Cellular and Molecular Biology

Research Article Open Access

NGF and CNTF Expression and Regulation Mechanism by miRNA in Acute Paralytic Strabismus

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Abstract

Nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) are well known neurotrophic factors and widely used in the clinical treatment for its promotion effect on peripheral nerve regeneration. And they were also recommended for the acute paralytic strabismus treatment. However, whether the NGF and CNTF have protective effect for the extraocular muscles of acute paralytic strabismus patients is still poorly understood. Thus, in this study, we want to evaluate the biological function of NGF and CNTF on the extraocular muscle cells and revealed the regulation mechanism behind it. Firstly, the relative expression of ngf and cntf was assessed by Quantitative Real-time Polymerase Chain Reaction (RT-PCR). Then, the influence of NGF and CNTF on the extraocular muscle cell proliferation was determined by Cell Counting Kit-8 (CCK-8). The inflammatory response in muscle cells after NGF and CNTF treatment was evaluated by ELISA and Reactive Oxygen Species (ROS) detection. In addition to this, the up-stream regulation of the ngf and cntf expression was also studied. The TargetScan was used for the predication of potential miRNAs targeting with ngf and cntf 3'-UTR, which is soon confirmed by luciferase activity assay. Taken together, all the results above indicated that NGF and CNTF could promote the muscle cells proliferation and inhibit the inflammatory levels, then exert protective effect on the muscle cells function. It was conceivable that let 7-5p was the up-stream regulator of ngf and cntf, and let 7-5p might serve as a potential molecular target for acute paralytic strabismus treatment.

Keywords: Acute paralytic strabismus; miRNAs; *let 7-5p*; *NGF*; *CNTF*

Introduction

Paralytic strabismus is a complete or partial paralysis of the single or multiple extraocular muscles caused by the nucleus that controls the movement of the eye muscles or the external of the extraocular muscles (extraocular muscle in dysfunctional condition but not complete paralysis) [1-3]. Paralytic strabismus is divided into congenital and acquired paralytic strabismus. Acquired paralytic strabismus is mostly acute paralytic strabismus, and its clinical causes can be divided into neurogenic, myogenic and mechanical. At present, the commonly drugs used for acute paralytic strabismus in the clinic include vitamin B3, vitamin C, creatinine, adenosine triphosphate, and coenzyme A, and so on [4]. ese drugs can improve neuromuscular function. However, the current clinical data show that the cure rate of acute paralytic strabismus is not very ideal, so we need to further reveal the pathogenesis of acute paralytic strabismus, and provide new targets for drug development in the future.

Nerve growth factor (*NGF*) and ciliary neurotrophic factor (*CNTF*) are two kinds of neurotrophic factors which are deeply and widely reported, and its promotion e ect on peripheral nerve regeneration has been widely con rmed [5,6]. In the previous study, we can see the *NGF* has a relatively strong promoting e ect on the ber regeneration, while the *CNTF* shows a relative stronger promotion on the motor ber regeneration. A large number of studies have shown that *NGF* and *CNTF* have protective e ects on sympathetic, sensory and motor nerves. However, the current research on *NGF* and *CNTF* pays more attention to its nutritional e ects on neurons. For example, the *NGF* and *CNTF* are given a er the peripheral nerve injury treatment. Generally, only the protective e ect of *NGF* and *CNTF* on neurons is noticed, its Non-neuronal protection was needed to be explored, especially the protection on skeletal muscle.

In recent years, more and more researches focused on the study of microRNAs (miRNAs) regulation on target genes expression. miRNAs are endogenous RNAs with 20-24 nucleotides [7], which could directly bind to the 3′-untranslated region (UTR) of its target gene at the posttranscriptional level and involved in post-transcriptional regulation, a ecting both the stability and translation of mRNAs, then regulating various cellular functions [8]. A growing number of studies have demonstrated that miRNAs participate in the progression processes of multiple diseases, and have the potential to be a promising target for the target for disease treatment.

