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Original Article

NDUFA4 enhances neuron growth by triggering growth factors and inhibiting neuron apoptosis through Bcl-2 and cytochrome C mediated signaling pathway

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Abstract: Dandy-Walker malformation (DWM) is the most prevalent congenital malformation in cerebellum, however, pathological mechanism of DWM has not been fully clarified. This study aims to investigate effects of *NDUFA4* on growth of neurons. LV5-NDUFA4 and LV3-NDUFA4-RNAi lentivirus were constructed and transfected to neurons. Ciclosporin A, together with the two lentivirus were applied to neurons to observe neuron growth, apoptosis, and related protein expression. MTT assay was used to observe neuron growth. Apoptosis was detected by using flow cytometry assay. Real-time PCR was utilized to examine NDUFA4 mRNA expression. Western blot and immunohistochemistry assay were used to detect nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), brain fibroblast growth factor (bFGF) and cytochrome C (Cyt C) expression. Results indicated that NDUFA4 significantly enhanced neuron activity and inhibited neuron apoptosis ($P<0.05$). NDUFA4 significantly increased Bcl-2 and decreased cleave caspase-3 expression compared to normal control group ($P<0.05$). NDUFA4 up-regulated growth factors, including NGF, BDNF, bFGF and Cyt C and inhibited Cyt C expression. NDUFA4 interfere inhibits antagonistic effect of ciclosporin A on apoptosis and decrease up-regulative effect of ciclosporin A on neuron growth. NDUFA4 over-expression enhances antagonistic effect of ciclosporin A on apoptosis and increases up-regulative effect of ciclosporin A on neuron growth. In conclusion, NDUFA4 enhances neuron growth by triggering NGF, BDNF and bFGF expression, inhibits neuron apoptosis by increasing Bcl-2 expression and decreasing cyto C expression. Meanwhile, NDUFA4 regulates the antagonistic effect of ciclosporin A on apoptosis and the up-regulative effect of ciclosporin A on neuron growth.

Keywords: Dandy-Walker malformation, *NDUFA4* gene, growth factor, ciclosporin A, apoptosis

Introduction

Dandy-Walker malformation (DWM) is the most prevalent congenital malformation in the cerebellum, which is also characterized as various meningeal anomalies, cerebellar hypoplasia, cystic dilatation of the fourth ventricle, and the occipital skull defects [1, 2]. The occurrence of the DWM even achieves to the 1/2500-1/5000 of the live births according to the previous studies [3, 4]. The DWM presents in the life early, about 80% to 90% within the first year with the characteristics of the raised intracranial pressure in clinical [5]. Generally, the DWM is composed of a large and complex of disorders, including the classical type of DWM (also named as isolated DWM, DWI), Dandy-Walker variant (DWV) and Dandy-Walker syndrome (DWS). It's well known that the DWS is closely correlated with the genetic mutations, such as FOXC1, ZIC1 and ZIC4 [6], and the other systemic anomalies, such as the cardiovascular malformation [7]. The MWV comprises the cystic posterior mass with the cerebellar vermis variable hypoplasia and the posterior fossa without enlargement [8]. However, among these three types of DWM, the pathological mechanism of DWI has not been fully clarified till now.

In the recent years, the chromosome microarray analysis (CMA) technology has been extensively applied in discovering the microdeletion and micro-duplication in some of the genomic disorders [9, 10]. Nowadays, the novel developed next generation sequencing (NGS) technology was also used in finding pathological gene sites in some of the diseases [11]. In our previous study [12], we used the CMA technology together with NGS technology, and discovered a critical region including NDUFA4 gene, which is associated with the DWM and is restricted to the 7p21.3. Another study [13] of our team showed that the NDUFA4 gene was insufficiency in chromosome 7p21.3 region of the DWM patients. The NDUFA4 protein is a critical nuclear DNA-encoded sub-unit, which is also associated with the mitochondrial complex enzyme IV (cytochrome C complex). What's most important is that the NDUFA4 mainly distributes in the brain [14]. We speculated that the amounts of the NDUFA4 gene may lead to the DWM mediated by the abnormal OXPHOS pathway. Therefore, we hold the view that NDUFA4 could act as a potential candidate for the DWM according to it's function and the expressing pattern.

In this study, we established the NDUFA4 gene over-expressed and NDUFA4 gene knockout lentivirus vectors, and both of which were used to infect the isolated neuron. In the following experiments, the effects of NDUFA4 gene on the neuron proliferation, neuron apoptosis, growth factors involving in neuron growth were investigated in this study.

