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ORMDL3 knockdown in the lungs alleviates airway inflammation and airway remodeling in asthmatic mice via JNK1/2-MMP-9 pathway

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ABSTRACT

Orosomucoid-like protein 3 (ORMDL3) is a common mutation in many asthma patients and its effects on the specific pathogenesis of asthma are still unclear. Therefore, in this study, we used a mouse that specifically knockout the mouse ORMDL3 gene to further study the mechanism. We used ovalbumin (OVA) to induce asthma in wild-type mice and ORMDL3 knockout mice. Lung ventilation resistance, airway inflammation, mucus hypersecretion, collagen deposition, the levels of inflammatory factors and the expression of ORMDL3 and JNK1/2-MMP-9 pathway were detected. The results showed that ORMDL3 gene was highly expressed in clinical asthmatic children and mouse asthma model. Knocking down the ORMDL3 gene in the lung tissue of asthmatic mice can reduce airway hyperresponsiveness, airway inflammation, mucus secretion, and collagen deposition around the airway. After knocking down the lung tissue of mice, the IL-4, IL-5 and IL-13 concentrations in broncho alveolar lavage fluid of asthmatic mice were significantly decreased, and the activation of JNK1/2-MMP-9 pathway was inhibited in mouse lung tissue. Collectively, our results demonstrate that the ORMDL3 gene may aggravate asthma symptoms by activating the JNK1/2-MMP-9 pathway, which indicates that the ORMDL3 gene may be the key molecule for the next step of asthma targeted therapy.

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1. Introduction

Asthma is a common chronic airway inflammatory disease. The main manifestations are airway hyperresponsiveness, reversible airflow limitation, and decreased lung function [1]. The pathogenesis of asthma is mediated by multiple factors such as immunity, environment and genetics. The Global Initiative for Asthma Committee reports that there are currently about 300 million asthma patients worldwide, and about 250,000 asthma patients die each year worldwide, with young people accounting for a large proportion [2]. However, its specific pathogenesis is still unclear, and the imbalance of Th1/Th2 subgroups and functional imbalance are still recognized as immunological pathogenesis [3]. The World Health Organization estimates that the global cost of asthma accounts for about 1% of the global disease burden, which is higher than the total number of tuberculosis and acquired immune deficiency syndrome, which increases the economic burden on society and individuals. Repeated wheezing attacks seriously affect the

physical and mental health of children [4]. Matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase-1 (TIMP-1) are closely related to the mechanism of airway remodeling [5]. The matrix metalloproteinase 9 is an important factor for the degradation of extracellular matrix, by regulating extracellular matrix metabolism and decomposing extracellular matrix and airway remodeling [6]. MMP-9 belongs to the metal ion - dependent proteinase, which is widely involved in the migration of cells and the process of extracellular matrix degradation [7]. Studies have shown that inflammation and injury of epithelial cells secrete MMP-9 can promote the decomposition of inflammatory cells, epithelial cell shedding, aggravation of respiratory inflammation and injury, leading to respiratory remodeling [8]. MMP-9 promotes smooth muscle cell proliferation and airway remodeling by mediating related cell factor and proteinase activity, ultimately leading to the onset of asthma [9].

The ORM gene is a new class of genes discovered during the sequencing of retinitis pigmentosa in 2002. The full name is ORM1-like Gene Family, which is highly conserved among members of the family [10]. The orosomucoid-like 3 (ORMDL3) gene is a member of the ORM family and was found to be closely associated with childhood asthma in 2007, encoding a transmembrane protein on

