

## Original Article

# MIR-194 is related to the pathogenesis of asthma by regulating TLR4 expression

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**Abstract:** Objective: Activation of Toll-like receptors on immune surveillance cells in the lung has been confirmed to be implicated in the pathobiology of allergic asthma. It is reported that TLR4 activation with LPS induced significant increases in IL-6 release and evoked pro-asthmatic like changes in the constrictor and relaxation responsiveness of isolated airway smooth muscle (ASM) tissues. IKK mediated NF- $\kappa$ B activation is also involved in the responses of ASM to LPS. Methods: In this study, we first screened 10 candidate miRNAs in the plasma samples of the patients with asthma and found that the expression of miR-375 and miR-194 was reduced significantly. Subsequently, we confirmed 2 target genes of miR-375 that are related to the hypersensitivity of the NF- $\kappa$ B signaling. Results: We found miR-194 and miR-375 were reduced and miR-21 was significantly up-regulated in the plasma samples of asthma patients. The same condition was also confirmed in the LPS stimulated ASM cells except the expression of miR-375. After prediction by bioinformatics tools; we noticed that TLR4 may be a direct target of miR-194. Conclusions: Confirmed by dual luciferase assay and Western blot, we identified that the expression of TLR4 was repressed by miR-194. Meanwhile, reduced expression of miR-194 is correlate with over activated NF- $\kappa$ B signaling and up-regulated cytokines from ASM cells when stimulated by LPS.

**Keywords:** microRNA, TLR4, asthma, cytokine, NF- $\kappa$ B

## Introduction

Asthma is a chronic inflammatory disease of the airways which was mostly induced by inhaled antigens. Recently, several reports indicated that airway smooth muscle (ASM) can directly respond to various pro-asthmatic stimuli [1, 2]. Meanwhile, it is well known that human ASM cells constitutively express TLR4 and TLR9 [3, 4]. After stimulation by LPS, activated TLR4 potently elicits release of the pleiotropic pro-inflammatory cytokine IL-6 and evokes significant pro-asthmatic like changes in rabbit ASM tissue, suggesting that ASM cells play important roles during the pathogenesis of asthma [5, 6].

MiRNA is a group of endogenous, short non-coding RNAs, which regulates genes expression through targeting the 3'UTR of mRNA. MiRNAs have been found in various organisms,

and many of them are evolutionary conserved. Meanwhile, it is estimated that more than a half of all human protein-coding genes are potentially regulated by miRNAs [7]. MiRNA is a group of indispensable immuno-response regulator that modulates the homeostasis of the immune system. Disturbed miRNAs expression has been found to be related to many kinds of auto-immune diseases such as lupus erythematosus [8], rheumatoid arthritis [9], systemic sclerosis [10] and so on. Recently there are reports indicated that altered miRNA profile was existed in the asthma patients [11]. However, the role of miRNAs during asthma pathogenesis needs to be further unveiled.

In this study, we first screened 10 candidate miRNAs in the plasma samples of the patients with asthma and found that the expression of miR-375 and miR-194 was reduced significantly. Subsequently, we confirmed 2 target genes

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of miR-375 that are related to the hypersensitivity of the NF- $\kappa$ B signaling.

### Materials and methods

#### Subjects

28 patients (17 males and 11 females) with bronchial asthma were recruited by the Respiratory Department, the Second Affiliated Hospital of Fujian Medical University. The inclusion criteria were as follows: 1) patients had previous history of paroxysmal wheezing, dyspnea, chest distress, and/or coughing; 2) according to the Global Initiative for Asthma, patients had reversible airflow limitation as measured by an increase in forced expiratory volume in one second (FEV1) of at least 15% after inhalation of 200  $\mu$ g salbutamol, or a decrease in FEV1 of over 20% after inhalation of <8 mg/mL acetylcholine; 3) skin prick tests showed patients were allergic to at least one of the following allergens: house dust mites, mixed grass pollens, mixed tree pollens, dog hair, feathers, cat hair, fine soft hair, cockroaches, or mold; 4) patients had no upper and lower respiratory tract diseases within 2 months and had no chronic heart or lung disease; and 5) within at least 4 weeks, patients had not systemically used corticosteroids, theophylline, long-acting  $\beta$ 2-agonists, leukotriene receptor antagonists, or antihistamines.

