

Abnormal expression of miR-135a in patients with depression and its possible involvement in the pathogenesis of the condition

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Abstract. At present, due to the increasing pressures on society and the stress of everyday living, the number of individuals suffering from depression has increased. Therefore, the treatment of depression has also received increasing attention. MicroRNA (miRNA/miR)-135a is a well-studied miRNA. It has been reported that miR-135a is significantly downregulated in patients with depression and may be a potential marker for the diagnosis of the condition. However, the specific mechanisms of action of miR-135a in patients with depression remain unclear. In the present study, it was found that miR-135a was downregulated in patients with depression, and in a mouse model of depression. The effects of miR-135a on depression-related symptoms in mice were then explored. In the mice with chronic unpredictable mild stress (CUMS) that were treated with miR-135a for 3 weeks, a significantly reduced level of weight gain was observed in comparison with the control group. In addition, treatment with miR-135a mimic significantly increased sucrose preference in the sucrose preference test in the mice, and reduced the immobility time in the forced swimming test and tail suspension test. Treatment with miR-135a mimic also inhibited CUMS-induced hippocampal cell apoptosis. Furthermore, treatment with miR-135a mimic and fluoxetine significantly reduced the CUMS-induced increase in the expression levels of inflammatory factors (IL-1 β , IL-6 and TNF- α) in the hippocampus of the mice. Subsequently, reverse transcription-quantitative polymerase chain reaction and western blot analysis revealed that treatment with miR-135a mimic significantly inhibited the expression of Toll-like receptor 4 in the mouse hippocampus. In conclusion, the

findings of the present study indicate that miR-135a may be a novel potential target for the treatment of depression.

Introduction

With the increasing amount of pressure on society and the stress of everyday living, the number of individuals suffering from depression is increasing (1). At present, depression is a common disease which is recognized by the World Health Organization and it is one of the main causes of psychiatric disability worldwide (2). Establishing trust and rapport is the primary intervention strategy for depression, followed by specific interventions according to each diagnosis and the individualized treatment care plan (3,4). Researchers have also highlighted the need for nursing training in order for nurses to perform initial assessment procedures and acquire appropriate evidence-based intervention skills and techniques (3). It has been reported that hippocampal volume and nerve density are markedly reduced in patients with depression through pathophysiological studies (4-6). In addition, a number of studies have demonstrated that the pathogenesis of depression is affected by several factors, such as neural and structural plasticity, neurotransmitter systems, and epigenetic and genetic susceptibility (7-11). Therefore, the study of depression has generally attracted the attention of researchers (12,13).

MicroRNAs (miRNAs/miRs) are a group of endogenous small and non-coding RNAs that regulate gene expression by partially binding to the 3'-untranslated region (3'-UTR) of targeted mRNAs (14-16). Studies have indicated that miRNAs are involved in a number of cellular biological processes in normal physiology and pathogenesis, such as differentiation, cell growth, apoptosis and inflammation (17). Previous research has indicated that miRNAs are highly expressed in the central nervous system and play a crucial role in brain functions, such as neurogenesis, neuronal metabolism, proliferation and apoptosis (18,19).

miR-135a has been found to be upregulated in hepatoma cells, and to facilitate cell proliferation, migration and invasion by targeting FOXO1 and TGFB1 (20,21). Moreover, miR-135a has been found to promote cell proliferation by targeting FOXO1 in malignant melanoma (22). In addition, a previous study demonstrated that miR-135a is related to prostate cancer (23). It has been reported that miR-135a is

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downregulated in the serum of patients with depression and may serve as a potential marker for the diagnosis of depression (24). In addition, Gheysarzadeh *et al* (24) demonstrated that miR-16 and miR-1202 expression was decreased in the serum of patients with depression. However, the mechanism of action of miR-135a in the development of depression remains unclear.

Currently, chronic unpredictable mild stress (CUMS)-induced mouse model of depression has been widely used to investigate depression *in vivo* (25-27). Therefore, the aim of the present study was to explore the role of miR-135a in CUMS-induced depression and to analyze its molecular mechanisms of action.

Materials and methods

Clinical samples. In the present study, peripheral blood was obtained from 50 patients with depression (male, 22; female, 28; age range, 37-53 years) and 50 healthy volunteers (male, 24; female, 26; age range, 38-51 years) between January 2018 and December 2019 at Binzhou Youfu Hospital (Binzhou, Shandong, China). Exclusion criteria were a history of bipolar or any psychotic disorder, the use of lithium or an antipsychotic within the prior 2 weeks; substance-use disorder within 3 months; pregnancy or lactation. The healthy controls had no lifetime history of any mental disorder. All patients signed informed consent forms. The present study was approved by the Ethics Committee of Binzhou Youfu Hospital.