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the endoplasmic reticulum. The ORMDL3 gene can also be expressed in mice and has a high degree of homology with humans [11]. The study of mouse ORMDL3 gene can reflect the relationship between human ORMDL3 gene and asthma to some extent. Although the ORMDL3 gene is closely related to the occurrence of asthma, it is still unclear how to regulate the pathogenesis of asthma [12]. This study constructed a specific ORMDL3 knockout mouse to explore ORMDL3 possible mechanisms involved in asthma, which provided a basis for animal experiments in the search for new targets for clinical treatment of asthma.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (6–8 weeks old) were brought from the Experimental Animal Center of Zhengzhou University. Club cells specific ORMDL3-knockout (CC10-Cre/ORMDL3^{fl/fl}) mice were generated by hybridizing CC10-rtTA/(TetO)7 transgenic mice (The Jackson Laboratory, USA) with ORMDL3^{loxP/loxP} mice. ORMDL3 deletion in CC10-Cre/ORMDL3^{fl/fl} mice was achieved by administering doxycycline (1 mg/ml) in drinking water containing 0.4% sucrose. The various control mice were ORMDL3^{fl/fl} mice without Cre that received doxycycline. Experimental mice were housed under specific pathogen-free conditions and subjected to a 12 h/12 h dark/light cycle. All the animal and samples in this study were approved by the Ethics Commitment of the First Affiliated Hospital of Zhengzhou University.

2.2. Establishment of an asthma model

Mice in the ovalbumin (OVA)-asthma group were sensitized on the first, eighth, and fifteenth days of the experiment, and 0.2 ml of OVA antigen mixture was intraperitoneally injected (in which OVA 20 µg, aluminum hydroxide 2 mg, and physiological saline to 0.2 ml). From the 22nd day, the mice were placed in a self-made atomization box, and 1% OVA (1 mg OVA, 100 ml saline) suspension was inhaled for asthma challenge once a day for 30 min each time and inspire for 14 days. Physiological saline was used instead of OVA in the sensitization and challenge phases in the control group. All mice were divided into four groups (n = 12 in each group): wild type mouse control group (control group), wild type mouse asthma group (OVA group), knockout mouse control group (ORMDL3^{fl/fl}-control group) and knockout mouse asthma group (ORMDL3^{fl/fl}-OVA group).

2.3. Mouse invasive lung function test

Six mice in each of the 4 groups were tested for invasive lung function, and the mice were taken out 24 h after the last challenge. Mice were anesthetized with 2% sodium pentobarbital, cannulated, and placed in a whole body plethysmography chamber to measure lung ventilation resistance. Mice were challenged with 20 µl methacholine (concentration 3.125, 6.25, 12.5, 25 or 50 mg/ml, respectively). The lung ventilation resistance of the mice was recorded every second, and finally statistical analysis was performed.

2.4. Lung tissue specimen collection

Each group of mice was anesthetized with ether anesthesia and fixed on the console within 24 h after the end of the last challenge. Open the chest to completely expose the lungs and heart, and use a hemostat to ligation the left hilum. The sterile surgical scissors were disconnected. At the distal end of the ligation of the hilar, the

upper and lower leaves of the left lung were taken out into a cryotube, and then placed in a −80 °C refrigerator for ELISA and RT-PCR; the right ventricle was intubated to the pulmonary artery. Rinse with normal saline until the lung tissue turns white. After no blood sample liquid flows out, apply 4% formaldehyde for internal fixation. The right lung was removed from the broken bronchi, and the right middle lobe was immersed in 4% formaldehyde for 24 h.

2.5. Tissue section preparation

The fixed lung tissue was paraffin-embedded, and the embedded wax blocks were marked again according to the grouping and serial number of the mice. The wax block was placed in a refrigerator at −20 °C, frozen for 30 min, and then sliced. The anti-skid slides are placed in the same position according to the wax block number, which are numbered sequentially. Set the slicer to adjust the thickness to 3 µm, slice each slice in succession, take 1 specimen from 3 sheets, place 40 °C warm water, and make the slice fully flattened. Select at least 3 slices of better shape for each wax block. Immediately fished on the anti-radiation slides and laid flat. To prevent peeling, the cut slices were baked in the oven for 2 h. After cooling at room temperature, keep it safe and use it as hematoxylin/eosin (HE) staining, Masson staining, periodic acid-Schiff (PAS) staining, and immunohistochemistry (IHC) staining. Antibody against ORMDL3 (1:500; Abcam, USA) was used for IHC staining. Tissues that were stained brown were recorded as being positive for ORMDL3. The intensity of IHC staining was quantified using Image-Pro Plus 5.0 software.