28 age and sex matched, healthy, non-asthmatic volunteers were recruited as control group. Normal volunteers were not allergic to anything or drugs investigated, had no respiratory tract diseases, and had normal lung function. This study met the relevant ethical requirements for human research, approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University, and all subjects signed a written consent form.

#### Blood sample collection

10 ml blood were obtained by venipuncture from patients and controls and then centrifuged at 1,000 g for 10 min at 4°C. 200  $\mu$ l plasma samples were collected for RNA extraction.

#### Cell culture

Human ASM cells, isolated from bronchial smooth muscle of asthma patients, were purchased from Lonza (Allendale, NJ, USA) and maintained in SmGM™-2 Bullet Kit™.

HEK293T and PC-3 cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO<sub>2</sub>.

K562 cells were cultured in RPMI + 10% fetal bovine serum (Hyclone, Logan, UT, USA) supplemented with penicillin/streptomycin and maintained at 37°C throughout the experiment.

#### RNA extraction and qRT-PCR

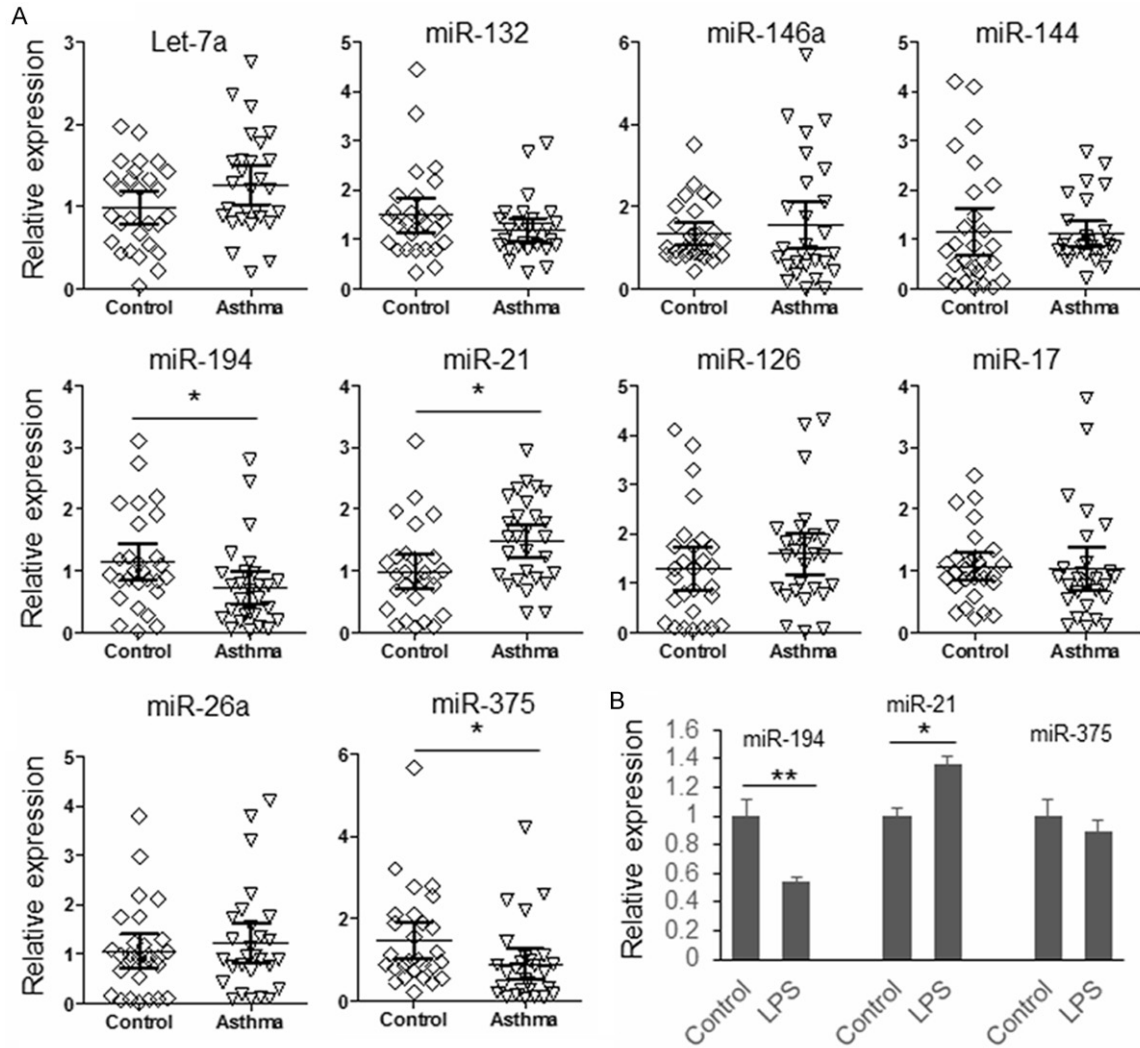
Total RNA was extracted from plasma samples by using TRIzol LS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression of miRNAs was detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) together with miRNA-specific TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA). The expression of miR-16 was used for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times.

