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Regulatory mechanism of microRNA-377 on CDH13 expression in the cell model of Alzheimer's disease

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Abstract. – OBJECTIVE: To explore whether microRNA-377 could participate in the development of Alzheimer's disease (AD) by regulating CDH13.

MATERIALS AND METHODS: In this research, AD model was constructed by the SH-SY5Y cells. The expression levels of microRNA-377 and CDH13 in the AD model were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The cell viability and apoptosis after knockdown of microRNA-377 and CDH13 were measured by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. The regulatory mechanism of microRNA-377 on CDH13 was confirmed by dual-luciferase reporter gene assay, qRT-PCR and Western blot.

RESULTS: Downregulated microRNA-377 and upregulated CDH13 were observed after successful construction of the AD model. Cell viability in the AD model group was significantly reduced compared with that of the control group. Moreover, downregulated microRNA-377 could further inhibit the cell viability, which was reversed by CDH13 knockdown. Cell apoptosis in the AD model group was enhanced after microRNA-377 knockdown, which was rescued by decreasing the expression level of CDH13. MicroRNA-377 was confirmed to regulate the expression level of CDH13 by dual-luciferase reporter gene assay, qRT-PCR and Western blot.

CONCLUSIONS: MicroRNA-377 could regulate the expression level of CDH13 by promoting cell proliferation and inhibiting cell apoptosis, thus participating in the occurrence of the Alzheimer's disease.

Key Words:
MicroRNA-377, CDH13, Apoptosis, Alzheimer's disease.

Introduction

Alzheimer's disease (AD), the most common form of dementia, is known as a neurodegenerative disease caused by the degenerative disorders of the central nervous system. Epidemiological researches[1,2] have shown that AD mainly occurs in elderly over 65 years old, the incidence of which increases with age. The pathogenesis of AD is complicated and affected by multiple factors. Many theories have been reported on the etiology of AD, such as free radical injury, beta-amyloid (Aβ) cascade hypothesis, cholinergic theory, Tau protein theory, calcium metabolism disorders and aluminum poisoning. However, Aβ cascade hypothesis and Tau protein theory are mostly of major concern to researchers. Establishment of an effective AD model is helpful to further study the pathogenesis and treatment of AD on the cellular level. Since Aβ can mimic AD damage, it is commonly used to induce the cell model of AD[3].

MicroRNAs are a class of small non-coding RNAs with 22 nucleotides (nt) in length. Mature microRNAs are formed by the precursor transcript with a hairpin structure that is cut through the Dicer enzyme[4]. In addition, mature miRNAs are complementarily bound to the 3'UTR sequence of the target mRNA, leading to the regulation of the target gene[5]. MiRNAs are widely existed in all kinds of species and highly conserved in evolution. It exerts a crucial role in regulating gene expression and therefore has been well recognized in recent years[6,7].

Studies have indicated that microRNA-377 can inhibit the proliferation and migration of gastric cancer cells by inhibiting VEGFA expression, and eventually participates in the development of gastric cancer[8]. Moreover, microRNA-377 has been proved to promote inflammation of white adipose tissues and reduce insulin sensitivity of obesity by inhibiting SIRT1 expression[9]. As to whether microRNA-377 is involved in the occurrence of AD still remain to be investigated. Therefore, we aim to explore the effect of microRNA-377 on

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cell viability and apoptosis by constructing the cell model of AD, which provides new ideas for exploring the AD pathogenesis.

Materials and Methods

Construction of the Cell Model of AD

The SH-SY5Y cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained in an incubator with 5% CO_2 and saturated humidity. After the cells grew by the logarithmic growth phase, 25-35 μL of Aβ (final dose of 20 μmol/L) and 10% dimethyl sulfoxide (DMSO) were added in the AD model group and the control group, respectively. The cells were continually cultured for 48 h.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription. Primers used in this study were as follows: CDH13 (F:5'-ACCAGCCTGTCCTAAACTTGA-3', R:5'-GTGTGGGCTTGAGACCTCG-3'), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F:5-'TGTGGGCATCAATGGATTTGG-3', R:5'-ACACCATGTATTCCGGGTCAAT-3').