In this present research, we aimed to explore the role of *NGF* and *CNTF* on the acute paralytic strabismus treatment [9]. Firstly, the relative expression of *NGF* and *CNTF* in the extraocular muscle samples was measured in both the mRNA and protein levels. e in uence of *NGF* and *CNTF* on extraocular muscle cell proliferation and in ammatory level was measured to prove the protective e ect on extraocular muscle. Subsequently, the bioinformatics prediction was performed for the searching of miRNA which could regulate the expression *NGF* and *CNTF*, and its biding with target genes was con rmed by luciferase activity assay. Finally, we want to clarify the potential mechanisms of

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Received September 15, 2019; **Accepted** September 24, 2019; **Published** September 30, 2019

Citation: Liu H, Tan N, Xu D, Li C, Xian G (2019) NGF and CNTF Expression and Regulation Mechanism by miRNA in Acute Paralytic Strabismus. Cell Mol Biol 65: 156.

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NGF and *CNTF* protective e ect on extraocular muscle. In conclusion, we convinced the important role of the *let 7-5p* in the acute paralytic strabismus by regulating *NGF* and *CNTF* expression.

Materials and Methods

Patients and tissue specimens collection

In this present research, the extraocular muscle samples were collected from 75 patients who were diagnosed as acute paralytic strabismus and underwent surgical resection treatment in our hospital from March 2018 to May 2019. All patients were informed about the detail perforation of this research and signed the patient consents before the collection of extraocular muscle samples. is experiment was approved by the Ethics Committee of Daping Hospital. All tissue specimens harvested were stored in liquid nitrogen at -80°C immediately a er surgical resection.

Cell cultures

Human 293-T cell line was obtained from American Type Culture Collection. e Hela cells were maintained in Dulbecco's Modi ed Eagle's Medium medium (DMEM; ermo Fisher Scienti c, Waltham, MA, USA) supplemented with 1% Penicillin and Streptomycin sulfate solution, 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies), as well as 2% L-glutamine. e 293-T cells were incubated in an incubator at 37°C, 5% CO_2, the culture medium was changed according to the cell state.

Cell transfection

e *let 7-5p* mimic, *let 7-5p* inhibitor, as well as the mimic-control and inhibitor-control were designed according to the sequence of *NGF* and *CNTF* mRNA, and then the let 7-5p mimics and inhibitors were synthesized by ShengGongPharma, Shanghai, P.R. China.

e PCR was performed to get the cDNA fragment sequence of the 293-T a er ampli cation the *ngf* and *cntf* sequence was cloned into pcDNA3.1 vector (Ruibobio, Guangzhou, China). In the parallel test group, the empty pcDNA-3.1 plasmid was used as negative control [10]. For miRNAs or pcDNA-3.1 plasmids transfection, the Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used following the manufacturer's instruction with little modi cation [11].

RNA isolation and quantitative RT-PCR

e transfection e ciency of let 7-5p and plasmid, as well as the relative expression of *ngf* and *cntf* was assessed by quantitative real-time RT-PCR according to the manufacture's protocol with some modi cation [12]. Generally, the TRIzol Reagent (Sigma, St. Louis, MO, USA) was used to extract the total RNA sample in the cells a er transfection or treatment under the instructions. en, the total RNA sample was qualitied and quanti ed with spectrophotometer, followed by reverse transcripted into cDNA following the manufacturer's proposal. Finally, to measure the transfection e ciency and genes relative expression, the RT-PCR was performed using SYBR Green Master Mix (Taraka), using the gapdh as the internal reference. e sequence of *ngf* and *cntf* were obtained from PUBMED/Nucleotide GeneBank, and the primer sequence was designed by Primer Premier 5.0 and synthesized by ShengGong (ShangHai, China). e primers used in this experiment are listed in Table 1. $2^{-\Delta\Delta Ct}$ method was recommended for the calculation of genes relative expression.

Genes	Sequences
let 7-5p	TCTGGCAAATTGAGGTAGTAGGT
	TCAAGCAAAGAAAGCTAGCACA
ngf	GGGAGCGCAGCGAGTTTTG
	GAGTGTGGTTCCGCCTGTAT
cntf	TGATTAGGCCCGCCAAACTT
	AACCTGGTATAAGCCGTGCC
gapdh	ATGTTGCAACCGGGAAGGAA
	AGGAAAAGCATCACCCGGAG

Table 1: The primers used in this experiment.

