MAPK1 knockdown ameliorated immune and inflammatory abnormalities in a mouse model of refractory asthma

Shuang Lin1, Xiaohong Yang2*
1Xinjiang Medical University, 2Department of Respiratory and Critical Medicine, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi 830000, Xinjiang Uygur Autonomous Region, China

*For correspondence: Email: baxianmei08436123@163.com

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Abstract

Purpose: To evaluate the potential molecular mechanisms involved in refractory asthma in an animal model, and the potential therapeutic effect of MAPK1 knockdown on the disease.

Methods: Eighteen female Institute of Cancer Research (ICR) mice, aged 8 - 10 weeks, were randomly divided into three groups: control, asthma model and refractory asthma, with 6 mice in each group. The expression of MAPK1 was knocked down in mice using an adenoviral vector. Subsequently, the methylation levels of MAPK1 promoter in mouse lung tissue were determined using methylation assays. Hematoxylin and eosin (H&E) staining and Periodic Acid-Schiff (PAS) staining were used to determine inflammatory and histological changes in lung tissues. Levels of immune cells were determined using flow cytometry, while Western blotting was used to measure the protein expression levels of ERK1/2, JNK, MEK1/2 and p38.

Results: Methylation assay results show that mean methylation level of cg11335969 locus was significantly reduced in the refractory asthma mouse model (p < 0.05). The levels of IgG1 and IgM in refractory asthmatic mice were reduced after MAPK1 knockdown. There was a significantly reduced degree of lung lesions in mice (p < 0.05), as was reflected in effectively decreased histopathological changes. Protein levels of ERK1/2, JNK, MEK1/2 and p38, and the levels of neutrophils, dendritic cells, and macrophages were significantly decreased (p < 0.05).

Conclusion: There is hypermethylated modification of MAPK1 at cg11335969 site in refractory asthma mouse model. Knockdown of MAPK1 attenuates inflammation and tissue damage, and reverses abnormal immune cell numbers in refractory asthma mice. Thus, MAPK1 inhibition may be a novel strategy for ameliorating immune abnormalities in refractory asthma.

Keywords: Refractory asthma, MAPK1, Mouse model, Immune, Inflammatory response

INTRODUCTION

Refractory asthma is a small category of asthma that does not respond to standard therapy, especially glucocorticoid therapy. Although it accounts for only 5% of all asthma cases, it is a costly and burdensome condition that is not easily controlled, and it causes great suffering. The etiology of refractory asthma has been associated with gender, decreased lung function, persistent eosinophilic inflammation, nasal polyps, and aspirin sensitivity [1]. Tobacco
smoke and ambient air pollution are also considered risk factors for refractory asthma [2]. Due to sustained interest in research on refractory asthma, the complexity of the disease is now well understood. However, very little is known about the mechanisms involved.

One of the major causes of refractory asthma is glucocorticoid resistance. Abnormal changes in the glucocorticoid receptor often lead to structural changes in the receptor which may also result in functional changes. In humans, glucocorticoids act through the glucocorticoid receptor which is activated by binding to a specific DNA sequence on a target gene in the nucleus, thereby regulating gene transcription and exerting various biological effects. It has been found that the glucocorticoid receptor may be regulated by the mitogen-activated protein kinase (MAPK) pathway. In eukaryotes, the physiology and immune response of cells are controlled by this group of serine/threonine protein kinases. In addition to being involved in the development of bronchial asthma, the MAPK pathway is involved in the development of asthmatic inflammation as well [3].

Among other roles, the MAPK1 protein is known to be involved in regulation of apoptosis, proliferation, inflammation, cell remodeling and differentiation. Although these biological processes are not unique to asthma, they play crucial roles [4]. When GR-β expression is increased in patients with refractory asthma, serum levels of cytokines, e.g., IL-17, are also increased, and sputum neutrophil levels are elevated, at which point the MAPK signaling pathway is activated. Animal studies have shown that inhibition of the MAPK pathway reversed the high expression levels of GR-β and IL-17 [5]. In an animal study, inhibition of the MAPK pathway was shown to reverse the high expression levels of GR-β and IL-17 [5]. This suggests that glucocorticoid resistance in patients with refractory asthma may be related to the regulation of glucocorticoid receptors by the MAPK signaling pathway. Therefore, MAPK1 inhibition may be a new strategy for reducing inflammation and remodeling in refractory asthma.