Western Blotting

Total proteins were extracted from cells by radioimmunoprecipitation assay (RIPA) method (Beyotime, Shanghai, China) and then quantified using bicinchoninic acid (BCA) (Beyotime, Shanghai, China) based on manufacturer's instructions. Proteins were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Cell Transfection

The cells in the logarithmic growth phase were seeded into the 6-well plates, and were transfected according to the instructions of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The microRNA-con, microRNA-337 mimics, anti-microRNA-337, si-CDH13 1 and si-CDH13 2 (Jima, Shanghai, China) were transfected to the cells. Complete medium was replaced 6 h after transfection. The interference sequences were: microRNA-337 mimics (5'-ATCACACAAAGGCAACTTTTGT-3'), anti-microRNA-337 (F:5'-CGGGATCCGAATTCACCAAGGGCAACCTCTTTTTTTAAGCTTGGG-3', R:5'-CCCAAGCTTAAAAAAAGAGGTTGCCCTTGGTGAATTCGGATCCCG-3'), si-CDH13 (5'-CGGGCGCUUCUAGUCGGACAA-3'), si-CDH13 2 (5'-GGACCAGUCAAUUCUAAAC-3').

Cell Viability

24 h after transfection, the cells were collected and seeded into 96-well plates at a dose of 2×10^3/mL, with 6 replicates of each group. The cell viability was measured by cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Briefly, 10 μL of CCK-8 solution were added into each well. Cells were incubated at 37°C for 2 h in the dark. Absorbance (OD) values at the wavelength of 450 nm were detected by the microplate reader.

Cell Apoptosis

Transfected cells in the logarithmic growth phase were seeded in 6-well plates at a density of 1×10^5/mL. Cells were harvested after culturing for 24 h. Annexin V-FITC (Invitrogen, Carlsbad, CA, USA) was used for cell staining for 15 min. Apoptosis was analyzed by a flow cytometer. Each experiment was repeated for three times and the average value was taken.

Dual Luciferase Reporter Analysis

The reporter plasmids containing the wild-type (WT) and/or mutant (MUT) sequences of the 3'UTR of CDH13 were constructed. Cells were plated in 96-well plates overnight prior to transfection. 50 pmol/L microRNA-377 mimics or negative control and 80 ng CDH13 WT or MUT plasmids were co-transfected to the cells. After 48 h of transfection, luciferase activity was detected using the Dual Luciferase Reporter Assay System.

Statistical Analysis

We used statistical product and service solutions (SPSS 19.0, IBM Corp., Armonk, NY, USA) software for statistical analysis. The quantitative

data were represented as $\bar{x}\pm s$. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). $p<0.05$ was considered statistically significant ($\alpha=0.05$).

Results

Construction of the Cell Model of AD and the Expression Level of microRNA-377 and CDH13

48 h after successful construction of the AD model, the protein expressions of total Tau, Tau (S404) and Tau (T231) in SH-SY5Y cells were detected by Western blot (Figure 1A). The protein expressions of total Tau, Tau (S404) and Tau (T231) in the AD model group were remarkably increased in comparison with those of the control group ($p<0.05$), indicating the successful construction of the AD model. Additionally, reduced expression level of microRNA-377 in the AD model was observed (Figure 1B). Bioinformatics assay and functional analysis revealed that the target gene of microRNA-377 was CDH13. Therefore, the expression level of CDH13 in the AD model was detected. Our results demonstrated that the expression level of CDH13 was remarkably increased in the AD cell model (Figure 1C).

