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Original article

MiR-93-5p targeting PTEN regulates the NMDA-induced autophagy of retinal ganglion cells via AKT/mTOR pathway in glaucoma

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ABSTRACT

Background: Glaucoma is hallmarked with the death of retinal neurons in the ganglion cell layer, which results in irreversible vision loss. The abnormal levels of miRNA have been associated with glaucoma. Our study purposed to explore the underlying molecule mechanism of miR-93-5p in NMDA-induced glaucoma.

Methods: The Sprague-Dawley (SD) rats were used for the establishment of glaucoma model with the injection of NMDA. Vision behavior test were performed on the glaucoma rats. MiR-93-5p expression was determined by real-time PCR. The levels of autophagy-related protein and PTEN were assessed by Western blot assays. TUNEL assay and flow cytometry were performed to analyze cell apoptosis in vivo and in vitro, respectively. And cell viability was examined by CKK-8 assay. The relationship between miR-93-5p and PTEN was confirmed by Dual-Luciferase reporter gene system.

Results: NMDA-induced glaucoma rats exhibited less time in the dark box, suggesting the recession of their vision. Moreover, the retinal ganglion cell (RGC) viability was reduced not only in the glaucoma rat models but also in the glaucoma RGC models. The autophagy-related protein was obviously increased in the NMDA-treated rats or RGCs. PTEN regulated the autophagy of RGCs through AKT/mTOR pathway in NMDA-treated RGCs. MiR-93-5p could target regulate PTEN negatively, and exhibit the similar effect of 3-MA on the survival of RGCs.

Conclusion: Up-regulation of miR-93-5p binding with PTEN suppressed the autophagy of RGCs through AKT/mTOR pathway in NMDA-induced glaucoma.

1. Introduction

Glaucoma is the second frequently leading cause of sight loss in the word after cataract, and the character of which is the progressive and irreversible vision loss resulted from the injury of optic nerve [1]. Pathological increase of intraocular pressure and insufficient blood supply of optic nerve are the pathogenetic risk factors of glaucoma. It is evaluated that more than 60 million people are impacted from glaucoma-caused blindness worldwide [2]. All forms of glaucoma are with a common and crucial pathophysiology element that is the death of retinal ganglion cells (RGCs), and the visual information was conveyed by neurons from retina to the brain [3]. It was demonstrated that *N*-methyl-D-aspartate (NMDA) receptors was over-stimulated conducing significantly to the apoptosis of RGCs not only in vitro but also in the animal models [4]. Hence, it was urgent to explore the novel therapy that protects against the death of RGCs induced by NMDA in glaucoma.

Autophagy, a stress response to the self-digestion of cells, serves on a vital role in homeostatic recycling mechanism of cells contributing to degrade the unnecessary or damaged organelles and proteins in still living cells [5]. Moreover, autophagy is with the feature of appearing large intracellular vesicles and strict control of autophagy-related protein. Sever evidences indicated that phosphatase and tensin homolog (PTEN) was involved in the autophagy. In Hepatocellular Carcinoma cells, PTEN activated by DNA damage induced the benefit autophagy in reaction to Cucurbitacin B [6]. PTEN up-regulated by the activation of Redox factor-1/Egr-1 promoted the apoptosis and autophagy-induced damage in lung cancer cells [7]. However, the mechanism relationship between PTEN and autophagy remained unclear in RGCs of glaucoma.

MicroRNA (miRNA), a kind of non-coding RNA with the length of about 20–22 NT, could negatively regulate gene at the post-transcriptional level via binding with the 3′-UTR of targeted mRNA on cell physiological process including the differentiation, proliferation and apoptosis of cells [8]. MiR-29a can inhibit scleral fibroblast and retinal pigment epithelium cell expression and secret MMP-2 [9]. It is no

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exception that miR-93 plays the important roles in many physiological processes and its deregulation was involved in many diseases. MiR-93 targeting Ang 2 modulated angiogenesis and lymph angiogenesis associated with the pathogenetic mechanism of malignant pleural effusion [10]. MiR-93inducd the autophagy leading to increase the sensitivity of pediatric leukemia cells to oxidative stress via binding the 3′-UTR of Beclin-1 mRNA [11]. According to the bioinformatics analysis, miR-93-5p was predicted to have the bind site on PTEN. Hence, we speculated that miR-93-5p might participate in the molecular mechanism of autophagy in the RGCs involving PTEN.

2. Methods

2.1. Experimental animals

Thirty-five Sprague-Dawley (SD) rats (gender: both sexes, weight: 200–250 g, age: 8–12 weeks) were provided by the Animal Center of Tongji University. SD rats were raised in the individual cages with the standard laboratory conditions that the temperature was held at 23–25 °C and free food and water were offered under the 12 h cycle of light and dark. All SD rats of our study were normal on both ophthalmic and systemic test and were suffered from acclimatization for a week before daily treatment. Experiments on animals were performed as national regulations and obtained the approval of the experimental animal ethical committee of Shanghai East Hospital of Tongji University.

2.2. Study scheme

The glaucoma rat model was established via NMDA-induced RGC death. SD rats were randomly divided into two groups: the sham group (n = 10) and the NMDA group (n = 2). The handlings were given as follows (Fig. 1A):

Day 0: the sham group with intravitreous injection of PBS, the NMDA group with intravitreous injection of NMDA.