ELISA assay

e release of IL-1β and TNF-α in extraocular muscle tissues of acute paralytic strabismus patients was detected in this experiment performed according to the protocols with some modi cation [13]. In brief, the extraocular muscle was isolated and cultured at 37°C, 5% CO_2, followed by *NGF* and *CNTF* addition. A er treatment, the cell supernatant was collected for the IL-1β and TNF-α content detection with ELISA. is experiment was repeated at least three times.

ROS detection

To detect the inhibitory e ect of *NGF* and *CNTF* on extraocular muscle cells in ammatory response, a er treatment, the ROS level in cells of di erent groups were measured by ROS detection kit following the instructions [14]. Brie y, the cells were isolated and cultured at 37°C, 5% CO_2, followed by NGF and CNTF treatment. Subsequently, the cells were harvested for the ROS detection in ow cytometry.

miRNA prediction and luciferase activity assay

To predicate the miRNA which could regulate the expression of *ngf* and *cntf* genes, as well as the binding sites of miRNA with the target genes, the TargetScan so ware was used in this study. Based on the prediction results by bioinformatics so ware, the binding of miRNA with *ngf* and *cntf* genes was further con rmed by luciferase activity assay [15]. In short, the pMIR-REPORT luciferase reporter plasmids (Promega Corporation, Madison, WI, USA) used in this experiment was inserted with the wild-type (WT) and 3'-UTR mutant *ngf* and *cntf* genes. e let 7-5p mimic and the luciferase reporter plasmids were co-cultured into 293-T cells. A er 24 h incubation in an incubator at 37°C, 5% CO_2, the in orescence intensity in each group were measured GloMax20/20 illuminometer (Promega Corporation). Data were showed as mean ± SD. is experiment was repeated at least three times.

Western blotting

e relative expression of NGF and CNTF in the extraocular muscles of acute paralytic strabismus patients was further measured by western blot. is experiment was performed according to the protocols previous described with some modi cation [16]. Generally,

the extraocular muscle samples harvested from patients were used in the detection, the radio-immunoprecipitation assay (RIPA) lysis bu er (Beyotime, Shanghai, China) containing protease inhibitor cocktail (ermo Fisher) was recommended to lysis the cells for the protein extraction. A er extraction, the BCA Protein Assay kit (Sigma, USA) was employed for the quanti cation of total protein concentration. Equal amounts of protein samples from di erent groups were separated on 12% sodium dodecyl sulfate-polyacrylamide denaturing gels and then transferred onto a polyvinylidene di uoride membrane (PVDF, Millipore, MA). A er that, the PVDF membranes were blocked in 5% blocking bu er at room temperature for 2 hours to exclude non-speci c binding. e primary antibodies against NGF and CNTF (Abcam) or GAPDH (Abcam) were incubated with PVDF membranes overnight at 4°C. Next, horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Finally, the chemiluminescence (ECL) detection kit (ermo Fisher Scienti c) was used for proteins expression visualized and quanti cation was performed using ImageJ so ware (BIO RAD) [17]. e experiment was in triplicate independently.

Statistical analysis

All the results were presented as means ± SD in this research from three di erent repeats. SPSS 17.0 So ware (IBM, Armonk, NY, USA) was recommended for the statistical analysis. Student t-test was used for the statistical comparisons of two groups, and one-way ANOVA was performed for statistical comparisons of more than three di erent groups. $p<0.05$ was regarded as has statistically signi cant between groups.

Results and Discussion

Aberrant expression of ngf and cntf in extraocular muscle tissue

As two important types of neurotrophic factors, the *NGF* and *CNTF* play a vital important role in the procession of peripheral nerve regeneration. However, the protection e ect of *NGF* and *CNTF* on injured skeletal muscle was still unknown. us, in this experiment, the relative expression of *ngf* and *cntf* in extraocular muscle tissue was measured by PT-PCR and western blot, respectively. As the results showed in Figure 1A, the relative expression of in the superior oblique muscle of the paralysis was signi cantly weaker than that in the upper oblique muscle of the control group. e expression of *ngf* and *cntf* in the inferior oblique muscle of the paralysis was signi cantly enhanced compared with that in the control group ($p<0.05$). e expression pro les of *ngf* and *cntf* in the superior and inferior oblique muscles of the control group showed no signi cant statistical di erences.