Animals and experimental design. A total of 75 male C57BL/6 mice (age, 8-10 weeks; weight, 18-22 g) were purchased from the Nanjing University Animal Research Center. The mice were housed in a standard environment (temperature, 22±2°C; humidity, 55±5%; light/dark cycle, 12 h) with free access to food and water. The mice were randomly divided into five groups (n=15 per group) as follows: i) Unstressed control; ii) CUMS; iii) CUMS + 0.5 nmol mimic control injected intraperitoneally (5'-UUUGUACUACACAAAAGUACUG-3'; Guangzhou RiboBio Co., Ltd.); iv) CUMS + 0.5 nmol miR-135a mimic injected intraperitoneally (5'-UAUGGCUUUUUAUCCUAUGUGA-3'; Guangzhou RiboBio Co., Ltd.); and v) positive control group injected intraperitoneally with 20 mg/kg/day fluoxetine (Sigma-Aldrich; Merck KGaA) for 21 days (CUMS + FLU). All animal experiments were performed according to a protocol approved by the Committee on Care and Use of the Laboratory Animal Committee of Binzhou Youfu Hospital.

A total of 3 weeks after mimic control/miR-135a mimic/FLU treatment, the mice were anesthetized with an intraperitoneal injection of pentobarbital (Sigma-Aldrich; Merck KGaA; 50 mg/kg) and sacrificed by cervical dislocation. Death was defined as the lack of heartbeat and breathing. The peripheral blood and hippocampus tissue were subsequently harvested following euthanasia as previously described (28).

Mouse model of CUMS. A mouse model of depression was established by inducing CUMS, as previously described (25). In brief, the CUMS procedures included the following various mild stress factors: Continuous night illumination (overnight),

cage tilt (7 h), water and food deprivation (24 h), swimming in cold water, noise, wet pads, foreign object exposure (6 h), tail clamp (1 min), hanging of the mice on the balance bar with a rope (10 min), physical restraint for 3 h and a 5-min oscillation. The mice were subjected to 2-3 kinds of stimuli each day; however, the same stressor was not applied again within less than 3 consecutive days. The CUMS procedure lasted for 6 weeks, and treatments with miR-135a mimic/mimic control/FLU were performed daily from the 4th to the 6th week. The body weights of the mice were monitored from the beginning of the experiment and measured each week.

Sucrose preference test (SPT). The SPT was performed using the method previously described by Iñiguez *et al* (29). SPT was performed every week. Briefly, the mice were deprived of water and food for 24 h and then tested for sucrose preference. Each mouse was given free access to two bottles for 12 h: One bottle contained 1% sucrose solution (w/v) and the other bottle contained tap water. To avoid the influence of the positioning, the two bottles were placed opposite each other. After 12 h, the consumed volumes of tap water and sucrose solution were recorded. The sucrose preference was calculated as (sucrose solution intake)/(sucrose solution intake + tap water intake) x100%.

Forced swimming test (FST). After 3 weeks of mimic control/miR-135a mimic/FLU treatment, FST was performed. The FST was carried out in a cylindrical container (height, 65 cm; diameter, 30 cm), which was filled to a height of 40 cm with water (temperature, 22-23°C). The FST lasted for 6 min and the immobility time (in sec) was recorded during the last 4 min. The immobility time was defined as the time when the mouse remained still and not struggling, using only the basic motion to maintain its head on the water, or touching the bottom for >1 sec.

Tail suspension test (TST). After 3 weeks of mimic control/miR-135a mimic/FLU treatment, the TST was performed as previously described (30). Briefly, the tape was placed at a distance of 1 cm from the extremity of the tail of the mouse to fix the position. The mice were suspended for 6 min and immobility was recorded during the last 4 min.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the murine hippocampus tissue or human and murine peripheral blood using TRIzol® reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Total RNA concentration was detected using a NanoDrop 2000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). Total RNA was stored at -80°C until use. The synthesis of cDNA was performed using the RevertAid™ First Strand cDNA Synthesis kit (Vazyme Biotech Co., Ltd.). The reaction conditions were as follows: 70°C for 5 min, 37°C for 5 min and 42°C for 60 min. SYBR-Green (Vazyme Biotech Co., Ltd.) qPCR assay was performed to measure the expression level of the target gene. The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 30 sec. Relative expression levels were calculated