Materials and methods

Neuron isolation and identification

The neuron was isolated from Sprague-Dawley rats brains (post-natal 24 h). The neuron was mixed and then distributed into cell culture plates (Corning Costar, NY, USA) at a density of 1.5×10^6 cells/well. Briefly, the rat brains were isolated and immersed into the ice-cold Hanks's salt solution (HSS) without Ca^{2+}/Mg^{2+}, and adjusting pH 7.5. Then, the meninges was removed, and the cerebral cortical regions were dissected and dissociated by using 0.25% trypsin (Sigma-Aldrich, St. Louis, Missouri, USA) and DNase I (100 U/mL, Gibco BRL.Co.Ltd., Grand Island, New York, USA) for 30 min. The cell fraction was suspended in Dulbecco's Modified Eagle Medium F12 (DMEM, Gibco BRL.Co.Ltd., Grand Island, New York, USA) containing 10% fetal bovine serum (FBS, ZSJQ Bio. Inc., Beijing, China), 1% ITS (Sigma-Aldrich, St. Louis, Missouri, USA), 0.1% streptomycin (100 U/ml, North China Pharmaceutical Factory, Shijiazhuang, China) and 0.1% penicillin (100 U/ml, North China Pharmaceutical Factory, Shijiazhuang, China). Subsequently, the neurons were seeded on the poly-l-lysine-coated six-well plates (Corning Costar, NY, USA) at a density of 1.5×10^6 cells/well. About 4 to 6 hous, the DMEM was substituted with the Neurobasal medium (Gibco BRL.Co.Ltd., Grand Island, New York, USA), including 0.5 mM GlutaMax, 1% sp, and 2% B-27 (Gibco BRL. Co.Ltd., Grand Island, New York, USA) for 12 h. Finally, the neurons were used for the following experiments.

Construction of LV5-NDUFA4 and LV3-NDUFA4-RNAi plasmids

Lentivirus vector for rat NDUFA4 gene encoding a GFP sequence was constructed by Western Bio. Tech. (Chongqing, China). The NDUFA4 gene sequence is listed as the followings: sense strand, 5'-GAATCGGCGGCCGCGCCACCATGCTCCG-3', anti-sense strand, 5'-GCTATGGGATCCTTAGAAGTCTGGGCCTTCTTTTTCAGTTTGCTG-3'. For the lentivirus vector for targeting NDUFA4 encoding GFP was also constructed by Western Bio. Tech. The target shRNA sequence is 5'-GGAACAAACTGGGTCCCAATG-3'. The following siRNA sequences targeting the NDUFA4 sequence were designed and illustrated as sense strand, 5'-GATCCGGAACAAACTGGGTCCCAATGTTCAAGAGACATTGGG ACCCAGTTTGTTCCTTTTTTG-3', and ant-sense strand, 5'-AATTCAAAAA AGGAACAAACTGGGTCCCAATGTCTCTTGAACATTGGGACCCAGTTTGTT CCG-3'. The blank lentiviral vector expressing GFP was considered as the negative control according to the previous study described [15].

Lentivirus package

The 293T cells purchasing from ATCC cell bank (Manassas, VA, USA) were plated in 6-well plates and transiently transfected with LV5-NDUFA4 (0.5 μg) and LV3-NDUFA4-RNAi plasmids (1.5 μg) per well, by employing the calcium phosphate precipitation method according the previous reported [16]. In this study, the packaging system includes the LV5/LV3 lentivirus (with GFP tag), PG-p1-VSVG, PG-P2-REV and

PG-P3-RRE, and the later three with virus packaging elements.

LV5-NDUFA4 and LV3-NDUFA4-RNAi lentivirus transfection

Then, the virus titer of the LV5-NDUFA4 and LV3-NDUFA4-RNAi lentivirus were tested, and were titered to 2×10^8 TU/ml as previously reported [17]. The neurons were seeded in the six-well plates before the lentivirus transfection. Then the lentivirus (0.1 ml) were mixed with the 0.1 ml complete medium, which contains 8 mg/ml polybrene (Sigma-Aldrich, St. Louis, Missouri, USA). The neurons were incubated with the above prepared lentivirus togenther at 37°C for 1 h. Frequently, the neurons were incubated with the fresh complete medium (containing polybrene with the final concentration of 8 mg/ml) for 24 h, and then incubated with the complete DMEM medium for 48 h. Finally, the neurons were harvested and applied in the following experiments.

Ciclosporin a treatment, lentivirus transfection and trial grouping

In order to investigate the mechanism for the NDUFA4 gene deficiency or deletion, the mitochondria associated apoptosis factor, cytochrome C (Cyt C) was studied. The Cyt C specific inhibitor, ciclosporin A [18], was applied to the neurons in this study. The neurons were treated with the ciclosporin A at the final concentration at 0.2 μg/L, 2 μg/L and 20 μg/L, and assigned as the 0.2 μg/L Ciclosporin A, 2 μg/L Ciclosporin A and 20 μg/L Ciclosporin A group, respectively. Meanwhile, neurons were treated with the LV5-NDUFA4 and LV3-NDUFA4-RNAi lentivirus, together with 0.2 μg/L, 2 μg/L and 20 μg/L Ciclosporin A, and assigned as the NDUFA4 RNAi+0.2 μg/L Ciclosporin A, NDUFA4 RNAi+2 μg/L Ciclosporin A and NDUFA4 RNAi+20 μg/L Ciclosporin A group, respectively. Moreover, the neurons without treatment was assigned as normal control group (NC group), neurons infected with LV5-NDUFA4 lentivirus was assigned as NDUFA4 group and neurons infected with LV3-NDUFA4-RNAi lentivirus was assigned as NDUFA4-RNAi group.

MTT assay

The neurons were cultured in the 96-well plates and transfected with LV5-NDUFA4 and LV3-NDUFA4-RNAi lentivirus due to above methods. The neuron viability was evaluated by observing the conversion of the MMT (Sigma-Aldrich, St. Louis, Missouri, USA) to the formazan product. About 24 h post-transfection, the MTT was added into the 96-well plates to achieve the final concentration of 5 mg/ml, and the neurons were incubated with MTT for 4 h at 37°C. Finally, the MTT reaction was terminated by utilizing the DMSO (200 μl) to dissolve formazan products. The 96-well plates were read by using the micro-ELISA plate reader (Bio-Tek Inc., Winooski, VT, USA) at 490 nm. Every MTT assay was performed at least six wells for duplication.