Day 1: five rats were killed in NMDA group, and the retinas of them were harvested and used to real-time PCR and Western blot assays.

Day 2: five rats were killed in NMDA group, and the retinas of them were harvested and used to real-time PCR and Western blot assays.

Day 3: five rats were killed in NMDA group, and the retinas of them were harvested and used to real-time PCR and Western blot assays.

Day 4: the last 10 rats in NMDA group and 5 rats in the sham group were performed with visual behavior test. Then five rats were killed in NMDA group, and the retinas of them were harvested and used to real-time PCR and Western blot assays.

Day 5: the last 5 rats in NMDA group and the rats in the sham group were killed and the retinas of them were harvested and used to TUNEL, real-time PCR and Western blot assays.

For the establishment of glaucoma rat model, the rats were injected intraperitoneally with 80 mg/kg ketamine and 12 mg/kg xylazine (Ilium Troy Laboratories, P.L, Australia) mixture to be anaesthetized. NMDA deliquesced by 0.1 M PBS at a concentration of 160 nmol was injected intravitreally in both eyes of rats, followed by the application of polymyxin and neomycin ointment [4].

2.3. TUNEL assay

The retinas were isolated from the eyes to detect the apoptotic cells in them. Apo-BrdU-IHCTM In Situ DNA Fragmentation Assay Kit (Biovision, USA) was used to the TUNEL assay in accordance with the manufacture's protocol [12]. The slides were handled with a sequence of dehydration and rehydration. The antigen was harvested again by Proteinase K deliquesced in 10 mM Tris (1:100, pH 8) for 20 min, and then be washed for 3 min in PBS. After subjected to the treatment of 100 μl of 3% H_2O_2 deliquesced by methanol at the volume ratio of 10% for 5 min, the sections were rinsed. And then the sections were maintained with Br-duTP at 37 °C overnight. After rinsed by PBS again, the sections were maintained with Anti-Brdu in the dark and humid chamber room. Sections with DBA solution were maintained for 15 min, followed by rinsed in PBS for 2 min 3 times. Methyl green was used for counterstaining and mounting. Light microscope was applied to observe the slides and the TUNEL positive cells were calculated in IMAGE J software (National Institutes of Health, MA, USA).

2.4. Visual behavior test

A dark-light box used for Visual behavior test, which was made up of a dark chamber (0.3 m × 0.5 m × 0.5 m) with the illumination of infrared light and a bigger white chamber (0.5 m × 0.5 m × 0.5 m) with the illumination of bright white light, was customized from Metronet Technology Ltd (Hong kong, China). There was a 10 cm × 12 cm aperture between two chambers of the dark-light box used for the free traverse of rats from one chamber to another. The activities of rats were captured by the camera of each chamber, which were connected to a Noldus recorder and monitor (German). Noldus EthoVision XT 8.0 software was used for the time of rats spending in the dark chamber was counted [13].

2.5. Cell culture and transfection

The rat retinal ganglion cells were obtained and cultured as described before [14,15]. Briefly, the retinol tissue isolated from the rats eyeball was incubated with 0.125% trypsin-EDTA and 15 U/ml papain solution (Worthington, NJ, USA) to digestion for 20 min at 37 °C. After centrifugation with 1000 r/min for 5 min, the remained cells were washed by PBS 2 times following the removal of supernatant. The appropriate DMEM complete culture solution was added into the cells to resuspension, followed by the suspension was then maintained in the culture vessel coated with rat anti-mouse Thy 1.1 antibody (1:50, abcam, USA) for 30 min at 37 °C. After rinsed by PBS 3 times, the 0.125% trypsin gently rinsed the culture vessel and DMEM complete culture solution terminated the digestion. After centrifugation with 1000 r/min for 5 min, the cells were grown in the petri dishes coated by poly D-lysine at 37 °C with a humid 5% CO_2. Half of the media was changed every third day.

MiR-93-5p mimic/miRNC (Negative control) and Si-PTEN/SiRNA NC (Negative control) which were synthesized by RiboBio (Guangzhou, China), were diluted with Opti-MEM and then mixed with Lipofectamine 2000 (Invitrogen, CA, USA) and then added into culture solution to transfect retinal ganglion cells.

2.6. The detection of miR-93-5p expression

Trizol reagent (Invitrogen) was chosen to isolate the total RNA. Then the PrimeScript RT Enzyme mix kit (Takara) was used to reverse transcription to obtain the cDNA. The prepared cDNA was used with Fast SYBR Green PCR kit (AppliedBiosystems, CA, USA) to quantitative real-time PCRonABI PRISM 7300 RT-PCR system (Applied Biosystems, CA, USA). $2^{-\Delta\Delta Ct}$ method was used to carry out the related quantitative expression of RNA.

2.7. Western blotting assay

The performance of western blotting assay was in accordance with previous report [16]. The antibodies against PTEN, pAKT, AKT, pMTOR, MTOP, LC3-I/II, Beclin-1, c-caspase3 and β-actin acted as loading control, were purchased from Cell Signaling Technology (Boston, MA, USA). The protein bands were imaged by a LI-COR Odyssey System (LI-COR Biotechnology, USA) and quantified in the IMAGE J software.

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