NGF and CNTF promotes the proliferation of extraocular muscle cells

In the previous studies, we have con rmed the reduced-expression of *ngf* and *cntf* in the superior oblique muscle and the over expression in the inferior oblique muscle. us, we proposed a hypothesis; the content of *NGF* and *CNTF* may have closely relationship with the function of extraocular muscle. So, in this experiment, the e ect of *NGF* and *CNTF* on muscle cell proliferation was determined by CCK-8 assay. As the results showed in Figure 2, both the *NGF* and *CNTF* showed promotion e ect on the cell growth, there was a signi cant di erence between the treatment group and the control group. is result revealed the protective e ect of *NGF* and *CNTF* on muscle cell for the rst time.

NGF and *CNTF* inhibit the ROS mediated apoptosis in extraocular muscle cells

It has been reported that during acute paralytic strabismus, there is usually an increased in ammatory response level in in extraocular muscle tissues, re ecting as the up-regulated level of IL-1β, TNF-α and ROS [18]. us, whether the aberrant expression of *NGF* and *CNTF* in extraocular muscle tissue has relationship with the in ammatory

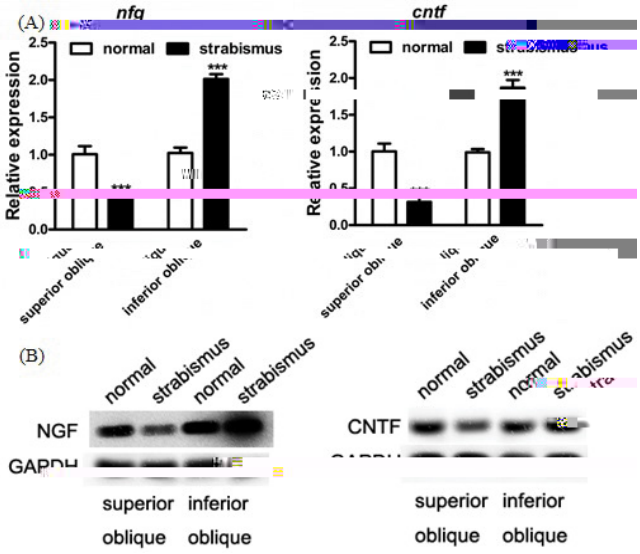

Figure 1: Non-neuronal protection of NGF and CNTF in extraocular muscle tissue. The relative expression of ngf and cntf in the superior and lower oblique in extraocular muscle tissue of acute paralytic strabismus patients was detected by RT-PCR (A) and western blot (B). ***means $p<0.001$.

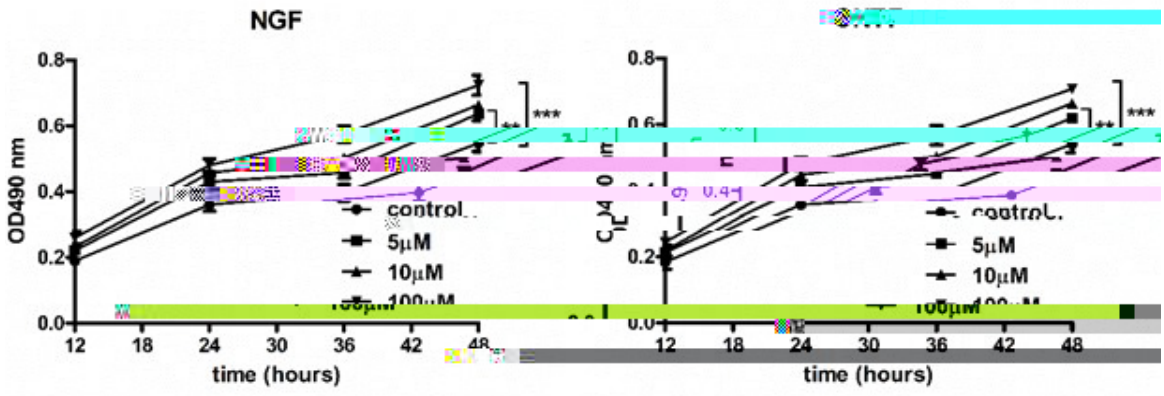

Figure 2 (a) At 77 K, 1a' s N_2 sorption isotherms; (b) At 273 K, 1a' s gas sorption isotherms; (c) At 296 K, 1a' s gas sorption isotherms; (d) The predicted IAST selectivity of 1a.

