

## Original Article

# MicroRNA-155 modulates the expression of pro-inflammatory cytokines in natural killer cells of rats exposed to chronic mild stress by regulation of ERK1/2 signaling pathway

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**Abstract:** Objectives: MicroRNAs (miRNAs) have been reported to be involved in depression. Besides, cell-mediated immunity (CMI) and inflammatory response system (IRS) are responsible for depression. The purpose of our study was to investigate the role of microRNA-155 (miR-155) in depression and the possible mechanism involving pro-inflammatory cytokines (PICs). Methods: Sprague-Dawley (SD) rats were randomly assigned to control and stress group. The rats in the stress group were subjected to chronic mild stress (CMS). Natural killer (NK) cells were isolated from peripheral blood mononuclear cells (PBMCs) of the rats in the both two groups. The expression levels of miR-155 in NK cells were determined by quantitative real-time RCP (qRT-PCR). Lentiviral vectors expressing a miR-155 sponge (Lv-miR155-sponge) and an empty vector (Lv-control) were then transfected into NK cells and confirmed by qRT-PCR. Quantitative analyses of supernatants levels for interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$  were performed before and after transfection using enzyme-linked immunosorbent assay (ELISA). In addition, the protein levels of phospho-extracellular signal-regulated kinase (ERK)1/2 and ERK1/2 were evaluated after transfection. Results: The relative levels of miR-155 and PICs were significantly increased in the stress group compared to the control group. But transfection with Lv-miR155-sponge significantly decreased the levels of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  (all  $P < 0.05$ ) and activated ERK1/2 signaling pathway. Conclusion: Our results suggest that miR-155 modulates the expression of PICs in NK cells of rats exposed to CMS by regulation of ERK1/2 signaling pathway.

**Keywords:** MicroRNA-155, pro-inflammatory cytokines, natural killer cells, depression, ERK1/2 signaling pathway

## Introduction

An increasing body of literature has demonstrated that depression serves as a potential risk factor in the morbidity and mortality of many human diseases [1]. Although considerable advances have been achieved over the past decades, the underlying pathophysiology of depression is still unknown. Recently, there is a substantial basic and clinical research documenting that depression has been accompanied by activation of inflammatory response system (IRS) and cell-mediated immunity (CMI) [2-4]. IRS is characterized by increased levels of pro-inflammatory cytokines (PICs), such as interleukin-1 (IL-1), IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [5], and increased levels of acute phase

(AP) protein, such as prostaglandin PGE<sub>2</sub>. CMI is correlated with cellular interactions between T cell and monocytes through cytokines derived from T cell [6], such as interferon- $\gamma$  (IFN- $\gamma$ ) could activate monocytes to produce IL-1, IL-12, and TNF- $\alpha$ . Moreover, natural killer (NK) cells, another lymphocyte subset, have also been observed in depression. Previous studies suggested that NK cell cytotoxicity and counts of NK cells were reduced in major depressive disorder (MDD) [7], however, other studies obtained opposing findings [8]. In addition, NK cell activity has been reported to be related to antidepressant response in MDD patients [9].

Moreover, increasing evidence indicates that altered neuronal and structural plasticity and

neurogenesis are responsible for depression. MicroRNAs (miRNAs), a class of small non-coding RNAs, play significant roles in neural plasticity and brain function, as well as the possible roles in neurodegenerative disorders including major depression [10-13]. In addition, miRNAs control both neuronal and immune responses [14]. Among miRNAs, miR-155 is involved in protective immunity by regulation of T cell, B cell, and dendritic cell differentiation [15]. Of interest, miR-155 has been reported to be over-expressed in NK-cell lymphoma/leukemia [16], and regulated the production of IFN- $\gamma$  in NK cells [17]. However, little information is available on the role of miR-155 in depression, and whether the possible mechanism involving in inflammatory cytokines in NK cells is unclear.

In consideration of the correlation between miR-155 and NK cells, the purpose of our study was to investigate the possible role of miR-155 in NK cells during depression, as well as the relationship between the expression of miR-155 and the expression of PICs.