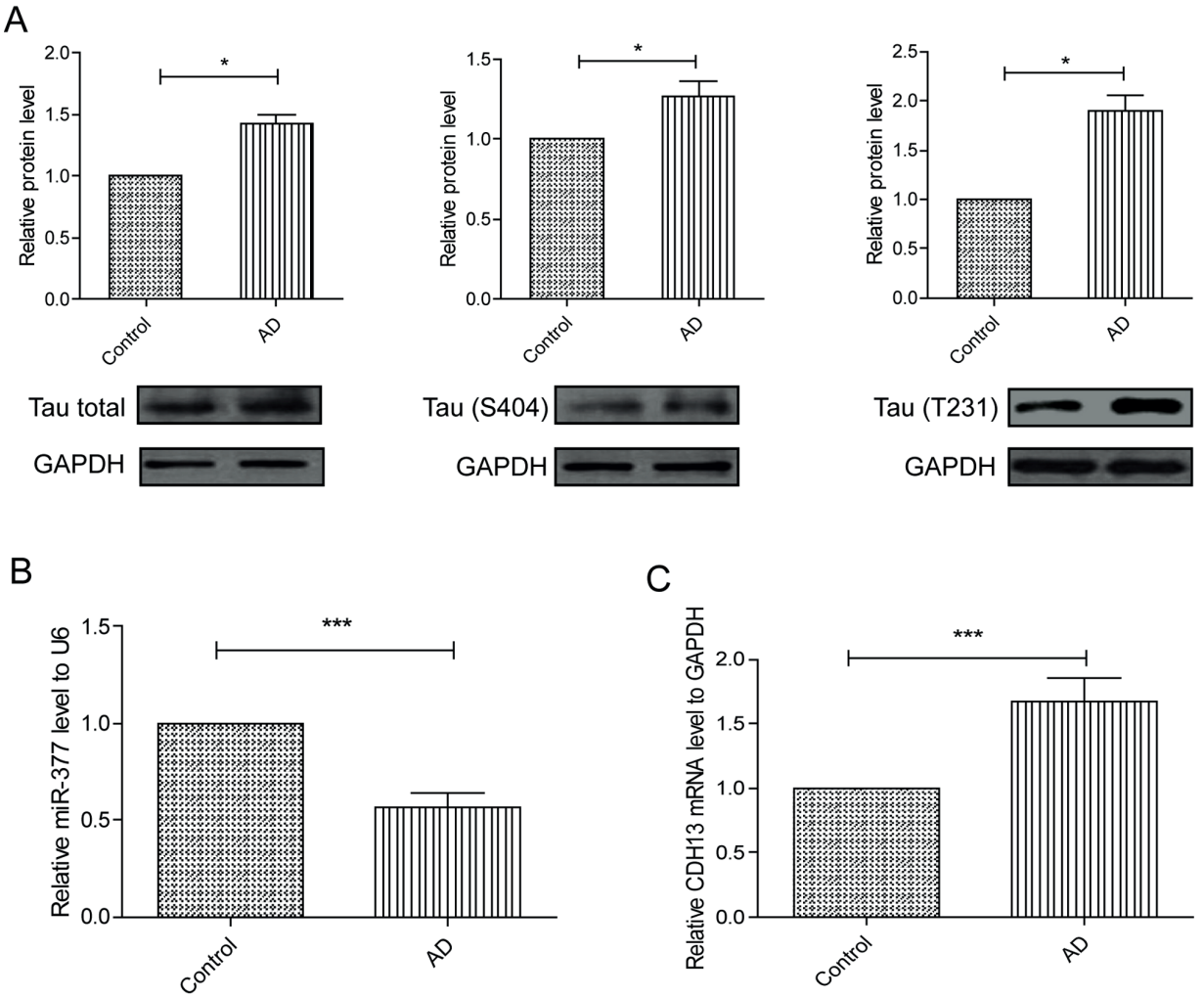

Figure 1. Construction of the AD cell model and the expression levels of microRNA-377 and CDH13. ***A,*** The expression levels and quantitative analysis of total Tau, Tau (S404) and Tau (T231) in SH-SY5Y cells. ***B,*** Compared with the control group, the expression level of microRNA-377 in the AD cell model group was significantly reduced. ***C,*** Compared with the control group, the expression level of CDH13 in the AD cell model group was significantly increased.

MicroRNA-377 Promoted Cell Proliferation, while CDH13 Inhibited Cell Proliferation

MicroRNA-377 mimics, anti-microRNA-377 and microRNA-con were transfected into the cells. Transfection efficiency was shown in Figure 2A. Similarly, si-NC, si-CDH131 and si-CDH132 were transfected into the cells. Our data showed that both siRNAs could inhibit the expression level of CDH13, while the effect of si-CDH131 was more significant (Figure 2B). Therefore, si-CDH131 was selected for the following experiments. Subsequently, cell viability in the control group, AD cell model group and AD cell model with microRNA-377 knockdown group was detected by CCK-8 assay. Our results indicated that cell viability was decreased in the AD cell model group and AD cell model with microRNA-377 knockdown group compared with that of the control group, which was more severe in the AD cell model with microRNA-377 knockdown group (Figure 2C). Additionally, cell viability in the control group, AD cell model group and AD cell model with CDH13 knockdown group was also detected by CCK-8 assay. Results indicated that cell viability was reduced in the AD cell model group compared with that of the control group, which was reversed by transfection of CDH13 siRNA (Figure 2D). Cell apoptosis in the control group, AD cell model group and AD cell model with microRNA-377 knockdown was accessed by flow cytometry. We found that the number of apoptotic cells was significantly increased in the AD cell model, which was further increased after transfection of anti-microRNA-377 (Figure 2E). Similar experiment was carried out in the control group, AD cell model group and AD cell model with CDH13 knockdown group. Interestingly, our results revealed that the number of apoptotic cells increased in the AD cell model, which was significantly reversed after transfection of CDH13 siRNA (Figure 2F). Taken together, these results suggested that microRNA-377 promotes cell proliferation, whereas CDH13 inhibits cell proliferation.