2.6. Broncho alveolar lavage fluid (BALF) analysis

BALF was collected three times in the right lung of each animal by washing with 0.5 ml of phosphate buffered saline. A recovery of more than 1.3 ml is considered successful in lavage. All cells in BALF were collected by centrifugation and treated with red blood lysis buffer, then counted using a cell counter and microscope. The remaining inflammatory cells were plated on glass slides, air dried and stained with Wright-Giemsa, and differential cell counts were determined according to conventional morphological criteria according to the manufacturer's instructions. The BALF supernatant was collected and the contents of IL-4, IL-5, IL-13 and MUC5AC in the supernatant were detected by ELISA kit. The experimental procedures and methods were carried out according to the instruction manual.

2.7. RT-PCR assay

The expression of MUC5AC mRNA and ORMDL3 mRNA in lung tissue was semi-quantitatively analyzed by RT-PCR. Total RNA was extracted from mouse lung tissue, and mRNA was used as a template to reverse-transcribe into cDNA using reverse transcriptase using Oligo (dT) or random primers. PCR amplification was performed using cDNA as a template to obtain MUC5AC and ORMDL3 gene expression.

2.8. Western blot assay

Mouse lung tissue whole protein was extracted using a whole protein extraction kit (KeyGen, China). Protein concentration was determined using the bicinchoninic acid (BCA) assay according to standard protocols. Protein samples (50 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking for 1 h, the first antibody was incubated overnight, and after the next day, the secondary antibody was

incubated for 1 h (1:2000, Proteintech, China). The primary antibodies included p-JNK1/2 (1:1000, CST, USA), JNK1/2 (1:1000, CST, USA), MMP-9 (1:500, Abcam, USA) and β -actin (1:1000, Proteintech, China).

2.9. Statistical analyses

GraphPad Prism software (version 5.0; GraphPad, San Diego, CA, USA) was used for statistical analysis. Two-way analysis of variance was performed to determine statistically significant differences between the different groups. Tukey's tests are used for comparison between groups. $P < 0.05$ was statistically significant.

3. Results

3.1. High expression of ORMDL3 gene in peripheral blood samples of children with clinical asthma and lung tissue of asthmatic mice

To test the expression of the ORMDL3 gene in children with asthma, we collected peripheral blood samples from clinical children for testing. The results of qPCR and Western blot showed that

the mRNA level and protein level of ORMDL3 in peripheral blood mononuclear cells of asthmatic children were higher than those in the normal group ($P < 0.05$, Fig. 1A and B). Similarly, the mRNA level and protein level of ORMDL3 in OVA group were higher than those in the control group ($P < 0.05$, Fig. 1C and D). The results of immunohistochemistry in mouse lung tissue also showed that the expression of ORMDL3 protein in lung tissue of asthmatic mice was significantly higher than that in control group ($P < 0.05$, Fig. 1E).

3.2. Knockdown of ORMDL3 gene reduces airway hyperresponsiveness and airway inflammatory cell infiltration in asthmatic mice

We used OVA to establish a mouse model of asthma. HE staining of pathological sections of mouse lung tissue also showed that the infiltration of bronchial inflammatory cells in ORMDL3^{fl/fl}-OVA group was significantly reduced compared with OVA group ($P < 0.05$, Fig. 2A). The lung function test showed that the lung ventilation resistance of ORMDL3^{fl/fl}-OVA group was significantly lower than that of OVA group ($P < 0.05$, Fig. 2B). The inflammatory cells in the alveolar lavage fluid were counted and the results