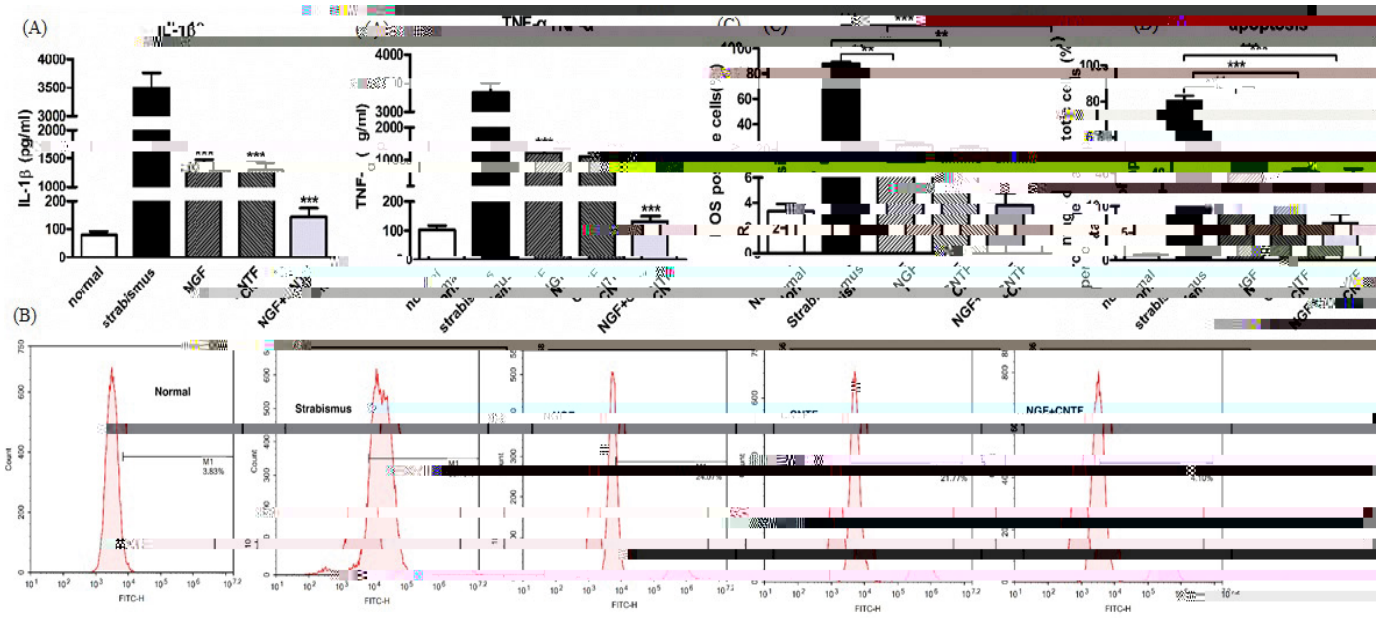

Figure 3: Reduced inflammatory response in extraocular muscle cells after NGF and CNTF. The extraocular muscle cells were isolated from patients' tissues and followed by NGF and CNTF treatment. (A) The IL-1β and TNF-α content was measured by ELISA assay. (B) ROS level determined by DCFH-DA detection in flow cytometry. (C) Percentage of apoptotic extraocular muscle cells after NGF and CNTF treatment. (D) Percentage of apoptotic extraocular muscle cells after NGF and CNTF treatment was measured by in flow cytometry.

response level was detected in this experiment. In Figure 3A, the ELISA was performed to measure the IL-1β and TNF-α content a er treated with *NGF* and *CNTF*, results indicated that the signi cant inhibitory e ect of *NGF* and *CNTF* on the IL-1β and TNF-α release. Besides, the ROS detection also con rmed that the *NGF* and *CNTF* could inhibit the in ammatory response in extraocular muscle tissues through reducing ROS production (Figure 3B). e percentage of apoptotic cells were measured in ow cytometry and the results were calculated in Figure 3C.

