akt, Akt, p-mTOR, and mTOR proteins in each group of SH-SY5Y cells were similar, and there was no significant difference among different groups, indicating that miRNA-29a-3p did not affect the total mRNA level of key proteins in the PI3K/Akt/mTOR signaling pathway. The expressions of p-Akt, Akt, p-mTOR, and mTOR proteins in each group of cells were detected, and the ratio was calculated. The phosphorylation levels of Akt and mTOR proteins significantly increased the activity of proliferation and regeneration for the SH-SY5Y cells. The Western blot assays demonstrated that the upregulation of miRNA-29a-3p could negatively regulate the expression of PTEN protein and enhance the phosphorylation of Akt and mTOR proteins. The proliferation activity and neurite length for SH-SY5Y cells in each group were tested, and the results indicated that the overexpression of miRNA-29a-3p could enhance the nerve cell proliferation ability and promote neurite growth. These findings suggested that miRNA-29a-3p may be a therapeutic target for the treatments of peripheral nerve injury in clinical practice.

5. Conclusion

The present study indicated that the upregulation of miRNA-29a-3p enhanced the proliferative activity of SH-SY5Y cells and promoted neurite growth by downregulating the expression of PTEN to reactivating the PI3K/Akt/mTOR signaling pathway. These study results supported the hypothesis that enhancing the expression of miRNA-29a-3p may be a desirable therapeutic approach for the treatment of peripheral nerve injury. However, our results were based on in vitro experiments. More in vivo experiments were needed to further confirm our findings prior to clinical application.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

All the authors declare no conflict of interests.

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