In this study, the level of methylation-associated modification of MAPK1 was studied in a mouse model of refractory asthma so as to further evaluate its mitigating effect on refractory asthma inflammation. A mouse model of refractory asthma was established, and MAPK1 was knocked down in the model. The inhibition of MAPK in refractory asthma was found to ameliorate inflammation and lung injury in the mouse model. The potential therapeutic significance of MAPK1 in refractory asthma was further validated in vivo.

**EXPERIMENTAL**

Establishment of a mouse model of asthma-like airway inflammation

Thirty female American Institute of Cancer Research (ICR) mice aged 8 - 10 weeks were purchased from Xinjiang Medical University. All experiments met ethical standards and were approved by Xinjiang Medical University. All mice were housed in a light-switched cycling facility from 7 am to 7 pm at a temperature range of 18 – 20 °C, with humidity maintained between 40 – 60 %. Feed and water were freely provided. The mice were acclimatized to laboratory environment and fed for 1 week prior to the experiment. After the experiment, mice were kept in individual cages. Six ICR mice were randomly selected as the control group. In the control group, 100 μL of 0.9 % saline was injected subcutaneously into the abdomen and subcutaneously into the left and right backs of the mice on days 1, 8, and 15. From day 22 onwards, the mice were nebulized with 0.9 % saline solution using an ultrasonic nebulizer inhaler for 30 minutes daily, for a total of 7 days.

A mixture of 20 μg OVA plus aluminum hydroxide gel was prepared and shaken on a shaker for two minutes to form a suspension. Then, 10 μL of the newly prepared suspension was injected subcutaneously into the abdomen and subcutaneously into the left and right back of the mice on days 1, 8, and 15. From day 22 onwards, the mice were nebulized with 5 % OVA solution using an ultrasonic nebulizer for 30 min daily for 7 days.

In the refractory asthma group, in addition to the treatment given to the asthma model mouse, the mice inhaled budesonide suspension by nebulization at a rate of 2 mL/20 min daily for a fortnight, followed by intraperitoneal injection of dexamethasone at a dose of 0.5 mg/kg at 100 μL injection volume daily for a fortnight. At the end of the 4 weeks of administration, the mice were observed to have developed refractory asthma. Finally, six mice were randomly selected as the refractory asthma group, and six mice were randomly selected from the asthma model as the asthma model group. This research was approved by the Animal Ethical Committee of Xinjiang Medical University (approval no. XJMUAR0369) and conducted according to “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 1985) [6].

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Sample collection

Mice with asthma and refractory asthma were anaesthetised via daily nebulised inhalation of budesonide suspension at a dose of 2 mL/20 min for 28 days after MAPK1 knockdown, followed by anesthesia with 300 μL of intraperitoneal ketamine (10 mg/mL) and xylazine (1 mg/mL). Blood was obtained through the retroorbital axis at volumes of 0.3 - 0.5 mL per mouse. The blood samples were left to stand for 2 h at 4 °C, followed by centrifugation at 1800 g for 5 min to obtain serum samples which were kept at -80 °C. The mice were fixed in 4 % phosphate-buffered formalin after excising the middle lobe of the right lung. Protein and DNA methylation analyses of the remaining lung tissue were carried out at 80°C.

MAPK1 knockdown

Adenoviral vectors encoding mouse MAPK1 short hairpin (sh) RNA (shRNA) (i.e., shRNA1, shRNA2, and shRNA3) and scramble shRNA (NC) from the MISSION library were purchased from Beijing Olinger. The oligonucleotides encoding the shRNA sequences were annealed in 1 x Taq buffer, and the annealed fragments were sub-cloned into the pSP72-E3 Ad vector to generate pSP72-E3/shMAPK. The newly constructed pSP72-E3/shMAPK shuttle vector was ligated into a linearized plasmid. Then, the plasmid was transformed into 293T cells using 20 μL of Lipofectamine 2000 for propagation. After 72 h of incubation at 37 °C, the medium was filtered through a 0.45-mm filter. Next, the medium was centrifuged at 25,000 rpm for 2 h at 4 °C. The supernatant was discarded, while the precipitate containing viral particles was dissolved in 5% glycerol and kept at -80 °C. The efficiency of MAPK1 transfection was determined using RT-qPCR, with GAPDH as an internal reference gene. The titer of viral particles was expressed as number of copies of the viral genome per mL. The mice were given adenoviral plasmids of shMAPK or NC through gavage with 1 x 1010 VP or 2 x 1010 VP on days 0, 2, and 4.