Apoptosis detection by using flow cytometry assay

The neuron apoptotic status was examined by employing the 7ADD/PE flow cytomery apoptosis detection kit (Beyotime Biotech. Inc., Beijing, China) with the PE/7-AAD double-staining according to the manufacturer's instruction. The neurons were harvested by utilizing the 0.25% trypsin, and rinsed for three times with the PBS, and then suspended with 500 μl binding buffer. The suspended neurons were incubated with the Annexin V-PE (5 μl) for 10 min at 4°C. Then, the neurons were incubated with the 7-ADD solution (10 μl) for 5 min at 4°C. Finally, the neuron apoptosis was analyzed by employing the FACS Vantage SE flow cytometer (Becton Dicknson Biosciences Inc. Brea, IN, USA).

Real time PCR

The total cellular RAN were extracted from the neurons by employing the Trizol kit and chloroform (Shanghai Chemical Reagent Factory, Shanghai, China) according to the previous study described [19]. Then, the SuperScipt first strand synthesis kit (Western Biotech. Inc. Chongqing, China) was used to synthesize the cDNA due to the manufacturer's instruction. Finally, cDNA was amplified by utilizing the PCR assay under the following conditions: 94°C for 4 min, following with 35 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 30 s, and terminated with 72°C for 10 min. The primers for the NDUFA4 were listed as the followings: forwards, 5'-GGGCAAGCCAAGAAACATC-3', reverse, 5'-GCTCTGGGTTATTCTTCCTGTC-3'. The primers for β-actin were listed as the followings: forwards, 5'-CCCATCTATGAGGGTTACGC-3', reverse, 5'-TT-

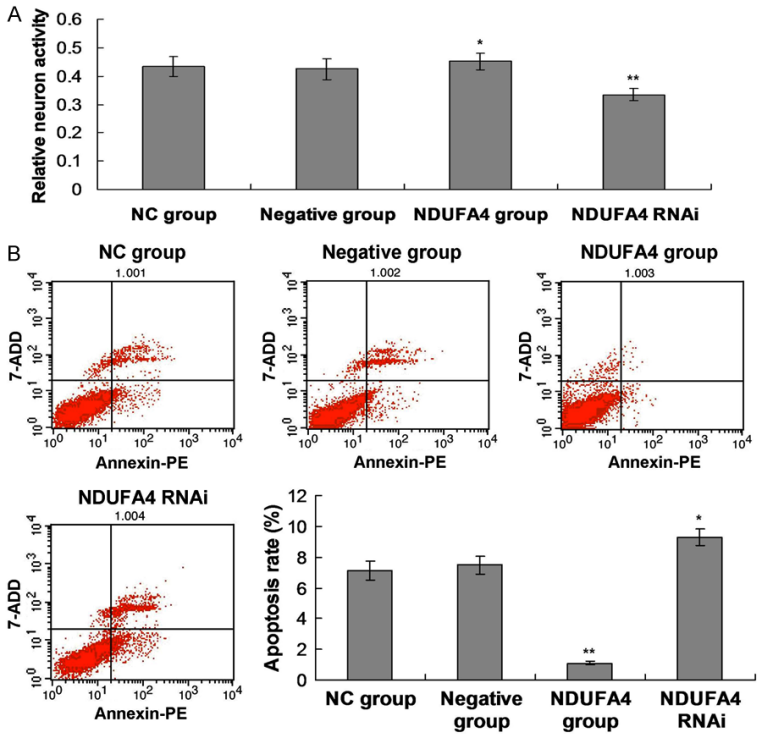

Figure 1. Observation for the effects of NDUFA4 on the neuron viability and apoptosis. A. Statistical analysis for the neuron viability examined by MTT assay. B. Neuron apoptosis examination by using the flow cytometry assay. *P<0.05 and **P<0.01 represent the neuron viability or apoptosis rate compared to the normal control (NC) group.

TAATGTCACGCACGATTTC-3'. Both of NDUFA4 and β-actin primers were synthesized by Western Biotech. Inc. (Chongqing, China). Then, the amplified products were loaded onto the 1.5% agarose gels, and the images were digitally captured by using the camera and analyzed with image analysis software.

Western blot assay

The neurons were lysed by employing the lysis buffer (Pplygen Biotech. Inc., Beijing, China). The lysed protein products were separated by employing 15% SDS-PAGE and electro-transferred onto the PVDF membranes (Millipore, Boston, MA, USA). The membranes were blocked with the 5% defatted milk in the PBS (Gibco BRL.Co.Ltd., Grand Island, New York, USA) supplementing with 0.05% Tween-20 at 4°C overnight. The membranes were incubated with the rabbit anti-rat Bcl-2 polyclonal antibody (Catalogue No: ab59348, Abcam Biotech., Cambridge, Massachusetts, USA), rabbit anti-rat cleaved caspase 3 polyclonal antibody (Catalogue No. ab13847, Abcam), rabbit anti-rat bFGF polyclonal antibody (Catalogue No. ab8880), mouse anti-rat Cyt C monoclonal antibody (Catalogue No. ab 13575), rabbit anti-BDNF monoclonal antibody (Catalogue No. ab108319), rabbit anti-rat NGF monoclonal antibody (Catalogue No. ab6199) and rabbit anti-rat GAPDH polyclonal antibody (Catalogue No. ab37168) for 2 h at the room temperature. Then, the membranes were incubated with the horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Catalogue No. ab-6789) and goat anti-rabbit IgG (Catalogue No. ab6721; Abcam Biotech., Cambridge, Massachusetts, USA) at 37°C for 1 h. The western blot bands were visualized and captured by using ECL kit (Pplygen Biotech. Inc., Beijing, China).