using the $2^{-\Delta\Delta C_q}$ method following normalization with reference to the expression of GAPDH or U6 (31). All experiments were performed in triplicate to ensure minimum deviation. The following primer sequences were used: GAPDH forward, 5'-TTTGGTATCGTGGGAAGGACTC-3' and reverse, 5'-GTA GAGGCAGGGATGATGTTCT-3'; U6 forward, 5'-GCTTCG GCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTC ACGAATTTGCGTGCAT-3'; Toll-like receptor 4 (TLR4) forward, 5'-CCTGACACCAGGAAGCTTGAA-3' and reverse, 5'-TCTGATCCATGCATTGGTAGGT-3'; miR-135a forward, 5'-ACACTCCAGCTCAGTATGGCTTTTATTCCCTATGT-3' and reverse, 5'-CTCAACTGGTGCCTGGAGTCGGCAAT TCAG-3'.

Western blot analysis. Protein was extracted using RIPA buffer with 1 mM protease inhibitor PMSF (Beijing Solarbio Science & Technology Co., Ltd.). A bicinchoninic acid protein assay kit (Sigma-Aldrich; Merck KGaA) was applied to determine protein concentration. Protein (30 mg per lane) was separated on 10% SDS gels and transferred onto PVDF membranes (EMD Millipore). The membranes were blocked with 5% skim milk with TBS containing 0.1% Tween-20 for 1 h at room temperature and incubated with the following primary antibodies: Anti-Bcl-2 (cat. no. 3498; 1:1,000), anti-Bax (cat. no. 2772; 1:1,000), anti-TLR4 (cat. no. 14358; 1:1,000) and anti-GAPDH (cat. no. 5174; 1:1,000) (all purchased from Cell Signaling Technology, Inc.) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit; cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. Subsequently, the protein bands were detected and visualized by RapidStep™ ECL Reagent (EMD Millipore). Band densities were quantified using Gel-Pro Analyzer Densitometry software (version 6.3; Media Cybernetics, Inc.).

ELISA. After treatment, mice were sacrificed and hippocampus were immediately dissected and then stored at -80°C. After the samples were homogenized and centrifuged at 5,000 x g at 4°C for 15 min, the supernatant was collected. ELISA was performed to examine the expression levels of TNF- α (cat. no. PT518), IL-6 (cat. no. PI330) and IL-1 β (cat. no. PI305) in the mouse hippocampus using ELISA kits according to the manufacturer's instructions (all manufactured by Beyotime Institute of Biotechnology).

Flow cytometric analysis. Cell apoptosis was analyzed using the Annexin V/PI Apoptosis Detection kit (BD Biosciences). The hippocampus was dissected as aforementioned and dissociated into a single cell suspension by enzymatic degradation using a neural tissue dissociation kit (Miltenyi Biotec, Inc.). The cells were then collected, centrifuged at low temperature and high speed (1,000 x g at 4°C for 5 min), and re-suspended in 100 μ l FITC binding buffer. Subsequently, the buffer was supplemented with ~5 μ l ready-to-use Annexin V-FITC and 5 μ l PI. In the dark, the cells were incubated for 30 min at room temperature. Annexin V-FITC and PI fluorescence were assessed using a BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (version 7.2.4; FlowJo LLC).

Statistical analysis. Data are presented as the mean \pm SD from at least three independent experiments. GraphPad 6.0 software (GraphPad Software, Inc.) was used for statistical analysis and unpaired Student's t-test was performed to determine whether differences between two groups were significant. Differences among multiple groups were analyzed by one-way ANOVA followed by a Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-135a in patients with depression and in the mouse model of depression. To explore the role of miR-135a in patients with depression and in the mouse model of depression, peripheral blood was collected from 50 patients with depression and 50 healthy subjects, and the expression of miR-135a was detected by RT-qPCR. The results of RT-qPCR revealed that compared with the healthy individuals, miR-135a expression was significantly reduced in patients with depression (Fig. 1A). Subsequently, a mouse model of depression was established by inducing CUMS for 6 weeks. Peripheral blood and hippocampal tissue samples were then collected from the mice, and the expression of miR-135a was detected by RT-qPCR. The results revealed that compared with the control group, the expression of miR-135a was decreased in mouse peripheral blood (Fig. 1B) and in the hippocampus (Fig. 1C) in the CUMS group. These results indicated that miR-135a expression was downregulated in patients and mice with depression.