RT-qPCR assay

The qPCR assay was performed using SYBR fluorescent qPCR kit and an ABI Stepone 96-well real-time PCR instrument. The amplification reaction conditions in the 14.5-μL assay system were as follows: activation at 94 °C for 30 sec; 94 °C for 5 sec, and amplification at 60 °C for 34 sec in a total of 45 cycles. Solubility curve temperatures were as follows: 95 °C for 15 sec; 60 °C for 1 min; 95 °C for 30 sec, and 60 °C for 15 sec. The internal reference gene was GAPDH, and CT values were normalized to those of the target genes. The ΔCT was calculated as follows:

\[ ΔCT = ΔCT_{\text{test sample}} - ΔCT_{\text{calibration sample}} \]

Finally, the relative mRNA expression levels were calculated using the 2-ΔΔCT method.

Quantification of DNA methylation using MassARRAY platform

The MassARRAY platform methylation assay technique was used to determine the differential methylation levels of MAPK1 in asthma at the methylation modification sites cg11335969 and cg00578437 in the MAPK1 promoter in mouse lung tissue. Genomic DNA was extracted from HPASMC using the QiAamp® DNAmini kit. The genomic DNA was subjected to bisulfite transformation reaction using the EpiTect Bisulfite kit. The CpG methylation status of the MAPK1 promoter was determined with MassARRAY platform. The results of the assay were calculated using the EpiTYPER™ software system version 1.0 (Agena, San Diego, USA) for quantitative DNA methylation analysis.

Histopathological examination of lung tissues

Inflammation and mucus secretion levels were visualized using H&E staining and PAS staining. Mesophylls fixed in 4% phosphate buffered formalin were embedded in paraffin. The paraffin-embedded tissue was sliced into 4-μm sections using a Leica RM2016 slicer and affixed onto slides. Then, the sections were stained with H&E, or with Schiff’s periodate. After washing with distilled water, the sections were dehydrated with anhydrous ethanol. After sealing with neutral gum, the tissue sections were examined under an IX71 inverted microscope.

Enzyme-linked immunosorbent assay (ELISA)

The levels of mouse IgM and IgG1 in serum were measured using Mouse IgM and IgG1 ELISA kits. A laboratory standardized ELISA procedure was followed.

Flow cytometry

Whole blood samples were collected and kept at room temperature for 2 h after addition of erythrocyte lysis buffer. A cell suspension was prepared. The cells were washed in PBS and stained with cisplatin, and Fc receptor blocker was added to the cell suspension in ice-cold staining buffer to stain and seal the Fc receptors for 15 min prior to surface staining. Cell
RESULTS

Established refractory asthma model and methylation analysis

During the establishment of the mouse models of asthma and refractory asthma, it was observed that at 3 weeks, the mice showed increased respiratory rate, nodding breathing and pulmonary rales, indicating that the OVA asthma-like airway inflammation model was successfully established. After nebulization and dexamethasone treatment, a small number of mice continued to have increased respiratory rate, nodding breathing, slow movement and lung rales on auscultation. These mice were identified as a refractory asthma mouse model.

The methylation levels of MAPK1 in lung tissues of six mice (two in each group) were studied using MassARRAY analysis. Four primers were designed according to the CpG island region of the MAPK1 promoter, corresponding to cg00578437 (MAPK1-1) and cg11335969 (MAPK1-12). The mean methylation level of cg11335969 locus was significantly lower in asthma and refractory asthma than in the control group (Figure 1). In addition, using agarose gel electrophoresis, the levels of cg00578437 and cg11335969 which were methylated in refractory asthma, were found to be reduced, and both cg00578437 and cg11335969 were not methylated (Figure 2). These results suggest that the hypomethylation status of the promoter region of MAPK1 in asthma regulates its expression level.