### Material and methods

#### *Animals and groups*

Twenty male Sprague-Dawley (SD) rats (45-days old) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd., China. All the animals were caged individually in an air-conditioned room with controlled temperature ( $22\pm 2^\circ\text{C}$ ) and humidity ( $60\pm 10\%$ ) under a 12-h light/dark cycles. Rats had access to food and water available *ad libitum*, and allowed to acclimatize for at least 2 weeks prior to experiments. The locomotor activity baseline was recorded and the general consummatory behavior was tested for water and sucrose. On the basis of sucrose intake in the final session and body weight, the rats were randomly assigned into two groups ( $n = 10$  in each group): control and stress group. The experiments were approved by the Ethics Committee on Animals at our University, and the animal use was in accordance with the standards for "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

#### *Chronic mild stress (CMS) protocol*

The rats in the stress group were subjected to an initial 28 days of chronic mild stressors. The stress procedure was performed with a fixed weekly schedule. Each week includes 14 h of

food deprivation, 2 h of limited food access, 14 h of cage tilt at  $45^\circ\text{C}$  or 36 h of continuous lighting, two-period 14 h of soiled cage, two-period 5 min of swim at  $30^\circ\text{C}$  followed by low-intensity stroboscopic illumination (120 flashes/min). This schedule was repeated over 4 weeks. The rats in the control group were housed in a separate room and received no special treatment.

#### *Isolation of NK cells*

Peripheral blood of the two groups was obtained immediately after the rats were executed. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll density gradient centrifugation. NK cells were isolated from PBMC using magnetic bead-based sorting with the DX5 antibody (AutoMACS, Miltenyi Biotech, Cambridge, MA) and cultured in the RPMI-1640 complete medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 300 mg/L L-glutamine, 10 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). NK cell viability was determined by an analysis of trypan blue exclusion. Cells were harvested for further analysis.

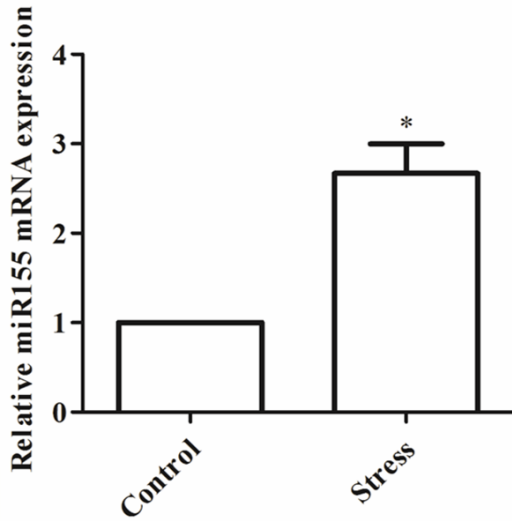
#### *Lentivirus preparation and transduction*

Lentiviral vectors expressing a miR-155 sponge (Lv-miR155-sponge), an empty vector (Lv-control), or luciferase were designed and generated by GenePharma (Shanghai, China) according to GenePharma's recommendations. To construct the miRNA-155 sponge lentivirus plasmid, a genomic fragment encompassing the miR-155 coding region was cloned into pMD18-T lentiviral vector (pMD18-T-miR155-Sponge). Thereafter, the miRNA-155 sponge fragment was digested and subcloned into pCDH-CMV-MCS-EF1-copGFP lentiviral vector. The lentiviruses were transduced into NK cells using Lipofectamine 2000, and the transduction efficiency was estimated by measuring the expression of green fluorescent protein (GFP). After incubation, the viral supernatant was replaced with fresh media and the culture supernatant was harvested and concentrated for further analyses.

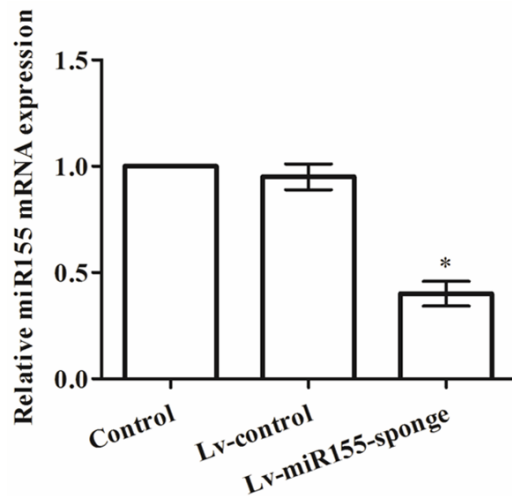
#### *RNA extraction and quantitative PCR analysis*

Total RNA was extracted from the cells transfected with or without expression vectors using Trizol reagent (Invitrogen, Carlsbad, CA, USA)

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**Figure 1.** Relative expression of miR-155 in NK cells. MiR-155, microRNA-155; NK, natural killer. \* $P < 0.05$  compared with the control group.