Effect of miR-135a on the body weights of mice and depressive-like behavior. The present study first confirmed that compared with the mimic control group, miR-135a mimic significantly enhanced the level of miR-135a in the hippocampal tissue of mice (Fig. 2A). At the beginning of the experiment, the weights of the mice in the different groups were similar. Following 2 weeks of CUMS, the body weights of the mice in the model group were significantly lower than those in the control group. Treatment with miR-135a mimic and FLU significantly attenuated the CUMS-induced reduction in weight gain (Fig. 2B). The effects of miR-135a on depression-related symptoms in mice were then examined. The SPT was determined every week. And following treatment of the mice subjected to CUMS with miR-135a mimic for 3 weeks, the FST and TST were used to evaluate the anti-depressant effects of miR-135a mimic. Compared with CUMS + mimic control group, treatment with miR-135a mimic and FLU markedly decreased CUMS-induced depression-like behavior at 3 weeks after treatment. Compared with the mice in the CUMS + mimic control group, miR-135a mimic significantly increased SPT (1, 2 and 3 weeks after treatment) in mice and reduced the immobility time in the FST and TST (Fig. 2C-E). These results indicated that miR-135a treatment relieved CUMS-induced depressive-like behavior.

Effect of miR-135a on CUMS-induced hippocampal cell apoptosis. Subsequently, the effects of miR-135a on the apoptosis of neural cells in the mouse hippocampus were examined by flow cytometry. Compared with the control group, hippocampal neuronal apoptosis was significantly increased in the

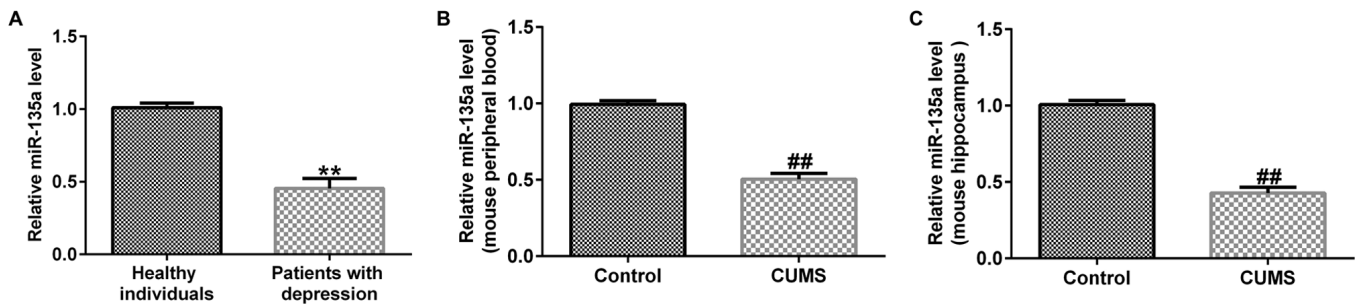


Figure 1. miR-135a expression is decreased in patients and mice with depression. (A) miR-135a expression levels were evaluated by reverse transcription-quantitative polymerase chain reaction in peripheral blood from 50 patients with depression and 50 healthy individuals. Expression of miR-135a in mouse (B) peripheral blood and (C) hippocampus in the control and CUMS groups. Data are presented as the mean \pm SD. ** P <0.01 vs. healthy individuals; ## P <0.01 vs. control. CUMS, chronic unpredictable mild stress; miR, microRNA.