MAPK1 knockdown ameliorated inflammatory and pathological changes in model mice

To further study the importance of MAPK1 in refractory asthma, MAPK1 expression was inhibited in mice via adeno-viral knockdown. Results from qRT-PCR showed that, out of the three shRNAs, the best knockdown effect was in shRNA-1. Therefore, shRNA-1 synthetic virus was selected for subsequent experiments (Figure 3). The serum levels of IgG1 and IgM in samples from each group of mice were determined with ELISA. The levels of IgG1 and IgM were higher in asthmatic and refractory asthmatic mice than in the control group transfected with NC, but were lower in asthmatic mice transfected with shMAPK1 than in refractory asthmatic mice transfected with NC. The levels of IgM were lower in asthmatic mice transfected with shMAPK1 than in refractory asthmatic mice transfected with NC. The levels of IgG1 and IgM were decreased in shMAPK1-transfected refractory asthmatic mice when compared to NC-
Figure 1: Quantitative determination of DNA methylation of MAPK1 in mice lung tissues. B13, B14, C13 and C14 are the control group, B15, B16, C15 and C16 are the asthma model group, B17, B18, C17 and C18 are the refractory asthma model group. Darker colors represent higher degrees of methylation.

Figure 2: Determination of methylation of MAPK1 in mouse lung tissues using agarose gel electropherogram.

Figure 3: Knockdown efficiency of MAPK1 adenovirus. *P < 0.05, **P < 0.01, compared with normal cells; ***P < 0.05, ****P < 0.01, compared with the empty group.

Figure 4: Levels of IgG1 and IgM in six groups of mice, as determined using ELISA method. ***P < 0.001, compared to control + NC; ###P < 0.001, compared to asthma + NC or refractory asthma + NC transfected refractory asthmatic mice (P < 0.001; Figure 4). To determine the effect of MAPK1 on the pathological changes in the model mice, H&E staining and PAS staining were performed on the lung tissues. The results showed that the alveolar structure of the control group was intact, and the alveolar wall was normal, with no obvious dilatation of blood vessels and erythrocyte exudation, and there was no obvious shedding of cells in the alveolar lumen. In contrast, lung structure was more severely damaged in the refractory asthmatic mice. However, MAPK1 knockdown reduced the extent of lung lesions (Figure 5). The results suggest that the histopathological changes in the model mice which were more severe in the refractory asthmatic mice, were effectively mitigated by MAPK1 knockdown.
MAPK1 knockdown affects MAPK signaling pathway

To investigate the effect of MAPK1 knockdown on MAPK signaling pathway in mouse model of asthma, the expressions of key MAPK signaling proteins were determined using Western-blot assay. The results are shown in Figure 6.

Figure 6: The protein levels of ERK1/2, JNK, MEK1/2 and p38 in lung tissues of mice in different groups. *P < 0.05, **P < 0.01, ***P < 0.001, compared with transfected NC group.

Compared with the control group transfected with NC virus, the protein expression levels of ERK1/2, JNK, MEK1/2 and p38 in control mice transfected with shMAPK1 were significantly down-regulated (P < 0.001). The protein expression levels of ERK1/2, JNK, MEK1/2 and p38 were significantly lower in asthmatic mice transfected with shMAPK1 than in asthmatic mice transfected with NC (P < 0.05). Moreover, the protein expression levels of ERK1/2, JNK, MEK1/2 and p38 were significantly lower in refractory asthmatic mice transfected with shMAPK1 than in refractory asthmatic mice transfected with NC (P < 0.01).

MAPK1 knockdown regulated immune response in asthma

A significant correlation between MAPK1 and immune cells was found in the results of the bioinformatics analysis in Part I. Therefore, in the context of suppressing MAPK1 expression in mice, this study further determined changes in levels of immune cells in the various groups. The levels of neutrophils in the MAPK1 knockdown mouse model of asthma were lower than those in the NC group of asthmatic mice, and the levels of neutrophils in the knockdown MAPK1 refractory asthmatic mice were significantly lower than those in the NC (Figure 7A).

Figure 7B shows that the levels of dendritic cells were lower in the knockdown MAPK1 mouse model than in the NC asthmatic mice, and the levels of dendritic cells were also lower in the knockdown MAPK1 refractory asthmatic mice than in the NC refractory asthmatic mice. In addition, the levels of macrophages were lower in the knockdown MAPK1 mouse model than in the NC group of asthmatic mice, and also lower in the knockdown MAPK1 refractory asthmatic mice than in the NC group of refractory asthmatic mice, as presented in Figure 7C.