#### Dual luciferase assay

A 523 bp segment of TLR4 3'UTR containing the predicted target region of miR-194 were cloned into pmirGLO vector separately, downstream of firefly luciferase coding region (Promega, Madison, WI, USA) to generate luciferase reporter vectors. For luciferase reporter assays, cells were seeded in 48-well plates. MiRNA mimics or inhibitors and luciferase reporter vectors were co-transfected into cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA USA). Two days post transfection, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

#### Immunoblotting

Protein extracts were boiled in SDS/ $\beta$ -mercaptoethanol sample buffer, and 20  $\mu$ g were loaded into each lane of 8% polyacrylamide gels. The proteins were separated by



**Figure 1.** Expression of candidate miRNAs. A. The expression of 10 candidate miRNAs in the plasma sample of 28 patients with asthma and 28 paired controls were detected by qRT-PCR; B. Human ASM cells were treated with LPS for 30 min and the expression of miR-194, miR-21 and miR-375 was detected by qRT-PCR. The results were analyzed by student's t-test and  $P < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ .

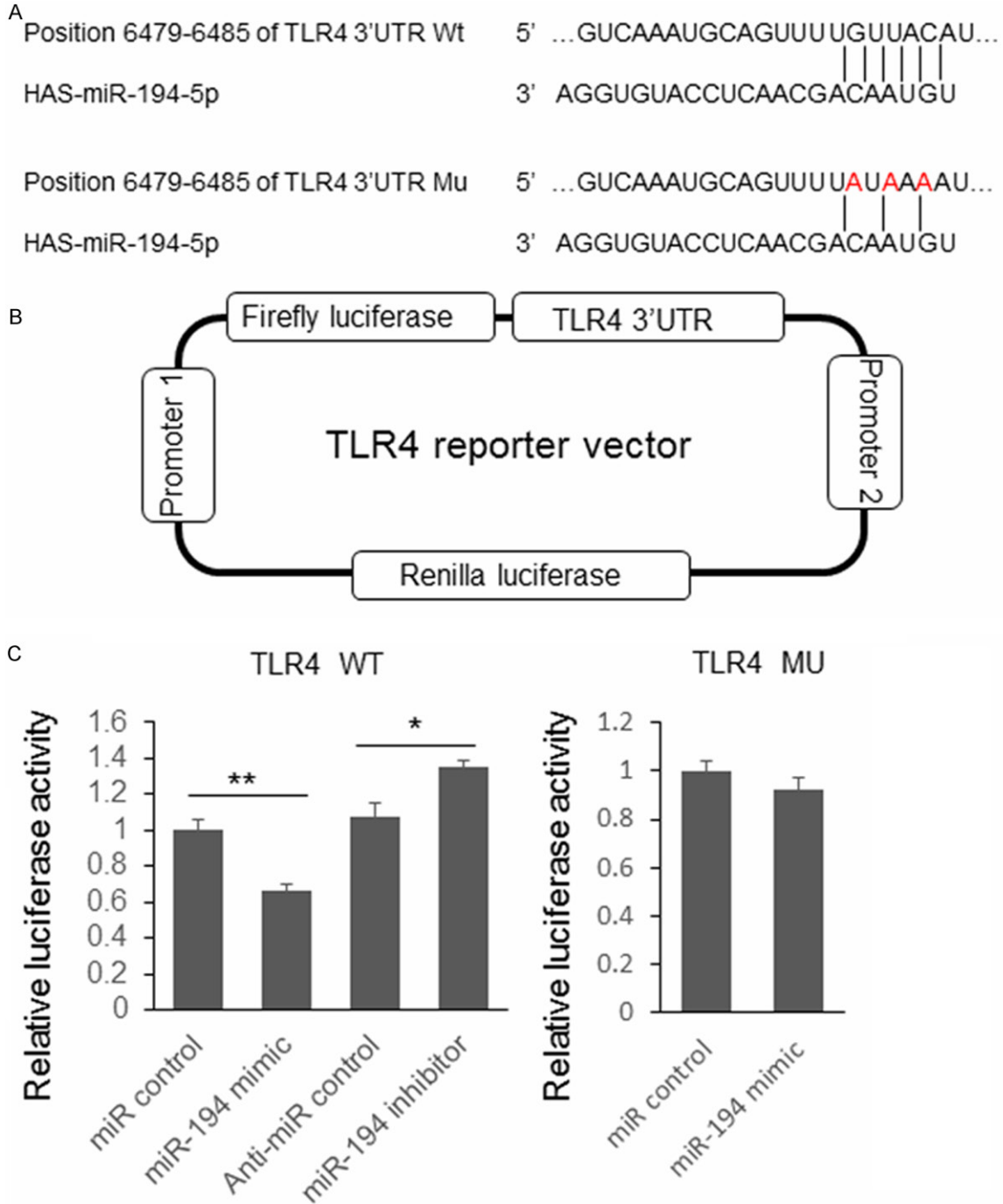
electrophoresis, and the proteins in the gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-TLR4 polyclonal antibody (Abcam, Cambridge, MA, USA) or rabbit anti-p65 polyclonal antibody (Abcam, Cambridge, MA, USA) or mouse anti- $\beta$ -actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 37°C. The specific protein antibody complex was detected by using horseradish peroxidase conjugated anti-rabbit or anti-mouse antibody. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The  $\beta$ -actin signal was used as a loading control.