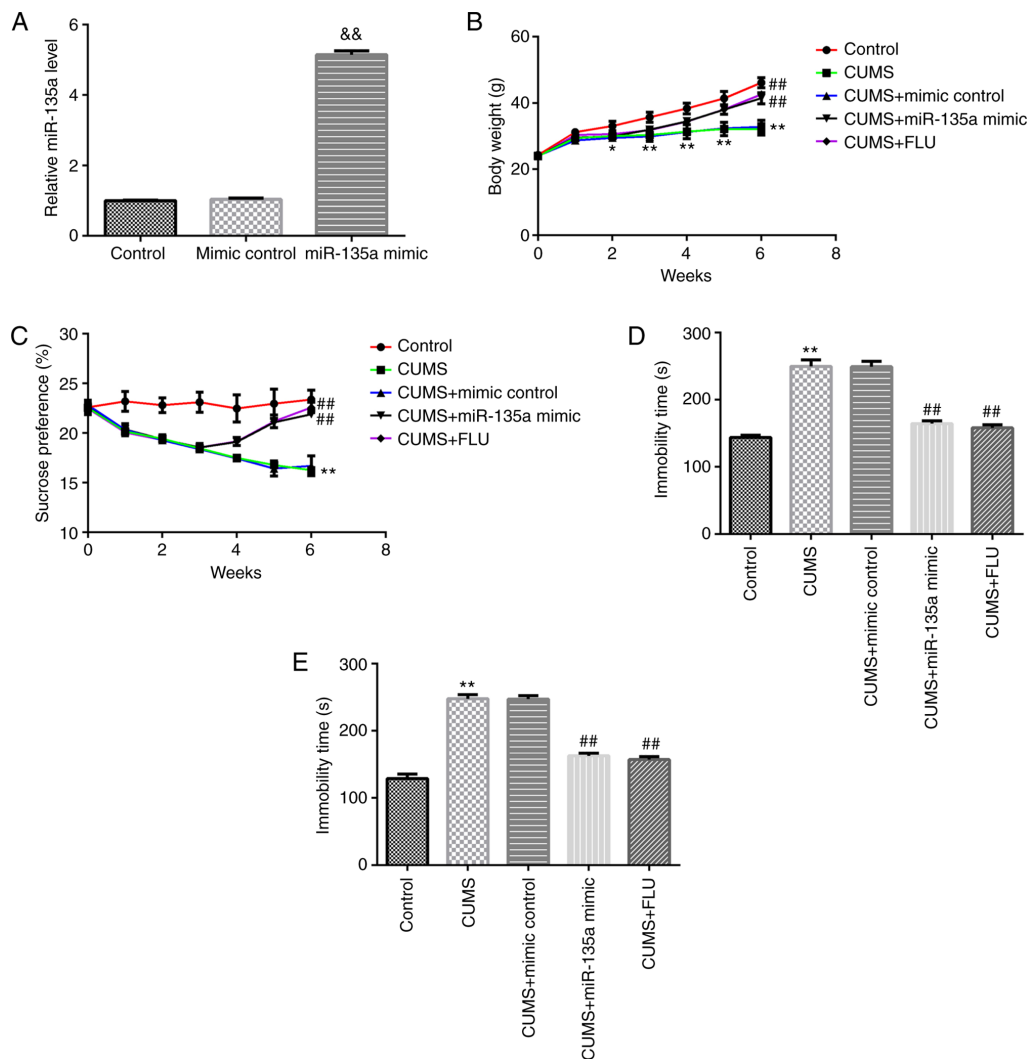


Figure 2. miR-135a attenuates CUMS-induced weight loss in mice and symptoms associated with CUMS in mice. (A) miR-135a level in the hippocampal tissue of mice was detected using reverse transcription-quantitative polymerase chain reaction. (B) Body weight of mice. (C) Sucrose preference test results. (D) Forced swimming test results. (E) Tail suspension test results. Data are presented as the mean \pm SD. && P <0.01 vs. mimic control group; * P <0.05 and ** P <0.01 vs. control group; ## P <0.01 vs. CUMS + mimic control group. CUMS, chronic unpredictable mild stress; FLU, fluoxetine; miR, microRNA.

CUMS group (Fig. 3A and B). However, the apoptotic rate in the hippocampus was decreased in the miR-135a mimic- and FLU-treated group compared with the CUMS group (Fig. 3A and B). In addition, compared with the control group,

Bax protein expression was increased, Bcl-2 protein expression was decreased, and the Bax/Bcl-2 ratio was increased in the CUMS group (Fig. 3C and D). However, miR-135a mimic and FLU treatment significantly reduced the expression of