DISCUSSION

People who have refractory asthma often experience persistent symptoms, frequent exacerbations, low quality of life, and eventual loss of lung function. Despite well-designed and accepted guidelines, effective pharmacological treatments and a growing number of new therapies, asthma remains uncontrolled in a significant proportion of patients. In particular, the SARS-CoV-2 epidemic has caused respiratory illness and led to an epidemic in recent years. The use of glucocorticoids is currently the standard and most effective treatment strategy for unremitting asthma. High concentrations of glucocorticoids exert non-genomic effects, inhibit mast cell degranulation by stabilizing the plasma membrane or by reducing Ca^{2+} levels, and enhance anti-inflammatory effects by negatively interfering with the MAPK signaling pathway.

With respect to specific MAPK-associated...
mechanisms in refractory asthma, IL-2 and IL-4 induce increased glucocorticoid receptor-β expression or decreased glucocorticoid receptor-α expression via the p38 MAPK pathway. The glucocorticoid-induced expressions of various anti-inflammatory genes are down-regulated by decreased MAPK activity: alveolar macrophages contain high levels of p38 MAPK activity [7]. The activation of p38 MAPK pathway induced OVA-mediated attack on glucocorticoids in mice, but p38 MAPK inhibitors improved glucocorticoid sensitivity in asthmatic mice by inhibiting the expression of p38 MAPK. On the other hand, patients with refractory asthma also exhibit increased levels of surfactant proteins [8]. The cytokine IL-10 suppresses airway hyperresponsiveness in steroid-insensitive asthmatic mice by inhibiting neutrophil infiltration.

Recently, DNA methylation has received increasing attention due to its important role in the regulation of gene expression and its role as an important epigenetic mechanism regulating gene expression [9]. It is believed that the most common epigenetic mechanism in the pathogenesis of asthma is DNA methylation which alters the expressions of genes in asthmatics. In refractory asthma, MAPK1 is under-methylated, a status which may play a role in the development of refractoriness via the MAPK signaling pathway. Studies have shown that the methylation of DNA may be associated with asthma persistence and remission. However, the DNA methylation markers associated with severity of asthma symptoms remain unknown.

MassARRAY analysis spans promoter regions from 209 to 280, including 50 CpG islands, and it is used to assess methylation levels [10]. In this study, using MassARRAY analysis, it was found that the cg11335969 locus had significantly lower mean methylation levels in asthma and refractory asthma, and may have a role in regulating MAPK1 overexpression. The MAPKs regulate inflammation not only in structural cells but also in immune cells. In the presence of inflammatory mediators or pathogens, MAPKs enhance cellular responses and regulate immune cell differentiation and survival. Significant increases in levels of phosphorylated p38 MAPK, ERK and JNK have been reported in allergic asthmatic airways, and the severity of asthma was correlated with increased gene expressions. In a previous study, p38 MAPK-γ was knocked down by RNA interference in U937 cells and PBMCs, and IL2/IL-4 exposure did not induce corticosteroid insensitivity. This is in agreement with our findings. In addition, intraperitoneal OVA sensitization and subsequent induction with trans-nasal OVA excitation induced significant increases in total serum concentrations of IgE.
and IgG1 [11]. In contrast, IgE and IgG1 concentrations were significantly decreased in MAPK1 knockdown refractory asthmatic mice. Exposure to airborne pollutants, particulate matter, pathogens, and pulmonary allergens and irritants leads to an inflammatory response which may exacerbate the production of endogenous oxidants, leading to chronic inflammation, tissue damage and remodeling associated with asthma. Inflammation is a key factor in asthma. Activation of MAPKs, particularly ERK and p38MAPK, plays a facilitative role in the airway inflammatory response associated with asthma [12]. Results from H&E staining confirmed that MAPK1 inhibition produced an anti-inflammatory effect and was effective in mitigating refractory asthma. In addition, results from PAS suggest that epithelial cell damage in refractory asthma mice was significantly reduced after MAPK1 knockdown. The high expression level of MAPK1 in refractory asthma mice may be regulated by methylation modifications, and knockdown of MAPK1 in refractory asthma mice model significantly mitigated the inflammatory and pathological changes.