MicroRNA-377 Could Target Bind to CDH13

To verify whether microRNA-377 could bind to CDH13, WT and MUT sequences of 3'UTR of CDH13 were constructed (Figure 3A). Luciferase intensity of the WT sequence of CDH13 was remarkably decreased after transfection of microRNA-377, while no difference was found between the MUT sequence of CDH13 and the blank vector (Figure 3B). It's therefore suggestive that CDH13 could target bind to microRNA-377.

MicroRNA-377 Regulated the Expression Level of CDH13

To further elucidate the relationship between microRNA-377 and CDH13, microRNA-con, microRNA-377 mimics, anti-microRNA-con and anti-microRNA-377 were transfected into the cells. Our findings suggested that microRNA-377 mimics inhibited the mRNA expression of CDH13, whereas anti-microRNA-377 showed the opposite effect (Figure 4A). Protein expression of CDH13 was consistent with its mRNA expressions (Figure 4B). These results further demonstrated that microRNA-377 could regulate the expression level of CDH13.

Discussion

Alzheimer's disease (AD) is a common neurodegenerative disease mainly occurs in the elderly. The main clinical manifestations of AD include memory impairment and progressive cognitive impairment[10]. Progressive loss of synapses and neurons in the hippocampus and cerebral cortex are characterized as pathological alterations of AD, which is related to short-term memory loss and cognitive dysfunction. At the molecular level, the typical pathological hallmarks of AD are the senile plaques formed by extracellular insoluble Aβ and the nerve fiber entanglement formed by highly phosphorylated Tau protein in nerve cells[11]. As a serious public health problem, limited treatment for AD leads to the difficulty in improving the disease condition. Therefore, it is urgent to find new therapeutic targets to investigate the related pathogenesis of AD. Multiple studies have shown that Aβ is one of the most important pathogenic factors of AD. Aβ can activate microglia and astrocytes, accompanied by inflammatory reaction, which results in progressive synaptic damage. It alters the balance of neurons and produces oxidative stress injury, thus gradually leading to dementia[3]. In this investigation, the AD cell model was constructed by treating SH-SY5Y cells with Aβ. Our results showed that the expression level of Tau protein was significantly increased after the cell model construction. MiRNAs have been found to exert a crucial role in a variety of biological functions, including cell proliferation, differentiation and apoptosis[12]. MiRNAs in the