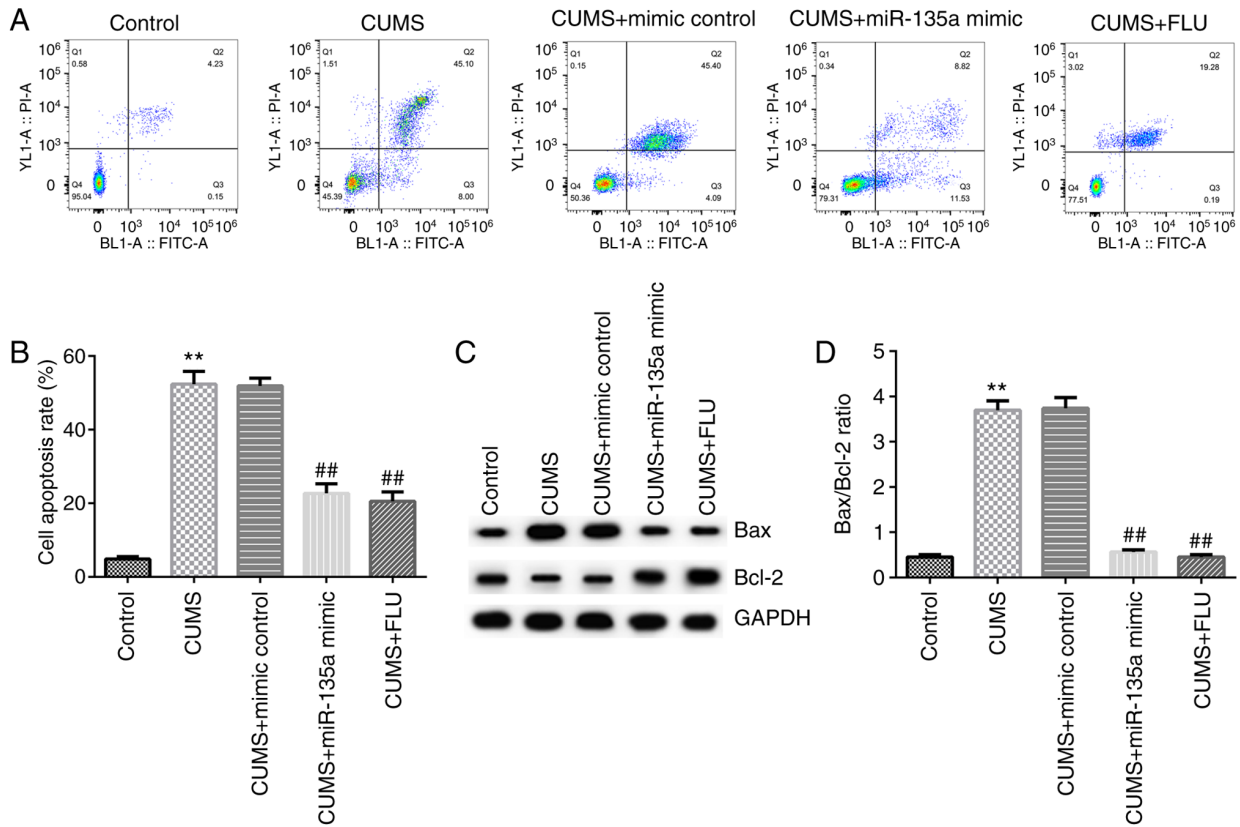


Figure 3. miR-135a inhibits CUMS-induced hippocampal cell apoptosis. (A and B) Flow cytometric analysis of cell apoptosis in mice in the different groups. (C) Western blot analysis of the protein expression of Bax and Bcl-2. (D) Ratio of Bax/Bcl-2. Data are presented as the mean \pm SD. ** P <0.01 vs. control group; ## P <0.01 vs. CUMS + mimic control group. CUMS, chronic unpredictable mild stress; FLU, fluoxetine; miR, microRNA.