**Figure 2.** Effect of lentivirus transfection with miR-155-sponge on the expression of miRNA-155 in NK cells. MiR-155, microRNA-155; NK, natural killer; Lv, lentivirus; \* $P < 0.05$  compared with the control group or Lv-control group.

according to the manufacturer's protocol. Each RNA sample was reversely transcribed to complementary DNA (cDNA) using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and quantified by QuantTect SYBR green real-time PCR Kit (Qiagen SA, Hilden, Germany). The relative expressions of miRNA-155 were determined using Hairpin-it™ miRNAs qPCR kit (GeneCopoeia, MD, USA) with the comparative  $2^{-\Delta\Delta CT}$  methods. Primers for miRNA-155 were de-

signed by the TaqMan miRNA assays and U6 snRNA was used for normalization.

### Enzyme-linked immunosorbent assay (ELISA)

After transfection, the supernatants were collected and centrifuged to remove cellular debris. Quantitative analyses of supernatants levels for IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  were performed using a commercial ELISA kit (Wuhan Eiaab Science, Wuhan, China) according to the manufacturer's instructions. All samples were prepared in duplicate.

### Western blotting analysis

Twenty-four hours after transfection, the cells in each group were collected for protein extraction. Total protein concentration was measured using Bio-Rad DC protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were separated on a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then were blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Inc.). Thereafter, the membranes were blocked in a sealed in 5% fresh nonfat dry milk in phosphate buffer saline (PBS) with 0.1% Tween 20 (PBST) and probed with the following primary antibodies: anti-extracellular signal-regulated kinase (ERK)1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phospho-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. GAPDH was used as a loading control for normalization. The immunoreactive protein bands were conducted to enhanced chemiluminescence and the immunoreactive bands were analyzed by densitometric analysis.

### Statistical analysis

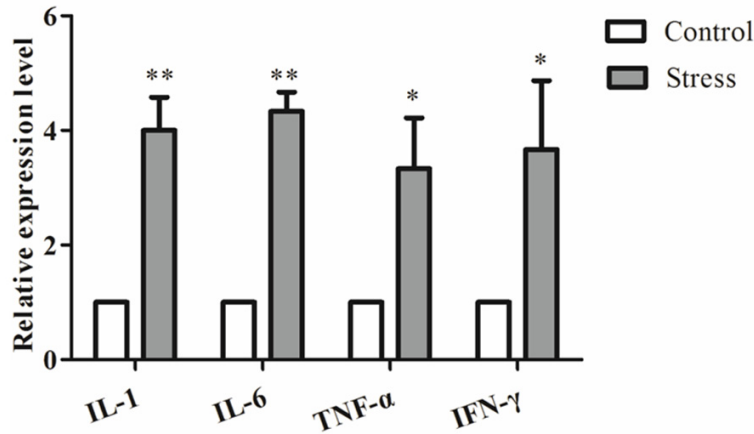
All experiments were performed three times. The data, presented as the mean  $\pm$  standard deviation (SD), were analyzed by statistical package for the social sciences (SPSS) 19.0 statistical software. One-way analysis of variance (ANOVA) was used to calculate the  $P$ -values. A  $P$ -value of  $< 0.05$  was considered as statistically significant.

## Results

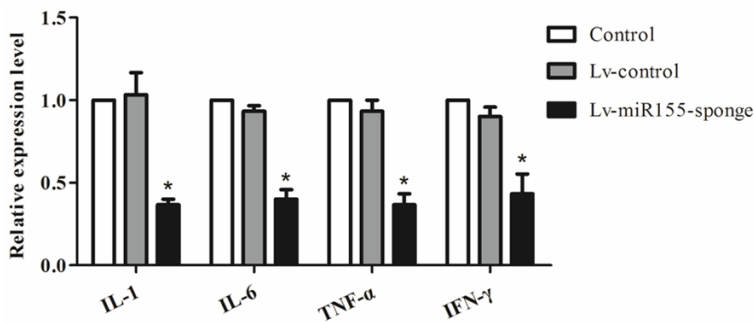
### Expression of miR-155 in NK cells

To explore the role of miR-155 in depression, we induced the CMS model of depression and

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**Figure 3.** Relative expression of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  in NK cells. IL, interleukin; TNF, tumor necrosis factor; and IFN, interferon; NK, natural killer. \* $P < 0.05$  compared with the control group; \*\* $P < 0.01$  compared with the control group.