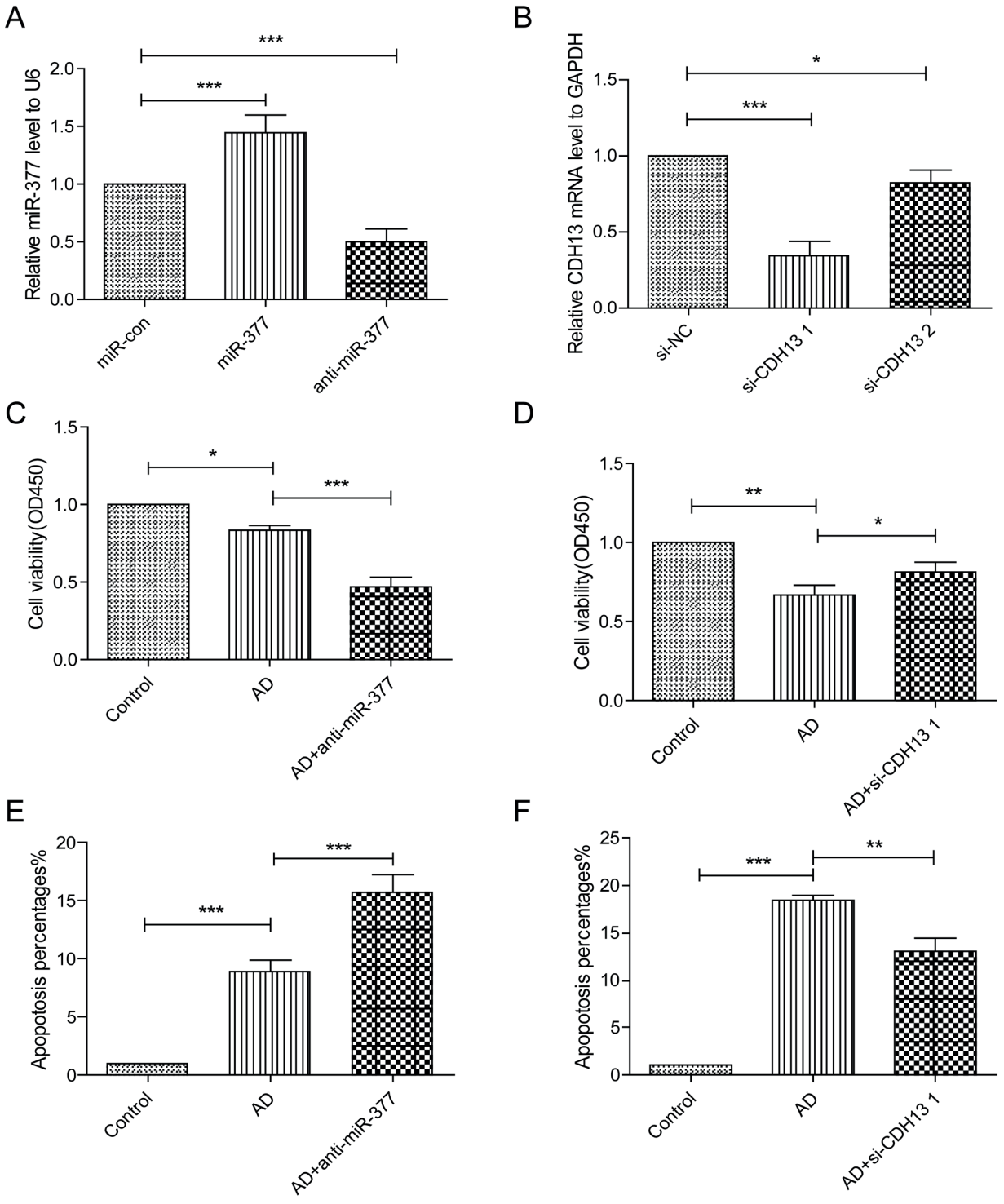

Figure 2. MicroRNA-377 promoted cell proliferation and CDH13 inhibited cell proliferation. ***A,*** The transfection efficiency after overexpression and knockdown of microRNA-377. ***B,*** The efficiency of two interference sequences after knockdown of CDH13. ***C,*** Cell viability in the control group, AD cell model group and AD cell model with microRNA-377 knockdown group. ***D,*** Cell viability in the control group, AD cell model group and AD cell model with CDH13 knockdown group. ***E,*** Cell apoptosis in the control group, AD cell model group and AD cell model with microRNA-377 knockdown group. ***F,*** Cell apoptosis in the control group, AD cell model group and AD cell model with CDH13 knockdown group.