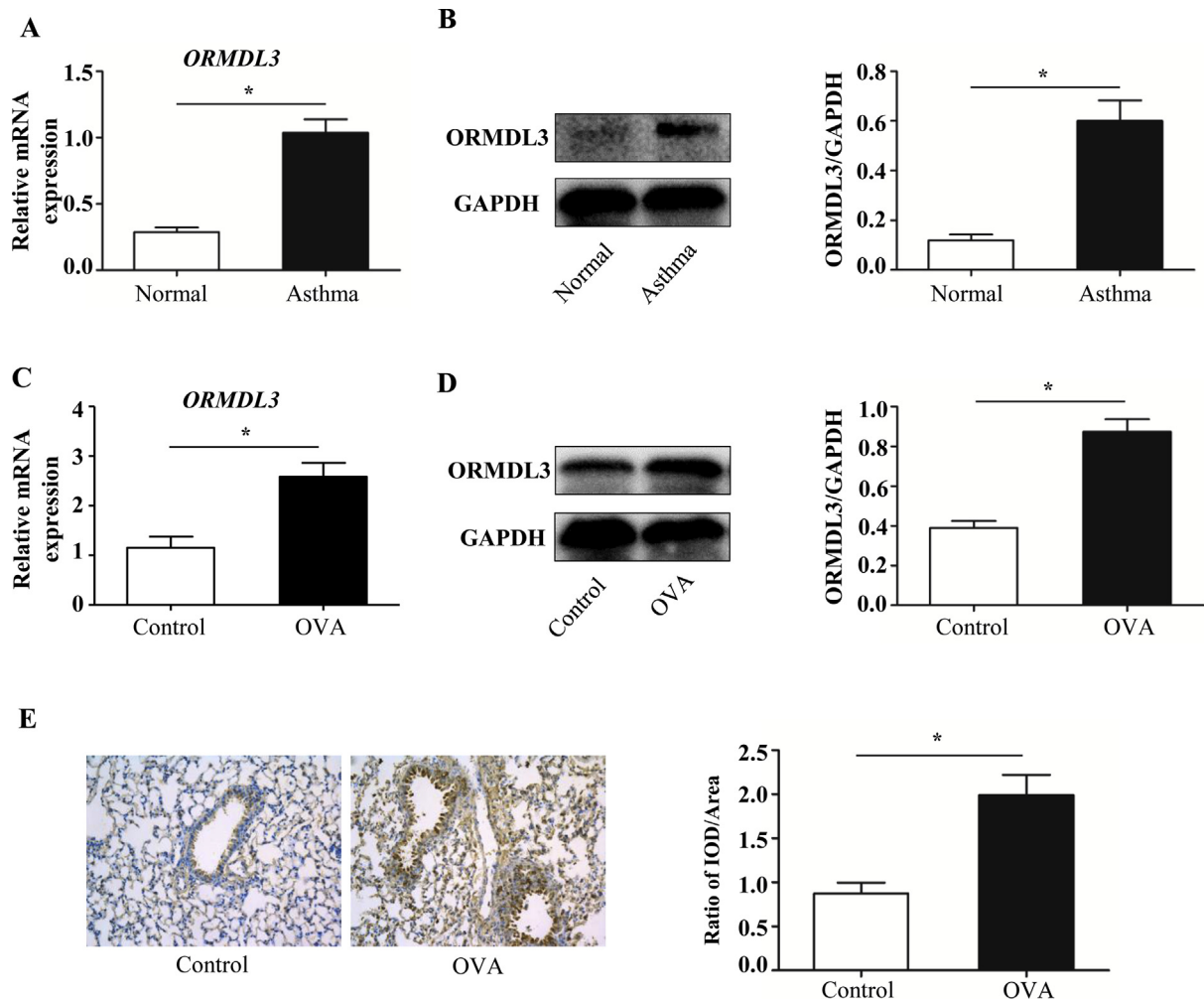


Fig. 1. High expression of ORMDL3 gene in peripheral blood samples of children with clinical asthma and lung tissue of asthmatic mice. (A) qPCR detection of ORMDL3 mRNA expression in peripheral blood of children with clinical asthma and control (*: Normal vs Asthma; $n = 6$ in each group). (B) Western blot analysis of ORMDL3 protein expression in peripheral blood of children with asthma and control (*: Normal vs Asthma; $n = 6$ in each group). (C) Detection of ORMDL3 mRNA levels in mouse lung tissue by qPCR (*: Control vs OVA; $n = 6$ in each group). (D) Western blot analysis of ORMDL3 protein expression in mouse lung tissue with OVA and Control (*: Control vs OVA; $n = 6$ in each group). (E) Immunohistochemical detection of ORMDL3 protein expression in mouse lung tissue (*: Control vs OVA; $n = 6$ in each group).