#### Supernatant protein analysis

TNF- $\alpha$  and IL-6 enzyme-linked immunosorbent assay. sandwich enzyme-linked immunosorbent assays (ELISAs) for TNF $\alpha$  and IL-6 were carried out on cell culture supernatants according to the manufacturer's instructions (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK). Supernatants were diluted with RPMI 1640 as appropriate for ELISA analysis.

#### Statistical analysis

Data were analyzed by using SPSS Statistical Package version 15. Independent two group's analyses are used t-test.  $P < 0.05$  was considered statistically significant.

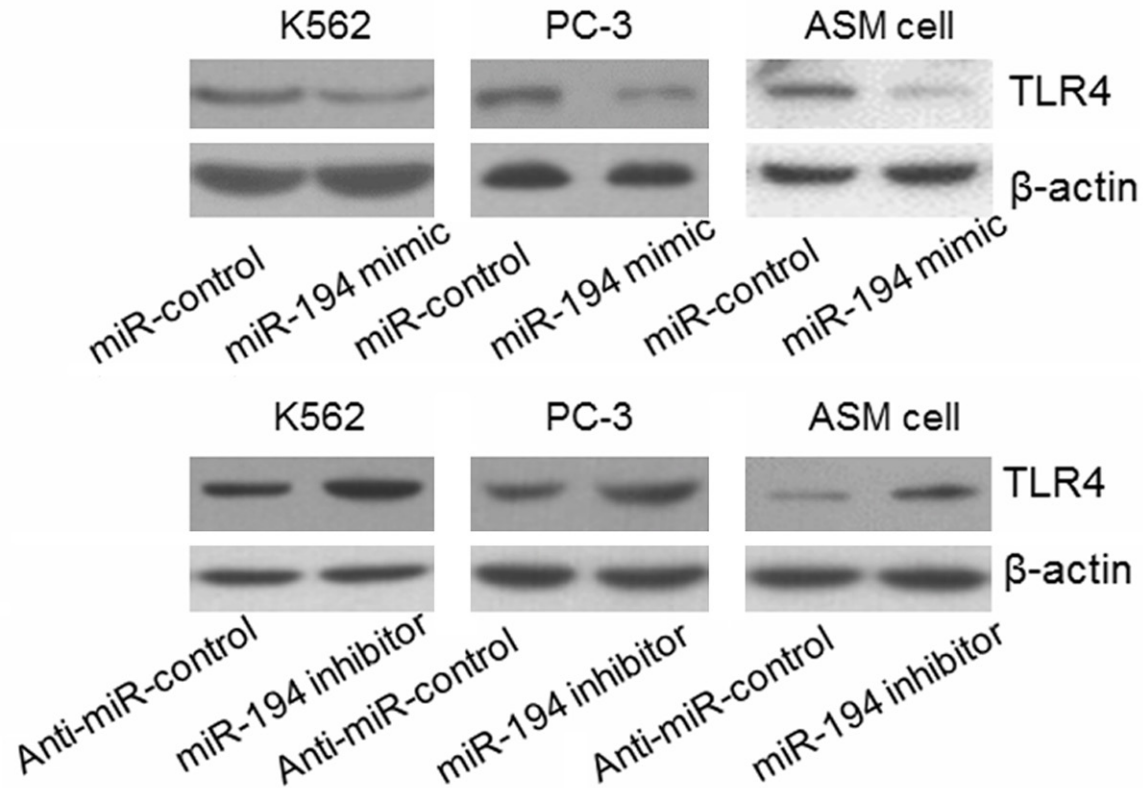


**Figure 2.** miR-194 target 3'UTR of TLR4. A. Schematic diagram of predicted interaction between miR-194 and the 3'UTR of TLR4. Red letters represent the mutated nucleotides; B. Schematic diagram for reporter vector construction; C. Dual luciferase assay. HEK293T cells were transfected with wild type or mutated TLR4 reporter vector and miR-194 mimic or inhibitor, with sequence scrambled single or double strand RNAs as control. 48 hours after transfection, the cells were lysed and luciferases activities were detected. The results were analyzed by student's t-test and  $P < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ .

## Results

To explore the roles of miRNAs during the pathogenesis of asthma, we first detected the

expression of 10 candidate miRNAs in the plasma sample of 28 patients with asthma and 28 paired controls. These miRNAs were reported to have a disturbed expression in asthma



**Figure 3.** miR-194 represses endogenous TLR4 expression. K562, PC-3 and ASM cells were transfected miR-194 mimic or inhibitor, 48 hours post transfection, the cells were lysed and the TLR4 expression was detected by immunoblotting.