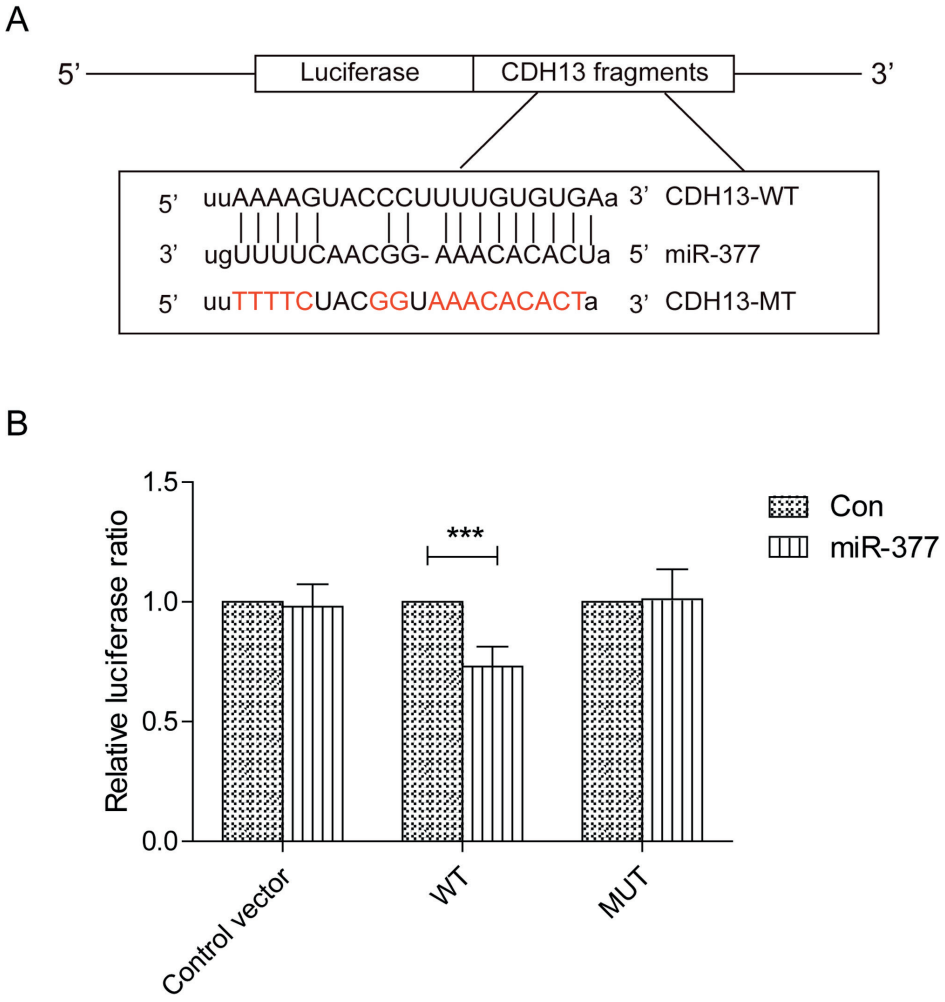

Figure 3. MicroRNA-377 target bound to CDH13. ***A,*** The binding site of microRNA-377 and CDH13. ***B,*** The luciferase reporter gene assay was used to test the binding of microRNA-377 and CDH13.

central nervous system are expressed in a time and stage specific manner, which regulate the development and function of the nervous system[13]. In recent years, differentially expressed miRNAs are found in AD patients. Another research[14] has illustrated that the expressions of miR-9 and miR-128 increased significantly in the brain tissues of AD patients through a gene chip method. Moreover, miR-146a expression was significantly elevated in the frontal cortex and hippocampus of AD patients[15,16]. Our findings indicated that the expression level of microRNA-377 in the AD cell model was significantly reduced, suggesting that microRNA-377 could promote cell proliferation and inhibit cell apoptosis.

CDH13 is an atypical cadherin family member. Although its extracellular cadherin structure is conserved, it lacks transmembrane and cytoplasmic regions and anchors on the cell membrane through glycosylphosphatidylinositol[17]. The mRNA and protein expressions of CDH13 are downregulated in multiple human tumor cell lines and tissues. For example, CDH13 is thought to be an early biomarker for lung cancer because of the decreased expression of methylation[18]. It is also a powerful prediction factor for the prognosis of lung cancer, ovarian cancer and esophageal cancer[19]. Accumulating genome related researches have found that CDH13 is associated with mental disorders, including drug dependence. In addition, the increased expression of CDH13 inhibits the growth of neurons. Huang et al[20] have shown that the expression level of CDH13 is increased after the *in vitro* astrocyte activity is inhibited. However, the cell growth is slowed down when CDH13 is stably expressed in the C6 glioma cell