The MAPK signaling pathway is involved in almost all aspects of asthma pathophysiology. Indeed, excessive MAPK signaling may lead to glucocorticoid resistance. Although many of the anti-inflammatory effects of glucocorticoids are mediated through glucocorticoid receptor-mediated inhibition of MAPK activity, the anti-inflammatory capacity of glucocorticoids is reduced in the presence of excessive MAPK activation. Therefore, in the context of inhibiting MAPK1 expression in mice, this study further determined alterations at the immune cell level. Inhibition of p38 MAPK and its downstream pathways resulted in the suppression of airway neutrophilia in steroid-resistant refractory asthma [13]. Significant attenuation of airway hyperresponsiveness and suppression of neutrophilic inflammation have been achieved through combination of targeting of p38 MAPK activation and oxidative stress. An association has been established between neutrophil inflammation and severe asthma/unremitting asthma, and sputum neutrophil counts were associated with a more severe asthma phenotype [14]. Refractory asthma patients may benefit from neutrophil-targeted therapy due to neutrophil inflammation. Targeting neutrophil cytotoxic weapons, e.g., MAPK1, may help to suppress host tissue damage and halt the vicious cycle of persistent neutrophil-associated airway damage in asthma patients.

Dendritic cells regulate the differentiation of helper T cells into antigen-presenting cells which are key drivers of allergic inflammation. Thus, designing ways to selectively increase dendritic cell cAMP may provide a novel approach to treating allergic asthma that could reduce dendritic cell-induced inflammation [15]. By presenting antigens, dendritic cells initiate allergic inflammation and propagate inflammation through the differentiation of helper T cells [16]. Under a variety of conditions, dendritic cells use p38 MAPK signaling to regulate the differentiation and functions of Th1 cells, Th17 cells, Tr1 cells, iTreg cells and γδ T cells. The p38 MAPK signaling pathway is a novel route in dendritic cells involved in driving the Th2 response to SEA stimulation. Depletion of CD11c+ dendritic cells during the sensitization phase in a mouse model of asthma eliminates the characteristic features of asthma.

In addition to regulating innate immune responses against invading pathogens, alveolar macrophages initiate inflammatory responses, repair tissues, and initiate further tissue repair. There are two polarized macrophage patterns: M1 (pro-inflammatory) and M2 (anti-inflammatory), depending on the microenvironment of the lungs. Bronchial macrophages from asthmatic patients express less CD16 and CD64 and show reduced phagocytic activity and anti-inflammatory capacity [17]. Macrophages upregulate genes involved in the IL-17 pathway and increase the release of neutrophil-associated mediators (including IL-8 and IL-17C), leading to the complex chronic inflammatory environment characteristic of refractory asthma. Macrophage polarization has been shown to be associated with the pathogenesis of asthma in adults and children, and it is a potential biomarker of severity of asthma. The p38 MAPK inhibitors enhance the anti-inflammatory effects of alveolar macrophages in corticosteroid-insensitive asthmatics [17]. Knockdown of MAPK1 significantly decreased infiltration of neutrophils, dendritic cells and macrophages. This may be a molecular mechanism for treatment of refractory asthma, and it may provide a new therapeutic strategy for neutrophil-associated inflammatory diseases of the lung.

**CONCLUSION**

A mouse model of refractory asthma has been successfully established, and it has been demonstrated that MAPK1 is hyper-methylated, with potential to undergo hypermethylation modifications. Knockdown of MAPK1 further validates the attenuation of inflammation and tissue damage in refractory asthmatic mice after MAPK1 inhibition. Findings in this study highlight...
the important role of MAPK1 knockdown in attenuating MAPK signaling in the pathogenesis of refractory asthma, which may be achieved through improved immune function. Thus, MAPK1 inhibition is a potential strategy for ameliorating immune abnormalities in refractory asthma. Therefore, MAPK1 has the potential of being an additional therapeutic target for refractory asthma.

DECLARATIONS

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Ethical approval

This study was approved by the Ethics Committee of Xinjiang Medical University, China.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shuang Lin designed the study, supervised the data collection, and analyzed the data. Shuang Lin and Xiaohong Yang interpreted the data and prepared the manuscript for publication. Xiaohong Yang supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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