Immunohistochemistry assay

The neurons were fixed with the 4% paraformldehyde, and then used for the immunohistochemistry assay. The neurons were blocked with 5% BSA for 30 min, and incubated with the rabbit anti-rat NGF, BDNF, bFGF and Cyt C polyclonal antibody (RD Systems, Minneapolis, MN, USA) at 4°C overnight. Then, the neurons were incubated with goat anti-rabbit HRP labeled IgG at room temperature for 1 h. The immunohistochemistry was conducted with commercial SP immunohistochemistry kit (RD Systems, Minneapolis, MN, USA). Finally, the neurons were immersed in alkaline phosphatase labeled diaminobenzidine (RD Systems, Minneapolis, MN, USA), and the images were captured by using the biological inverted fluorescence microscope (Olympus, Japan).

Statistical analysis

The data were analyzed by using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The data were illustrated as the mean ± standard deviation. The comparisons were analyzed by utilizing the Student' *t* test. The P<0.05 was considered as the statistically significant.

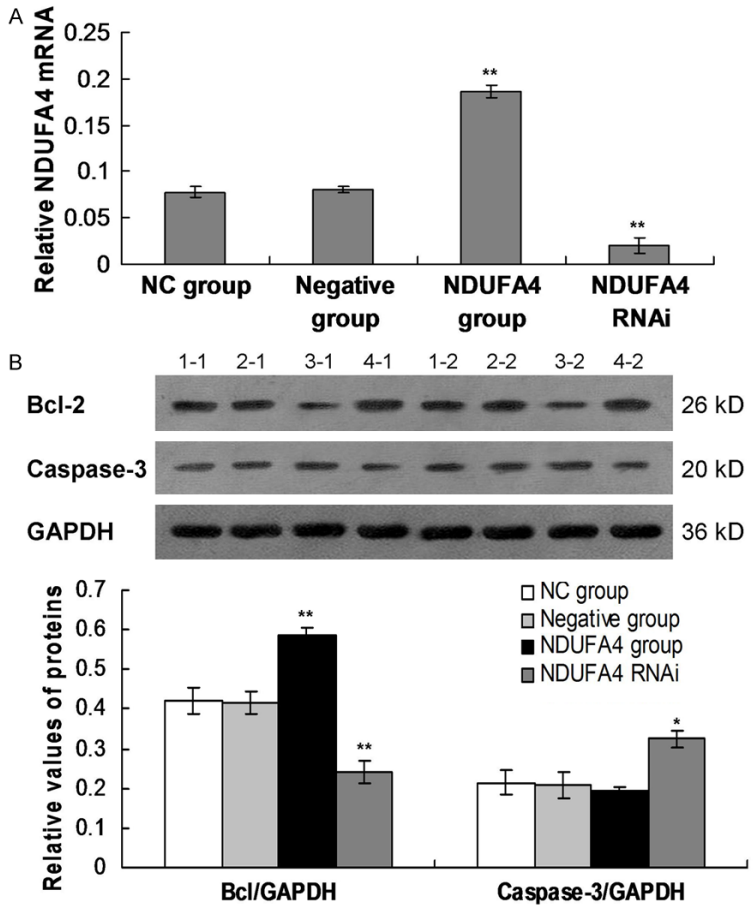

Figure 2. Examination for the apoptosis associated key molecules. A. NDUFA4 mRNA examination by using RT-PCR assay. B. Observation for Bcl-2 and cleave caspase-3 expression by using western blot assay. *P<0.05 and **P<0.01 represent the NDUFA4 mRNA or Bcl-2/cleaved caspase-3 expression compared to normal control (NC) group. 1-1 and 1-2 represent NC group, 2-1 and 2-2 represent Negative group, 3-1 and 3-2 represent NDUFA4 group, 4-1 and 4-2 represent NDUFA4 RNAi group.

Results

NDUFA4 enhances the neuron activity and inhibits neuron apoptosis

In order to observe the effects of NDUFA4 treatment on the neuron activity, MTT assay was performed. The results indicated that the NDUFA4 over-expression significantly enhanced the neuron activity compared to NC group (**Figure 1A**, $P<0.05$). Meanwhile, the NDUFA4 interfere (NDUFA4 RNAi group) could also significantly decreased neuron activity compared to the NC group (**Figure 1A**, $P<0.01$).

Furthermore, NDUFA4 over-expression significantly decreased the apoptosis rate of neurons ($P<0.01$), and NDUFA4 RNA interfere significantly increased the apoptosis rate of neurons ($P<0.05$), compared to the NC group (**Figure 1B**).

NDUFA4 increases Bcl-2 expression and decreases cleave caspase-3 expression

Firstly, the NDUFA4 mRNA expression was evaluated by using the RT-PCR assay. The results indicated that the NDUFA4 overexpression significantly enhanced the NDUFA4 mRNA expression ($P<0.01$), and NDUFA4 interfere significantly decreased the NDUF-A4 mRNA expression ($P<0.01$), compared to the NC group (**Figure 2A**).