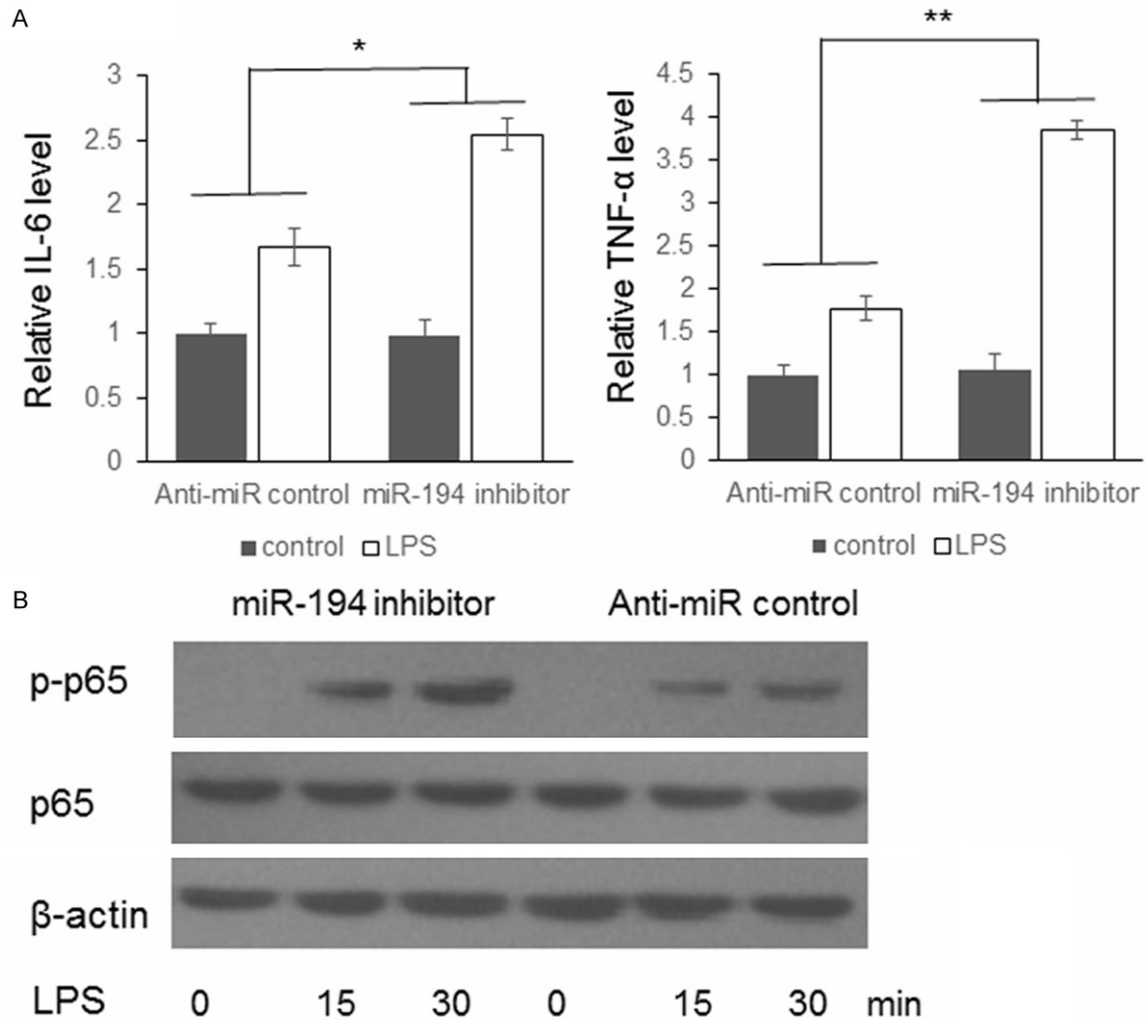
patients or function as immune system regulators [12-14]. As shown in **Figure 1A**, compared with healthy controls, there are three miRNAs have significant changed expression in the plasma of asthma patients (miR-194 is down regulated; miR-21 and miR-375 are overexpressed). To investigate whether there is the same condition in airway smooth muscle; ASM cells were treated with LPS for 30 min and then collected for RNA extraction. The expression of miR-21, miR-194 and miR-375 were detected by qRT-PCR. As exhibited in **Figure 1B**, only the expression of miR-21 and miR-194 was significantly changed after LPS stimulation. Since the up-regulated miR-21 in bronchial epidermal cells and plasma samples of asthma patients has been reported by other researchers, this study was focused on unveiling the function of miR-194 during the pathogenesis of asthma [15, 16].

To explore the biological function of miR-194, we first predicted the target genes of miR-194 by using online bioinformatics tool: TargetScan

(<http://www.targetscan.org>). We found TLR4 is a potential direct target of miR-194 (**Figure 2A**). To confirm this prediction, we constructed the dual luciferase reporter vector, by using 523 bp segment of TLR4 3'UTR that containing the predicted target region of miR-194 as the 3'UTR of firefly luciferase gene (**Figure 2B**). HEK293T cells were transfected with TLR4 reporter vector and miR-194 mimic or inhibitor, with sequence scrambled single or double strand RNAs as control. 48 hours after transfection, the cells were lysed and luciferase activities were detected. As shown in **Figure 2C** left panel, the relative luciferase activity was reduced significantly ( $P < 0.01$ ) in the cells transfected with miR-194 mimic. Meanwhile, when being transfected with miR-194 inhibitor, the luciferase activity was reduced ( $P < 0.05$ ). These results indicated that miR-194 repressed firefly luciferase expression by targeting TLR4 3'UTR. To confirm the predicted target site of miR-194 in TLR4 3'UTR, 3 nucleotides in the target region were mutated. As shown in **Figure 2C** right panel, the luciferase activity was not sig-



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**Figure 4.** LPS induced NF-κB activation was enhanced by miR-194 inhibitor. A. ASM cells were transfected with miR-194 inhibitor or anti-miR-control. 48 hours after transfection, the cells were treated with 500 ng/mL LPS for 30 minutes. The concentration of IL-6 and TNF-α in the supernatant was detected by ELISA; B. The phosphorylated p65 in the cell lysate was detected by immunoblotting.

nificantly changed ( $P>0.05$ ) when 3 nucleotides were mutated indicating that the predicted miR-194 target site is the real target site.