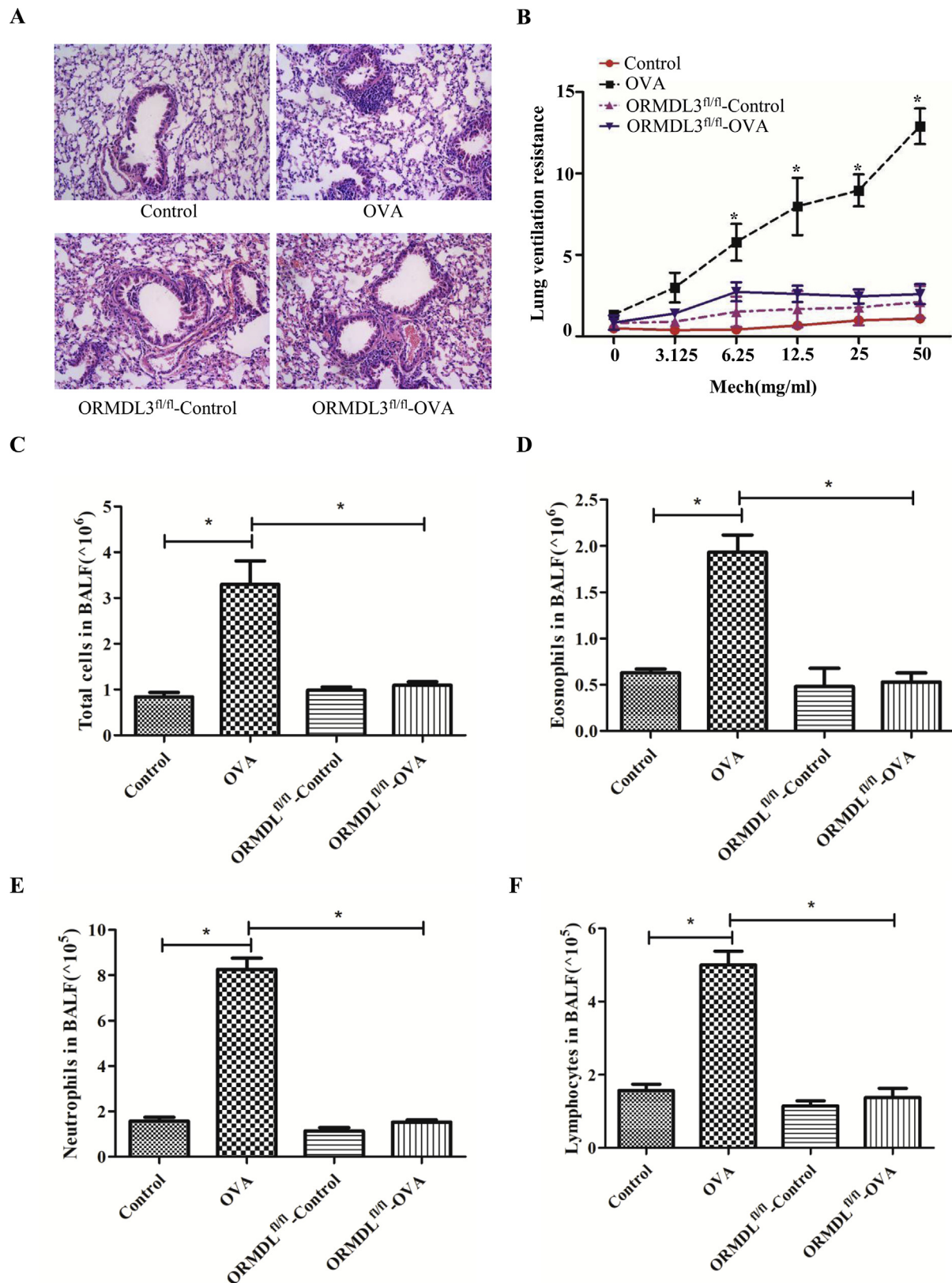


Fig. 2. Knockdown of ORM DL3 gene reduces airway hyperresponsiveness and airway inflammatory cell infiltration in asthmatic mice. (A) Infiltration of inflammatory cells around the bronchi in mouse lung tissue by HE staining. (B) Invasive pulmonary function test for detecting lung ventilation resistance in mice (*: ORM DL3^{fl/fl}-OVA vs OVA; $n = 6$ in each group). (C–F) Inflammatory cell statistics in BALF, total number of cells (C), eosinophils (D), neutrophils (E), lymphocytes (F) (*: ORM DL3^{fl/fl}-OVA vs OVA; $n = 6$ in each group).

showed that the total number of cells, eosinophils, neutrophils and lymphocytes in the BALF of the ORMDL3^{fl/fl}-OVA group were significantly lower than those in OVA group ($P < 0.05$, Fig. 2C–F).