To clarify the pathway caused the neuron apoptosis, the mitochondria associated apoptosis key factor, Bcl-2, was examined in this study. The results showed that NDUFA4 over-expression significantly increased the Bcl-2 expression ($P<0.01$), and NDUFA4 interfere significantly decreased the Bcl-2 expression ($P<0.01$), compared to the NC group (**Figure 2B**). The NDUFA4 over-expression slightly decreased cleaved caspase-3 expression compared to NC group (**Figure 2B**, $P>0.05$). However, the NDUFA4 RNA interfere significantly increased cleaved caspase-3 expression compared to NC group (**Figure 2B**, $P<0.05$).

NDUFA4 up-regulates growth factors and inhibits Cyt C expression

The western blot results (**Figure 3A**) indicated that the growth factors, including NGF, BDNF, bFGF, were significantly increased undergoing of the treatment of NDUFA4 compared to the NC group (**Figure 3B**, all $P<0.05$). Meanwhile, the NDUFA4 interfere significantly decreased the NGF, BDNF and bGFG expression compared to NC group (**Figure 3B**, all $P<0.05$). Moreover, the levels of Cyt C was also examined by using western blot assay. The results showed that

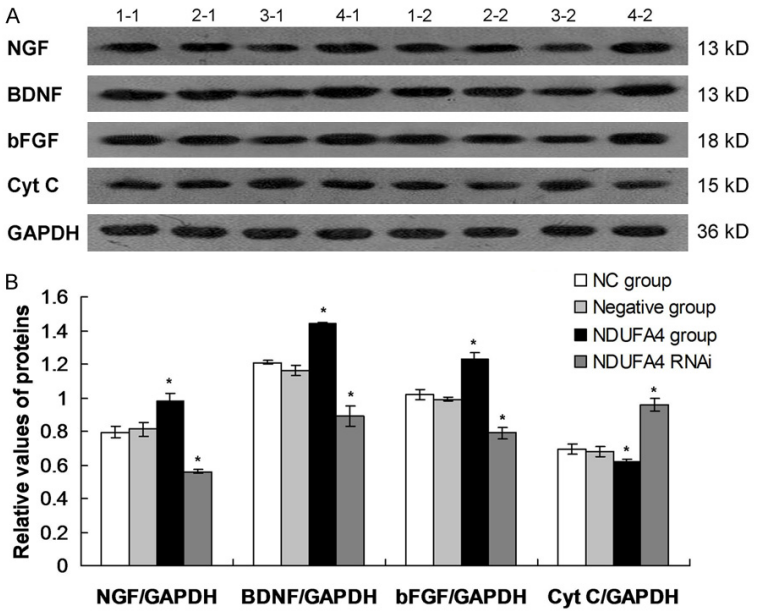

Figure 3. Evaluation for the effect of NDUFA4 overexpression or NDUFA4 RNAi on NGF, BDNF, bFGF and Cyt C expression by using western blot assay. A. Western blot assay for protein expression. B. Statistical analysis for the protein expression according to western blot assay bands. *P<0.05 represents the protein expression compared to normal control (NC) group. 1-1 and 1-2 represent NC group, 2-1 and 2-2 represent Negative group, 3-1 and 3-2 represent NDUFA4 group, 4-1 and 4-2 represent NDUFA4 RNAi group. NGF: nerve growth factor, BDNF: brain derived neurotrophic factor, bFGF: brain fibroblast growth factor, Cyt C: cytochrome C.

NDUFA4 significantly decreased Cyt C expression (P<0.05), and NDUFA4 interfere significantly increased Cyt C expression (P<0.05), compared to NC group (**Figure 3B**).

Furthermore, the immunohistochemistry assay (**Figure 4A**) also illustrated the same results as the above western blot assay results. The results also indicated that NDUFA4 overexpression significantly significantly increased above growth factors (P<0.01), and NDUFA4 interfere significantly decreased growth factors (P<0.01), compared to NC group (**Figure 4B**).

NDUFA4 interfere inhibits antagonistic effect of ciclosporin A on apoptosis and decrease up-regulative effect of ciclosporin A on neuron growth

From the **Figure 5**, we found that the ciclosporin A significantly increased the Bcl-2 and bFGF expression compared to NC group, at the concentration of 2.0 μg/L and 20 μg/L, respectively. Meanwhile, the ciclosporin A also significantly decreased cleaved caspase-3 and Cyt C expression compared to NC group. The Bcl-2 and bFGF expression in NDUFA4 interfere combing with ciclosporin A group is increased significantly compared to NDUFA4 interfere group at both concentration of 2.0 μg/L and 20 μg/L, but not at concentration 0.2 μg/L. Meanwhile, cleaved caspase-3 and Cyt C expression in NDUFA4 interfere combing with ciclosporin A group is decreased significantly compared to NDUFA4 interfere group at both concentration of 2.0 μg/L and 20 μg/L, but not at concentration 0.2 μg/L. Moreover, the Bcl-2 and bFGF expression significantly decreased in NDUFA4 interfere combining ciclosporin A group compared to ciclosporin A group at all concentration of ciclosporin A. And cleaved caspase-3 and Cyt C expression significantly increased in NDUFA4 interfere combining ciclosporin A group compared to ciclosporin A group at all concentration of ciclosporin A. These results showed that the NDUFA4 interfere could inhibit the antagonistic effect of ciclosporin A on apoptosis and decrease up-regulative effect of ciclosporin A on neuron growth.