**Figure 4.** Effect of lentivirus transfection with miR-155-sponge on the expression of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  in NK cells. MiR-155, microRNA-155; NK, natural killer; Lv, lentivirus; IL, interleukin; TNF, tumor necrosis factor; and IFN, interferon. \* $P < 0.05$  compared with the control group or Lv-control group.

isolated the NK cells from PBMCs of all the animals. The expression levels of miR-155 in the stress group and the control group were determined using qRT-PCR. As shown in **Figure 1**, the relative miR-155 mRNA levels were significantly increased in the stress group compared to the control group ( $P < 0.05$ ), indicating that miR-155 might play a significant role in depression.

### *Effect of lentivirus transfection with miR-155-sponge on the expression of miRNA-155 in NK cells*

The expression of miR-155 was silenced by transfection with Lv-miR155-sponge, and the lentiviral vectors expressing an empty vector were used as a control. After transfection, the

mRNA levels of miRNA-155 in NK cells were determined using qRT-PCR. The results showed that there were no significant differences between the control group (without transfection) and the Lv-control group. However, transfection with Lv-miR155-sponge resulted in significantly lower expression levels of miRNA-155 than the control group or the Lv-control group ( $P < 0.05$ ) (**Figure 2**).

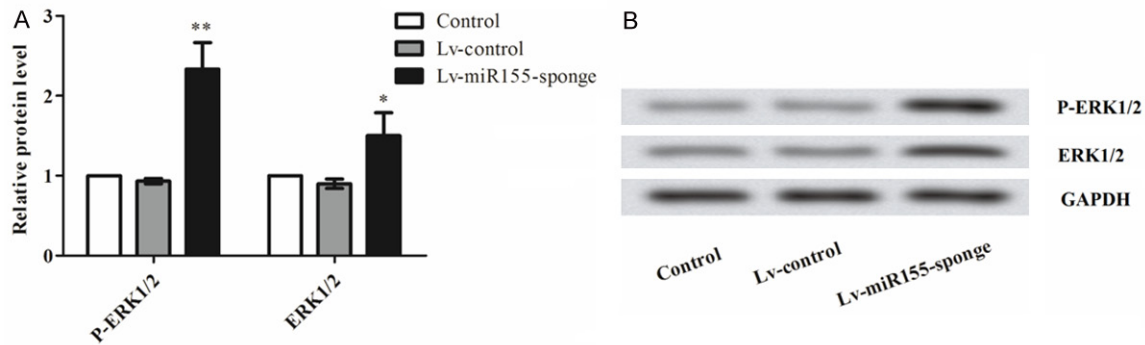
### *Expression of IL-1, IL-6, TNF-α, and IFN-γ in NK cells*

To explore the role of inflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$ ) in depression, the NK cells from PBMCs of all the animals were isolated and the expression levels of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  in the both two groups were determined using ELISA. We found that all the relative levels of IL-1 ( $P < 0.01$ ), IL-6 ( $P < 0.01$ ), TNF- $\alpha$  ( $P < 0.05$ ), and IFN- $\gamma$  ( $P < 0.05$ ) were significantly elevated in the stress group compared to the control group (**Figure 3**). The results suggested that inflammatory cytokines were highly expressed during depression.

### *Effect of lentivirus transfection with miR-155-sponge on the expression of IL-1, IL-6, TNF-α, and IFN-γ in NK cells*

To investigate the effect of lentivirus transfection with miR155-sponge on the expression of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  in NK cells, the expression levels of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  were measured by ELISA after transfection. As shown in **Figure 4**, no significant differences were found in the levels of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  between the control group and the Lv-control group. But all the levels were significantly down-regulated by transfection with Lv-miR155-sponge (all  $P < 0.05$ ), demonstrating that the expression of inflammatory cytokines were controlled and correlated with the expression of miR-155.

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**Figure 5.** Effect of lentivirus transfection with miR-155-sponge on ERK1/2 signaling pathway in NK cells. MiR-155, microRNA-155; NK, natural killer; Lv, lentivirus; p-ERK, phospho-extracellular signal-regulated kinase. \* $P < 0.05$  compared with the control group or Lv-control group.

### *Effect of lentivirus transfection with miR-155-sponge on ERK1/2 signaling pathway in NK cells*

To further investigate whether the effects of lentivirus transfection with miR-155-sponge on the expression of inflammatory cytokines were involved in the ERK1/2 signaling pathway, the protein levels of p-ERK1/2 and ERK1/2 were assessed. As shown in **Figure 5A** and **5B**, the protein levels of p-ERK1/2 ( $P < 0.01$ ) and ERK1/2 ( $P < 0.05$ ) were statistically increased by transfection with miR-155-sponge compared with the control group or the Lv-control group. These results suggested that the effect of lentivirus transfection with miR-155-sponge on the expression of inflammatory cytokines by activation of ERK1/2 signaling pathway.