Although miR-194 can bind with the 3'UTR of TLR4, it is still unknown whether miR-194 can regulate endogenous TLR4 expression. K562, PC-3 and ASM cells were transfected with miR-194 mimic or inhibitor in order to detect the effect of miR-194 on endogenous TLR4 expression (Figure 3). Compared with miR-control, the expression of TLR4 was significantly reduced in the cells transfected with miR-194 mimic. Meanwhile, the expression of TLR4 was up-regulated in the cells transfected with miR-194 inhibitor. These results further confirmed that TLR4 is a direct target of miR-194.

To further understand the function of miR-194 during TLR4 induced immune response, ASM cells were transfected with miR-194 inhibitor or anti-miR-control. 48 hours after transfection, the cells were treated with 500 ng/mL bacterial lipopolysaccharide (LPS) (Sigma Aldrich) for 30 minutes. The negative controls were treated by DMSO. The supernatant was collected for cytokines detection. As shown in Figure 4A, IL-6 and TNF-α levels were up-regulated higher in the miR-194 inhibitor transfected cells compared with negative control. Meanwhile, the phosphorylated p65 protein level in the ASM cells were also examined by immunoblotting. As exhibited in Figure 4B, the p-p65 level in the cells transfected with miR-194 inhibitor up-reg-

ulated to a higher level than the control cells, which confirmed the relation between repressed miR-194 and stronger immune response.

### Discussion

Asthma is a chronic inflammatory disease of the airways which was mostly induced by inhaled antigens. Recently, it has been reported that disturbed miRNAs was detected in the plasma and tissue samples of asthma patients [17-19]. However, the function of most of these miRNAs is not well understood. To unveil the functions of miRNAs during the pathogenesis of asthma, we first detected the expression of 10 candidate miRNAs in the plasma samples of 28 asthma patients and LPS treated ASM cells and found miR-194 and miR-21 have an altered expression. Predicted by bioinformatics tool and confirmed by dual luciferase assay and immunoblotting, we confirmed that miR-194 represses the expression of TLR4 by targeting 3'UTR. Meanwhile, we also confirmed a stronger activated NF- $\kappa$ B signal in the ASM cells. We unveiled the relationship between miR-194 repression and enhanced immune response in ASM cells for the first time and provide a potential target for asthma treatment.

Unlike other well documented effects in the innate immunity system of cells, toll like receptors can stimulate a variety of non-immune cells types, such as endothelial cells, cardiomyocytes and pulmonary epithelial cells [4, 20-22]. Moreover, it has recently been demonstrated that ASM cells from different species, including humans, also express various TLRs [4]. TLR4 is constitutively expressed in the ASM cells and the activation of TLR4 by LPS was found to elicit release of IL-6, a pro-inflammatory cytokine. In this study, we construct the relation between down regulated miR-194 and overexpressed TLR4 of ASM cells, which partially explained the hyper-sensitivity of asthma patients. However, our conclusion depends on in vitro study and clinical samples. Further in vivo research has to be employed to explore the roles of miR-194 in respiratory system.

In conclusion, we constructed the relationship between reduced miR-194 level and up-regulated TLR4 expression in ASM cells. This research partially explained the hyper-sensitivity of asthma patients to pathogens and may provide a new target for clinical treatment.

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### Disclosure of conflict of interest

None.