NDUFA4 over-expression enhances antagonistic effect of ciclosporin A on apoptosis and increases up-regulative effect of ciclosporin A on neuron growth

From the **Figure 6**, we found that the Bcl-2 and bFGF expression in NDUFA4 combing with ciclosporin overexpression group is increased significantly compared to NDUFA4 overexpression group at both concentration of 2.0 μg/L and 20 μg/L, but not at concentration 0.2 μg/L. Meanwhile, cleaved caspase-3 and Cyt C expression in NDUFA4 overexpression combing with ciclosporin A group is decreased significantly compared to NDUFA4 over-expression group at both concentration of 2.0 μg/L and 20 μg/L, but not at concentration 0.2 μg/L. Moreover, the Bcl-2 and bFGF expression sig-

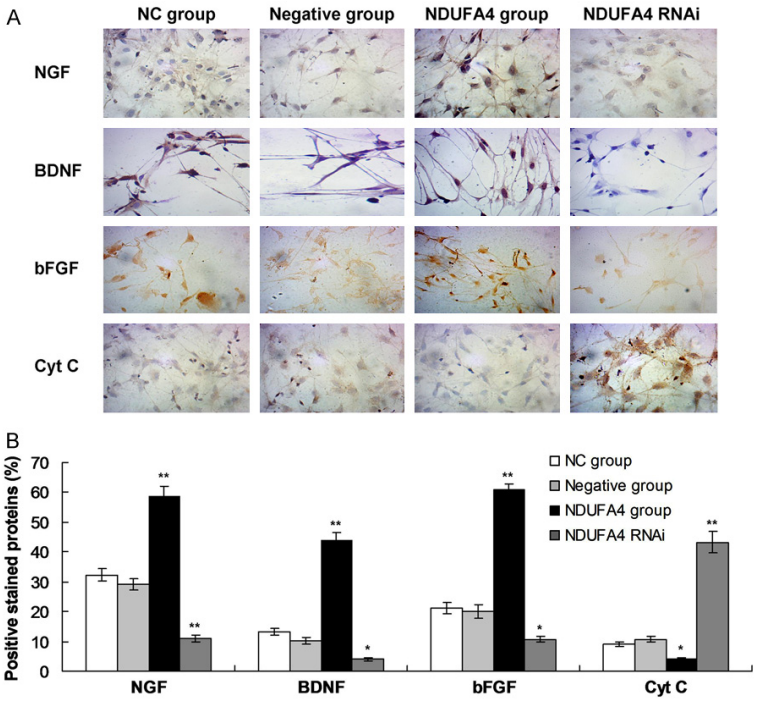

Figure 4. Examination for effect of NDUFA4 overexpression or NDUFA4 RNAi on NGF, BDNF, bFGF and Cyt C expression by using immunohistochemistry assay. A. Immunohistochemistry assay was employed to examine protein expression. B. Statistical analysis for the protein expression according to immunohistochemistry assay. $^{*}P<0.05$ and $^{**}P<0.01$ represent positively stained neurons compared to normal control (NC) group. 1-1 and 1-2 represent NC group, 2-1 and 2-2 represent Negative group, 3-1 and 3-2 represent NDUFA4 group, 4-1 and 4-2 represent NDUFA4 RNAi group. NGF: nerve growth factor, BDNF: brain derived neurotrophic factor, bFGF: brain fibroblast growth factor, Cyt C: cytochrome C.

nificantly increased in NDUFA4 over-expression combining ciclosporin A group compared to ciclosporin A group at all concentration of ciclosporin A. And cleaved caspase-3 and Cyt C expression significantly decreased in NDUFA4 over-expression combining ciclosporin A group compared to ciclosporin A group at all concentration of ciclosporin A. These results showed that the NDUFA4 over-expression could enhance antagonistic effect of ciclosporin A on apoptosis and increase up-regulative effect of ciclosporin A on neuron growth.

Discussion

DWM is the most prevalent and frequently occurred congenital malformation of human cerebellum, however, the mechanism of the developmental and genetic pathology has not been fully elusive [20, 21]. More than 85% of the DWM patients are diagnosed before 1 year old [22], and the about 86% to 94% patients occurred the hydrocephalus [23]. Till now, various neurological abnormalities related with the DWM, including microcephaly, hydrocephaly, ventriculomegaly and the agenesis for the corpus callosum, have been discovered and studied [24, 25]. The previous study [26] have been described the neuro-developmental outcomes in a series of typically disease developmental children. Sasaki-Adams et al. [27] reported that the co-occurrence of the aberration with the other neurological associated syndrome triggers the potential of the development delay. Therefore, we discussed the mechanism of the DWM, and investigated the effects of NDUFA4 expression on neurons in this study.

Meanwhile, Imataka et al. [6] reported that the genetic and teratogenic factors are considered as the reason for the cerebellar developmental disease, which is consistent with the multi-factorial inheritance pattern. There have been discovered a series of abnormalities of the chromosomes, including chromosome 3, 5, 8, 9, 13, 18, which were also proved to be correlated with the development of DWM [6, 27-29]. Our team discovered a critical region associated with DWM on the 7p21.3, including cerebellar disorder related gene, NDUFA4 gene [12]. Grinberg et al. [30] also discovered a region on the position of 3q24-25.1, which is commonly removed in the DWM patients. Therefore, we found that the gene deletion or deficiency may play critical role in DWM pathology, and may be selected as the therapeutic target for DWM.