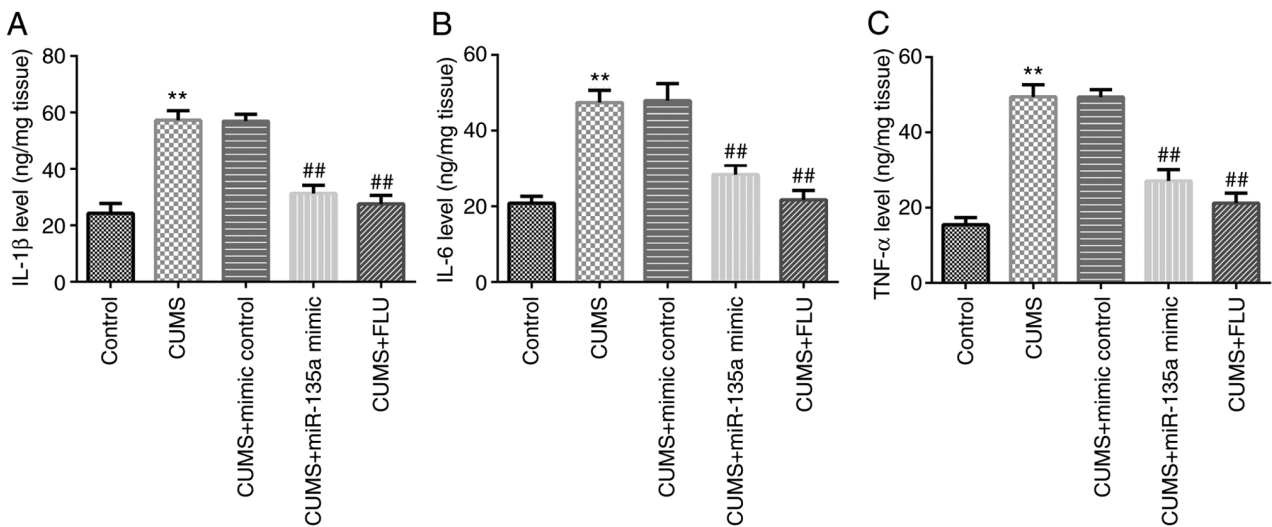


Figure 4. miR-135a attenuates the CUMS-induced hippocampal inflammatory response. ELISA of the protein levels of (A) IL-1 β , (B) IL-6 and (C) TNF- α in the hippocampus of mice. Data are presented as the mean \pm SD. ** P <0.01 vs. control group; ## P <0.01 vs. CUMS + mimic control group. CUMS, chronic unpredictable mild stress; FLU, fluoxetine; miR, microRNA.

Bax, increased Bcl-2 expression and decreased the Bax/Bcl-2 ratio (Fig. 3C and D). Taken together, these results indicate that miR-135a inhibited CUMS-induced hippocampal cell apoptosis.

Effects of miR-135a on the CUMS-induced hippocampal inflammatory response. Subsequently, to examine the effects of miR-135a

treatment on neuroinflammation, ELISA was performed to detect the levels of inflammatory factors in the hippocampus. The results revealed that the levels of IL-1 β , IL-6 and TNF- α were increased in the CUMS group compared with the control group (Fig. 4A-C). However, miR-135a mimic and FLU treatment significantly attenuated the CUMS-induced increase in the levels of inflammatory factors in the hippocampus of mice (Fig. 4A-C).

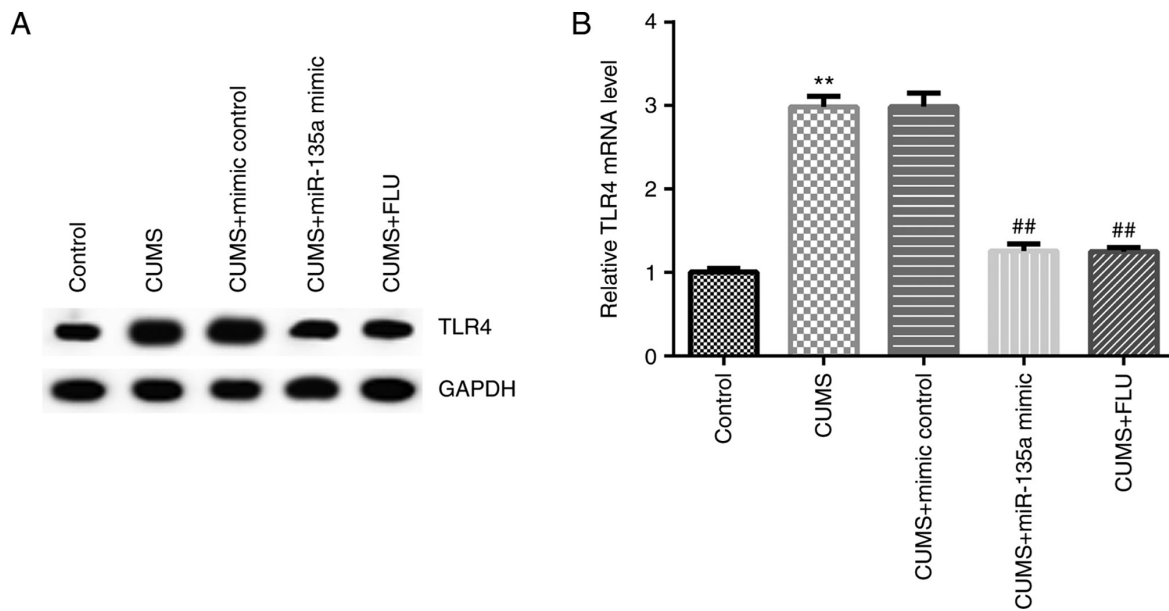


Figure 5. Expression of TLR4 in mice with depression. (A) Western blot analysis of TLR4 protein expression. (B) Reverse transcription-quantitative polymerase chain reaction assay of TLR4 mRNA expression. Data are presented as the mean \pm SD. ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. CUMS + mimic control group. CUMS, chronic unpredictable mild stress; FLU, fluoxetine; miR, microRNA; TLR4, Toll-like receptor 4.