Let 7-5p targets directly and negatively regulates ngf and cntf expression

As to the vital role of *NGF* and *CNTF* in the in extraocular muscle tissue during acute paralytic strabismus, we proposed a new question, how the *ngf* and *cntf* expression was regulated? To solve this problem, we used the TargetScan so ware to predicate the protentional regulator and reveal the novel treatment target. In Figure 2A, *let 7-5p* was identi ed as one of the most potential target regulators of *ngf* and *cntf*, with the highest scores. en, the expression of *let 7-5p* in the extraocular muscle samples were detected via RT-PCT (B) and the correlation between *let 7-5p* and *ngf* and *cntf* was further explored, as the results showed in Figure 2C, let 7-5p negatively regulates the expression of *ngf* and *cntf* expression in extraocular muscle tissue ($r= -0.9943$ and $r= -0.9948$, $p<0.0001$). For the further con rmation of the directly binding of let 7-5p and ngf and cntf, the luciferase reporter assay was performed according to the protocols. e result in Figure 2D suggested that *let 7-5p* mimic signi cantly reduced the luciferase activity, and *let 7-5p* inhibitor obviously elevated the luciferase activity ($p<0.005$). Above all, the *let 7-5p* was the up-stream of the *ngf* and *cntf*, which could negatively regulate ngf and cntf expression.