The present study, as a continuous study of our former study [12], employed the NDUFA4 gene as the investigated subject to discuss the role of gene deletion in DWM development. We constructed the LV5-NDUFA4 and LV3-NDUFA4-RNAi lentivirus firstly, and transfected into the

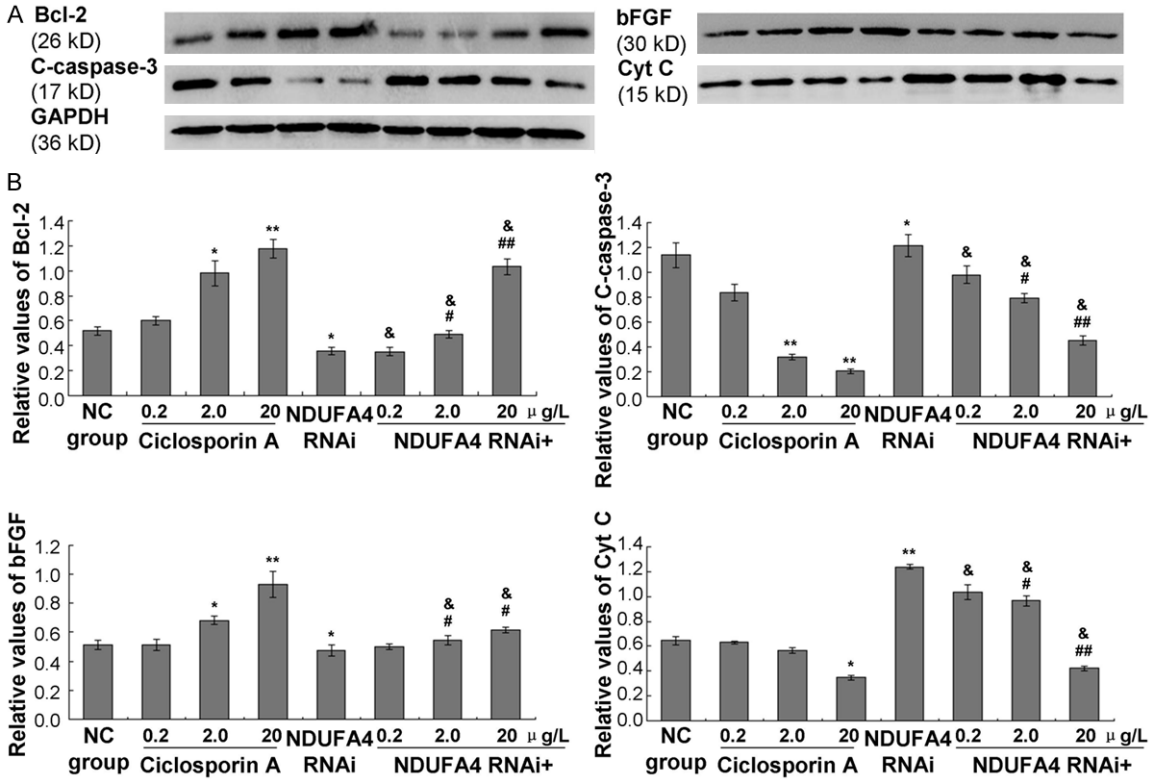

Figure 5. NDUFA4 interfere inhibits effect of ciclosporin A on apoptosis and neuron growth. A. Western blot assay bands of the Bcl-2, cleaved caspase-3, bFGF and Cyt C expression undergoing the NDUFA4 interfere and/or ciclosporin A treatment. B. Statistical analysis for the above proteins expression. *P<0.05 and **P<0.01 represent relative protein expression compared to normal control (NC) group. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 represent relative protein expression compared to NDUFA4 RNAi + 0.2 μg/L ciclosporin group. $^{\$}$P<0.05 represents relative protein expression compared to ciclosporin group without treatment of NDUFA4 RNAi at different concentration.

neurons to obtain the over-expressed NDUFA4 and knocked-down NDUFA4. The results of the MTT and flow cytometry assay suggest that the over-expression of NDUFA4 could significantly enhance the neuron activity and inhibit the neuron apoptosis, and inhibition of the NDUFA4 expression could decrease neuron activity and trigger the neuron apoptosis, which findings were consistent with the previous published reports [31, 32]. In order to investigate the pathway that triggers the neuron apoptosis, the Bcl-2 and cleaved caspase-3 expression were examined in this study. As all is known, the Bcl-2 is the key biomarker for the mitochondria mediated apoptosis [33], and the cleaved caspase-3 is the direct inducer for the cell apoptosis [34]. Our results indicated that the NDUFA4 over-expression significantly increased the Bcl-2 expression and NDUFA4 interfere significantly decreased the Bcl-2 expression. Meanwhile, the NDUFA4 interfere significantly increased the cleave caspase-3 expression.

Furthermore, we speculated that the NDUFA4 mediated neuron apoptosis may be caused by the inhibition of the growth factors. Therefore, we examined the growth factors by using western blot assay and immunohistochemistry assay, including NGF, BDNF, bFGF, in both NDUFA4 over-expression and NDUFA4 interfere group. The results showed that the NDUFA4 over-expression increased above growth factors expression and NDUFA4 interfere decreased the above growth factors expression.