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### References

- [1] Hakonarson H and Grunstein MM. Autologously up-regulated Fc receptor expression and action in airway smooth muscle mediates its altered responsiveness in the atopic asthmatic sensitized state. *Proc Natl Acad Sci U S A* 1998; 95: 5257-5262.
- [2] Hakonarson H, Maskeri N, Carter C, Hodinka RL, Campbell D and Grunstein MM. Mechanism of rhinovirus-induced changes in airway smooth muscle responsiveness. *J Clin Invest* 1998; 102: 1732-41.
- [3] Bachar O, Adner M, Uddman R and Cardell LO. Toll-like receptor stimulation induces airway hyper-responsiveness to bradykinin, an effect mediated by JNK and NF-kappa B signaling pathways. *Eur J Immunol* 2004; 34: 1196-207.
- [4] Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, Hunninghake GW. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* 2003; 278: 53035-44.
- [5] Savov JD, Brass DM, Lawson BL, McElvania-Tekippe E, Walker JK and Schwartz DA. Toll-like receptor 4 antagonist (E5564) prevents the chronic airway response to inhaled lipopolysaccharide. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L329-37.
- [6] Shan X, Hu A, Veler H, Fatma S, Grunstein JS, Chuang S, Grunstein MM. Regulation of Toll-like receptor 4-induced proasthmatic changes in airway smooth muscle function by opposing actions of ERK1/2 and p38 MAPK signaling. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L324-33.
- [7] Friedman RC, Farh KK, Burge CB and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; 19: 92-105.

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- [8] Qu B, Shen N. miRNAs in the Pathogenesis of Systemic Lupus Erythematosus. *Int J Mol Sci* 2015; 16: 9557-72.
- [9] Singh RP, Massachi I, Manickavel S, Singh S, Rao NP, Hasan S, Mc Curdy DK, Sharma S, Wong D, Hahn BH, Rehimi H. The role of miRNA in inflammation and autoimmunity. *Autoimmun Rev* 2013; 12: 1160-5.
- [10] Li Y, Huang J, Guo M and Zuo X. MicroRNAs Regulating Signaling Pathways: Potential Biomarkers in Systemic Sclerosis. *Genomics Proteomics Bioinformatics* 2015; 13: 234-41.11.
- [11] Liu Y, Yang K, Sun X, Fang P, Shi H, Xu J, Xie M, Li M. MiR-138 suppresses airway smooth muscle cell proliferation through the PI3K/AKT signaling pathway by targeting PDK1. *Exp Lung Res* 2015; 41: 363-9.
- [12] Kabesch M and Adcock IM. Epigenetics in asthma and COPD. *Biochimie* 2012; 94: 2231-41.
- [13] Tay HL, Plank M, Collison A, Mattes J, Kumar RK and Foster PS. MicroRNA: potential biomarkers and therapeutic targets for allergic asthma? *Ann Med* 2014; 46: 633-9.
- [14] Kabesch M. Epigenetics in asthma and allergy. *Curr Opin Allergy Clin Immunol* 2014; 14: 62-8.
- [15] Lu TX, Munitz A and Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J Immunol* 2009; 182: 4994-5002.
- [16] Wu XB, Wang MY, Zhu HY, Tang SQ, You YD and Xie YQ. Overexpression of microRNA-21 and microRNA-126 in the patients of bronchial asthma. *Int J Clin Exp Med* 2014; 7: 1307-12.
- [17] Wang Y, Yang L, Li P, Huang H, Liu T, He H, Lin Z, Jiang Y, Ren N, Wu B, Kamp DW, Tan J, Liu G. Circulating microRNA Signatures Associated with Childhood Asthma. *Clin Lab* 2015; 61: 467-74.
- [18] Sawant DV, Yao W, Wright Z, Sawyers C, Tepper RS, Gupta SK, Kaplan MH, Dent AL. Serum MicroRNA-21 as a Biomarker for Allergic Inflammatory Disease in Children. *Microna* 2015; 4: 36-40.
- [19] Comer BS, Camoretti-Mercado B, Kogut PC, Halayko AJ, Solway J and Gerthoffer WT. MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2014; 307: L727-34.
- [20] Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, Kubes P. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 2003; 111: 1011-20.
- [21] Frantz S, Kelly RA and Bourcier T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *J Biol Chem* 2001; 276: 5197-203.
- [22] Cheng T, Liu Q, Zhang R, Zhang Y, Chen J, Yu R, Ge G. Lysyl oxidase promotes bleomycin-induced lung fibrosis through modulating inflammation. *J Mol Cell Biol* 2014; 6: 506-15.