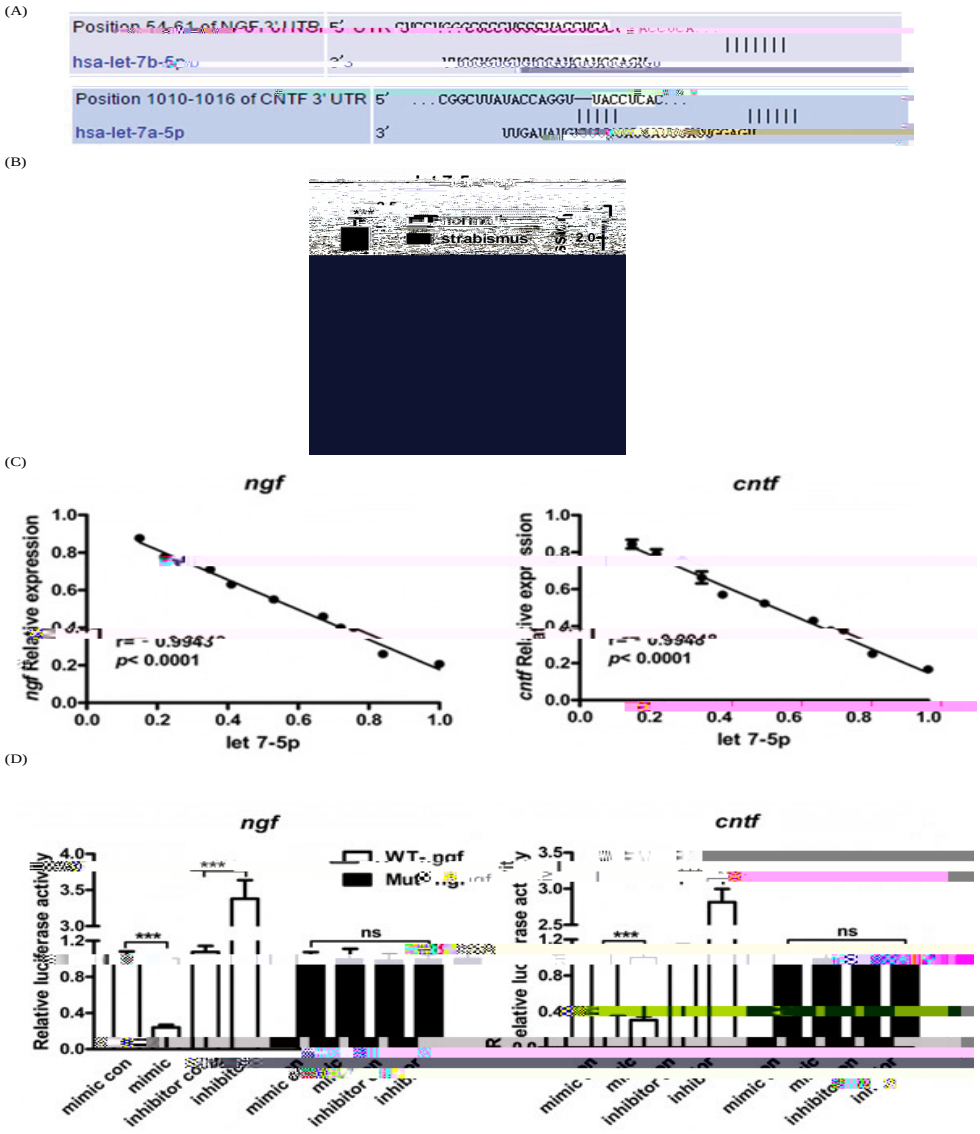

Figure 4: Let 7-5p negatively regulates ngf and cntf expression by directly binding. (A) The potential binding sites between let 7-5p and ngf and cntf predicated by TargetScan software. The sequence of let 7-5p is shown allied with the ngf and cntf mRNA 3'-UTR sequence. (B) The expression of let 7-5p in the extraocular muscle samples detected by RT-PCT. (C) The negative correlation between let 7-5p expression and ngf and cntf expression level. (D) Direct binding between let 7-5p and ngf and cntf verified by luciferase activity assay.

Conclusion

Neurotrophic factors (NTFs) secrete peptides have been proved could promote axonal regeneration and play important roles in maintaining survival of neurons. Among these *NTFs*, the best studied are the NGF and CNTF [19]. In 2006, Li et al. have reported the di erent function of *NGF* and *CNTF*, the *NGF* could promote for the regeneration of sensory bers, while the *CNTF* could signi cantly promote the regeneration of motor bers. In addition to the synergetic e ects of *NGF* and *CNTF* on survival and growth of sensory neurons, its protective e ect on muscle tissues was still unknown. So, in this experiment, we rstly detect the expression of *ngf* and *cntf* in the upper oblique and oblique muscle of the paralysis via RT-PCR. We found there was a down-regulation of *ngf* and *cntf* expression level in superior oblique muscle, and an up-regulated level in inferior oblique, which suggested that the content of *NGF* and *CNTF* may has a closely relationship with the function of extraocular muscle. Next, we further explore the promotion e ect of *NGF* and *CNTF* on the extraocular muscle cells proliferation and the inhibition on the in ammatory response in muscle cells. Consistent with what we have envisaged, the results in Figure 2 and Figure 3 proved the extraocular muscle cell growth could be promoted by the addition of *NGF* and *CNTF*, and the in ammatory response level in cells was also be reduced by the *NGF* and *CNTF*, re ecting as the down-regulated level of IL-1β and TNF-α release, as well as the ROS accumulation. And a new problem came followed, how the production of NGF and CNTF in the extraocular muscle tissues was regulated. To solve this problem, we searched related references and performed the following researches.

As a special kind of noncoding single-stranded RNA, the miRNAs

are widely exist in eukaryotes, which could bind to the 3'-UTR of the target genes mRNA and in uence the genes expression by mRNA cleavage or translation regulation. us, in the further mechanism, we aimed to explore the up-stream miRNA, which regulates the *ngf* and *cntf* expression in extraocular muscle cells. For the potential miRNA predication, the TargetScan so ware was employed in our research, and the results revealed the *let 7-5p* won the highest rating, becoming the most likely regulator. For further veri cation, the *let 7-5p* expression level in in isolated from acute paralytic strabismus patients was measured (Figure 4A) and the correlation between *let 7-5p* and ngf and cntf was calculated (Figure 4B). What surprises us is that there is a perfect negative correlation between *let 7-5p* expression and ngf and cntf expression level. Subsequently, the luciferase activity assay further con rmed the *let 7-5p* was the negative regulator of ngf and cntf in extraocular muscle cells.

Above all experiments, we not only proved the protective e ect of *NGF* and *CNTF* on the treatment of acute paralytic strabismus by promoting the proliferation of extraocular muscle cells and inhibiting the IL-1β and TNF-α release and ROS production, but also revealed the up-stream regulatory mechanism of ngf and cntf in extraocular muscle cells, proposed the important role of *let 7-5p* in the acute paralytic strabismus therapy. is research is the rst time completely disclosure the protective e ect of *NGF* and *CNTF* on extraocular muscle cells during acute paralytic strabismus, providing the new targets for the development of potential drugs in the further.

Con icts of Interest

e author(s) declare(s) that there is no con ict of interest regarding the publication of this paper.

Data Availability

e data used to support the ndings of this study are included within the article.

Acknowledgements

The research did not receive any specifc funding.

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