Cyt C acts as an electron transporting molecule and is critical part for the respiratory chain, which locates in inner of the mitochondrial membrane [35]. Therefore, the Cyt C is also a key biomarker for the mitochondria mediated apoptosis. Our results showed that the NDUFA4 over-expression significantly decreased Cyt C expression, and NDUFA4 interfere significantly increased Cyt C expression. May be the Cyt C molecule plays an important role in the neuron

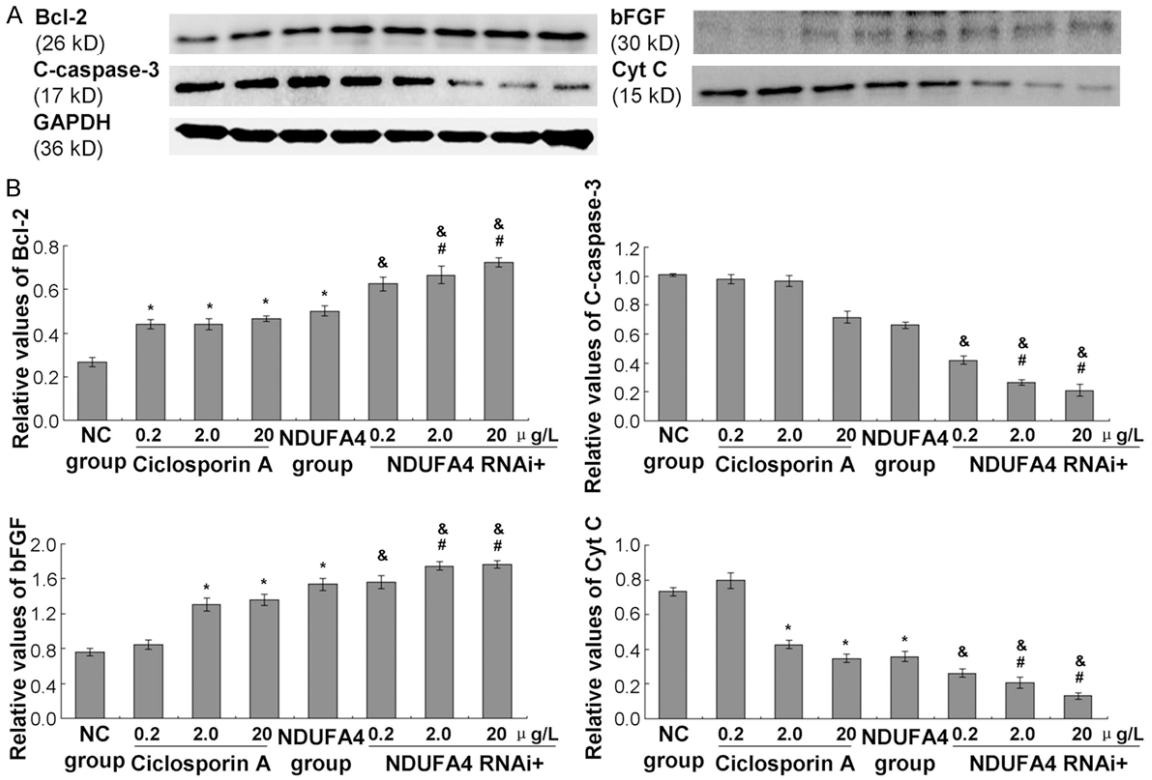

Figure 6. NDUFA4 overexpression enhances antagonistic effect of ciclosporin A on apoptosis and neuron growth. A. Western blot assay bands of the Bcl-2, cleaved caspase-3, bFGF and Cyt C expression undergoing the NDUFA4 interfere and/or ciclosporin A treatment. B. Statistical analysis for the above proteins expression. $^{*}P<0.05$ and $^{**}P<0.01$ represent relative protein expression compared to normal control (NC) group. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ represent relative protein expression compared to NDUFA4 RNAi + 0.2 μg/L ciclosporin group. $^{\$}P<0.05$ represents relative protein expression compared to ciclosporin group without treatment of NDUFA4 RNAi at different concentration.

growth and apoptosis. Therefore, we applied the Cyt C specific inhibitor, ciclosporin A, to observe the effects of NDUFA4 on neuron growth. The results indicated that the NDUFA4 interfere could inhibit the antagonistic effect of ciclosporin A on apoptosis and decrease up-regulative effect of ciclosporin A on neuron growth (illustrating from the NGF, BDNF and bFGF, which were the key biomarkers for the neuron growth [36, 37]. Meanwhile, the NDUFA4 over-expression enhances antagonistic effect of ciclosporin A on apoptosis and increases up-regulative effect of ciclosporin A on neuron growth. The present study is the first one that involves the NDUFA4 in the neurons growth and the apoptosis in the DWM.

In conclusion, the NDUFA4 enhances the neuron growth by triggering NGF, BDNF and bFGF expression, inhibits the neuron apoptosis by increasing Bcl-2 expression and decreasing cytochrome C expression. Meanwhile, the NDUFA4 regulates the antagonistic effect of ciclosporin A on apoptosis and the up-regulative effect of ciclosporin A on neuron growth.

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Disclosure of conflict of interest

None.

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