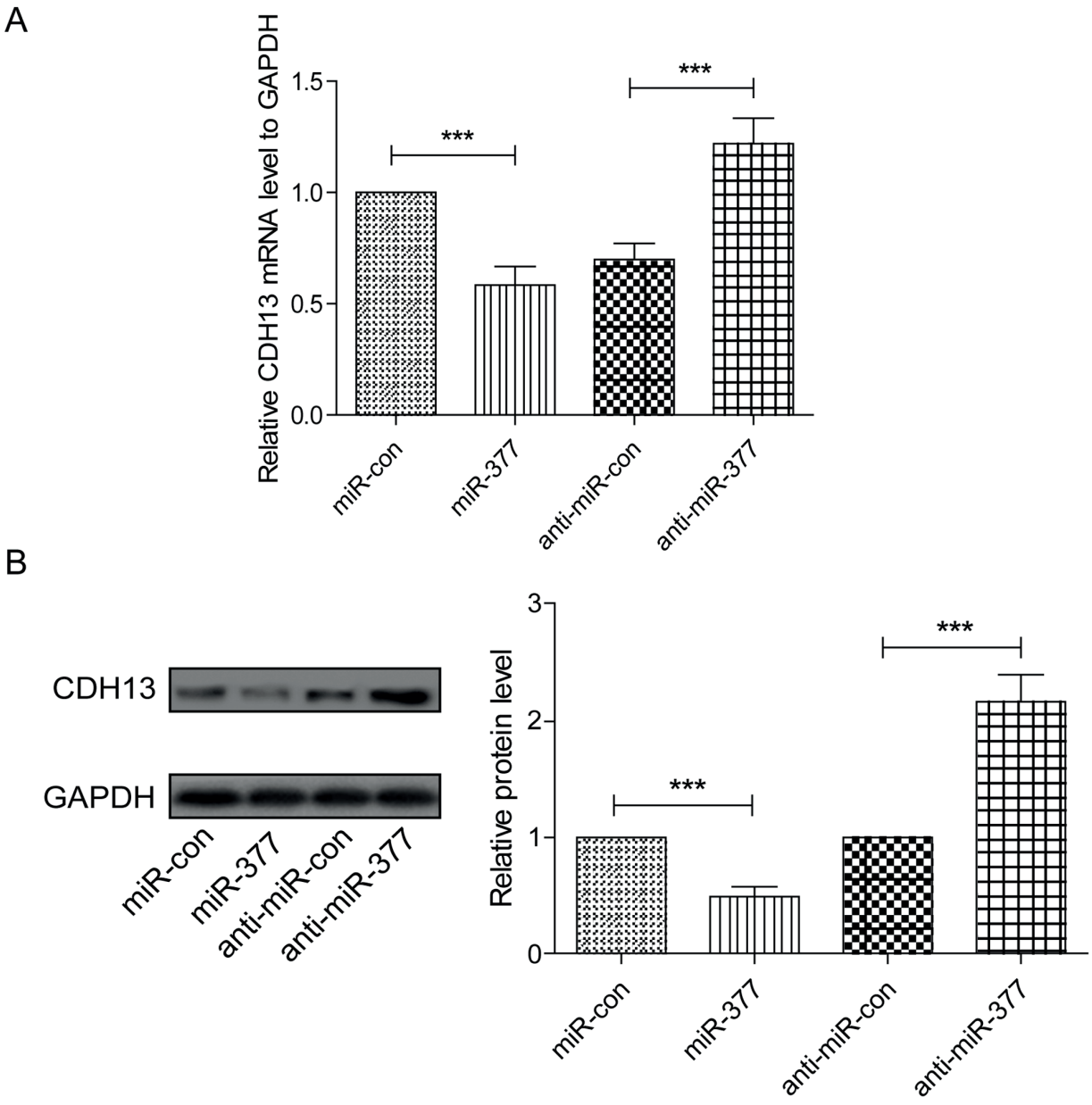

Figure 4. MicroRNA-377 regulated the expression level of CDH13. ***A,*** The mRNA expression of CDH13 after overexpression and knockdown of microRNA-377. ***B,*** The protein expression of CDH13 after overexpression and knockdown of microRNA-377.

line. Takeuchi et al[21] have pointed out that CDH13 is upregulated in the nervous system, which can inhibit the development of neuronal cells and induce G2 phase arrest in astrocytoma by regulating p21. In this study, we found that the expression level of CDH13 was significantly increased in the AD cell model. Additionally, CDH13 could inhibit cell growth and promote cell apoptosis by target binding to microRNA-377.

Conclusions

We observed that microRNA-377 is downregulated in the AD cell model. MicroRNA-377 could promote cell growth and inhibit cell apoptosis by regulating CDH13 expression. Therefore, it can provide a new research direction for exploring the mechanism of Alzheimer's disease.

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Conflict of Interest
The Authors declare that they have no conflict of interest.

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