Therefore, miR-135a decreased the CUMS-induced hippocampal inflammatory response.

miR-135a may protect against depression in mice by regulating TLR4 expression. A previous study demonstrated that TLR4 was a target gene of miR-135a (32). Therefore, in order to elucidate whether miR-135a affects depression by regulating the expression of TLR4, the expression of TLR4 was examined in the mice following treatment. After 3 weeks, the expression of TLR4 was detected in the hippocampus of the mice in each group by RT-qPCR and western blot analysis. The results revealed that compared with the control group, the mRNA expression of TLR4 was significantly increased in the CUMS group, and this increase was significantly inhibited by miR-135a mimic (Fig. 5A and B).

Discussion

Depression is a common illness that severely influences the quality of life. In recent years, an increasing number of studies have focused on the exploration of the role of miRNA in the pathological mechanism of depression (33-35). It has previously been reported that miR-135a exerts an inhibitory effect in prostate cancer and lung cancer (36,37). Furthermore, miR-135a has been reported to function as an oncogene in gastric and colorectal cancer (38,39). In addition, Shi *et al* (40) indicated that miR-135a expression was downregulated in glioma tissues and cells. However, to the best of our knowledge, there are few studies available on the expression and mechanisms of action of miR-135a in patients with depression. Gheysarzadeh *et al* (24) demonstrated that miR-135a expression was decreased in patients with depression. CUMS-induced animal models are currently considered as one of the most appropriate animal models for studying depression (25-28,41). CUMS exposure can induce

depression-like behavior in animals, such as behavioral despair, a lack of pleasure and reduced exercise tolerance (42). In the present study, it was found that the expression of miR-135a was downregulated in patients with depression and in a mouse model of CUMS-induced depression.

Wan *et al* (42) demonstrated that miR-29b-3p overexpression improved depressive-like behaviors in rats with CUMS. In the current study, the effects of miR-135a on depression-related symptoms in mice with depression were examined. It was found that miR-135a attenuated CUMS-induced in mice. In addition, miR-135a mimic significantly increased SPT in mice and shortened the immobility time in the FST and TST. The results of the present study also demonstrated that miR-135a inhibited CUMS-induced hippocampal cell apoptosis. There is increasing evidence to indicate that inflammation is the primary pathological mechanism of depression (43). Psychological and physical stressors can stimulate immune and inflammatory processes (44). Previous studies have demonstrated that the increase in the levels of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) in certain regions of the brain, such as the hippocampus of depressed mice, is associated with the pathophysiology of depression (45,46). In the present study, miR-135a attenuated the CUMS-induced hippocampal inflammatory response and reduced the CUMS-induced increase in the levels of inflammatory factors, including IL-1 β , IL-6 and TNF- α in the hippocampus of mice.

All the aforementioned results indicate that miR-135a has a certain alleviating effect on depression. Du and Lu (47) demonstrated that TLR4 was the direct target gene of miR-135a, and miR-135a suppressed oxidative stress and vascular inflammatory events through TLR4 in atherosclerosis. TLR4 plays an important role in the development process of atherosclerosis (48) and acts as a potential target of miR-590 (49). The findings of the present study demonstrated that miR-135a may protect mice against depression by regulating TLR4 expression.

In conclusion, the present study demonstrated that miR-135a regulated the apoptosis and inflammatory response in mouse hippocampal neurons by regulating the expression of TLR4, thereby alleviating the depressive behavior of mice and playing a protective role in depression.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YXD conceived and designed the current study, acquired, analyzed and interpreted the data, and prepared the manuscript. MZ, BJQ, CPL and JFW contributed to the acquisition and analysis of the data. JL contributed to acquisition and analysis of the data and prepared the manuscript. YXD and JL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients signed informed consent forms. The present study was approved by the Ethics Committee of Binzhou Youfu Hospital (Binzhou, China). All animal experiments were performed according to a protocol approved by the Committee on Care and Use of the Laboratory Animal Committee of Binzhou Youfu Hospital.

Patient consent for publication

All patients consented to the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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