### **Discussion**

In the present study, the role of miR-155 in depression was investigated, as well as the possible mechanism. We found that the expression of miR-155 and PICs in NK cells isolated from the depressed rats were significantly higher than those in the normal control group. To further explore the correlation between the expression of miR-155 and PICs in NK cells, we suppressed the expression of miR-155 in NK cells and determined the expression levels of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$ . The results showed that the expression levels of these cytokines were significantly decreased by suppression expression of miR-155, demonstrating that miR-155 might regulate the release of inflammatory cytokines in NK cells. Further study confirmed that the ERK1/2 signaling pathway was

activated by suppression expression of miR-155. Our results suggested that miR-155 played a significant role in depression. The effects might be related to the regulation of PICs by miR-155 in NK cells through ERK1/2 signaling pathway.

Major depression, a severe psychiatric disorder, has been reported to be the fourth leading cause of disability worldwide. Existing evidence has convincingly established that depression is an inflammatory disorder accompanied by CMI activation [2, 18]. A recent meta-analysis found that depression represented increased plasma IL-6 and TNF- $\alpha$  levels [5]. The increased plasma PIC levels are markedly related to the number of previous depressive episodes [18], and may be associated with treatment resistance to antidepressants [19-21]. In addition, previous studies found that repeated administration of PICs (e.g. IL-1, IL-6 and TNF- $\alpha$ ) showed depressive-like behaviors including anhedonia, anorexia, memory impairments, and anxiety-like behaviors [22-25]. However, sensitization of the inflammatory response has been suggested to be a new drug target in depression [26]. IFN- $\gamma$  is one of characteristic cytokine that is involved in CMI activation of depression. The mRNA expression of TNF- $\alpha$  and IFN- $\gamma$  has been suggested be related to the scores of depression during acute attacks of multiple sclerosis (MS) [27], while depressed patients who received treatment with antidepressants significantly decreased the elevated production of IFN- $\gamma$  [28].

Moreover, changes in the number and activity of NK cell is another altered immunity involved



in depression, however, these changes are not always consistent [29, 30]. Age differences, severity of depression, and previous history of pharmacological treatment might be contributed to the differences [31]. In addition to cytolytic activity, NK cells could produce cytokines and chemokines, which play an important role in immunoregulatory function. Many regulatory mechanisms have been suggested to regulate the potent responses of NK cells at both the cellular and molecular level. Recently, miRNAs have been implicated as important regulators in the modulation of NK cell development and function [32, 33]. Additionally, lacking miRNAs shows defects in NK cell proliferation and IFN- $\gamma$  secretion after viral infection [34]. MiR-155 and its gene targets have been well demonstrated in immune system [35]. It has been reported that miR-155 is overexpressed in many immune cells including NK cells. For example, Zawislak *et al.* has suggested that miR-155 plays a critical role in maturation, maintenance, and responses to viral infection of NK cell [36]. In consideration of the role of NK cells in depression, the role of miR-155 in NK cells, and the role of miRNAs and PICs in depression, we therefore speculated that miR-155 might be responsible for depression by regulating PICs in NK cells.

To confirm the speculation, we first induced CMS model of depression and isolated NK cells from all rats. Our study showed an elevated expression of miR-155 and PICs in the depressed rats compared to the normal rats. The results were in line with previous studies in which there were also higher levels of PICs in depression. We further explored the relationship between the expression of miR-155 and PICs. The expression of miR-155 was suppressed using lentivirus transfection, and then the expression of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  were confirmed. The results suggested that after suppressing the expression of miR-155, the elevated levels of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  were also decreased in NK cells, indicating that miR-155 regulates the expression of PICs in NK cells. To further understand the possible signaling pathway mechanism, we determined the expression levels of p-ERK1/2 and ERK1/2. It has been well demonstrated that ERK1/2 signaling is an important player in neural plasticity and neurogenesis processes, which is involved in depression [37, 38], as well

as the activation of NK cell [39]. Similarly, our results found that ERK1/2 signaling was stimulated by suppression of miR-155 in NK cells exposed to CMS, indicating that the effect of miR-155 on depression by regulating the expression of PICs in NK cells through ERK1/2 signaling pathway.

In conclusion, our results suggest that miR-155 regulates the expression of PICs in NK cells of depressed rats by modulation of ERK1/2 signaling pathway. Suppressing the expression of miR-155 might be a new targeted therapeutic remedy for depression.

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### Disclosure of conflict of interest

None.

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