3.3. Knockdown of mouse lung tissue ORMDL3 gene to alleviate airway mucus hypersecretion and collagen deposition around airway in asthmatic mice

We used glycogen staining to detect mucus secretion in the bronchus of mice. The results showed that the mucus secretion in ORMDL3^{fl/fl}-OVA group was significantly reduced compared with the OVA group (Fig. 3A). MUC5AC protein is a key protein secreted by mucus. The level of MUC5AC mRNA in lung tissue of ORMDL3^{fl/fl}-OVA group was significantly lower than that in OVA group ($P < 0.05$, Fig. 3B). The MUC5AC protein content in mouse BALF was detected by ELISA kit, and the results showed that the MUC5AC protein concentration in the ORMDL3^{fl/fl}-OVA group was significantly lower than that in the OVA group ($P < 0.05$, Fig. 3C). Masson staining showed that the collagen deposition around the

bronchioles in the lung tissue of the ORMDL3^{fl/fl}-OVA group was lower than that in the OVA group (Fig. 3D).

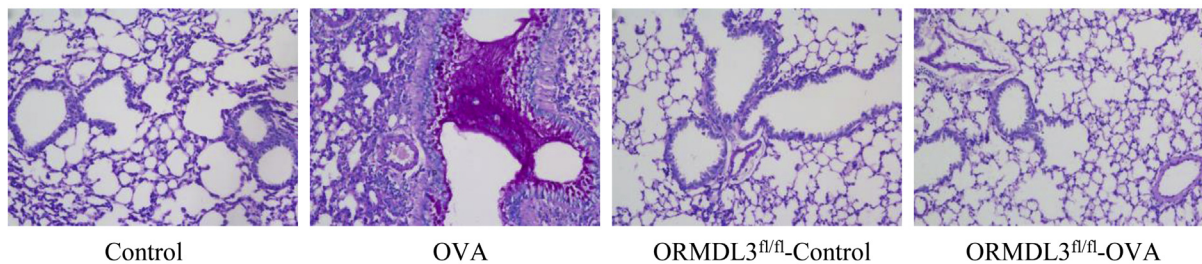
3.4. Knocking down ORMDL3 gene reduces the expression of Th2 cytokines in asthma

At present, the pathogenesis of asthma is relatively recognized as the airway inflammation caused by Th1/Th2 cell immune imbalance [13]. The inflammatory factors such as IL-4, IL-5 and IL-13 secreted by Th2 cells are significantly increased. Therefore, we used ELISA kit to detect IL-4, IL-5 and IL-13 levels in BALF of mice in this study. We found that IL-4, IL-5 and IL-13 concentrations in BALF of ORMDL3^{fl/fl}-OVA group were lower than OVA group ($P < 0.05$, 4A–C).

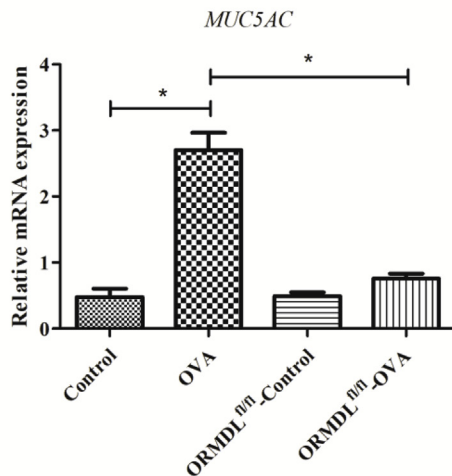
3.5. Knocking down ORMDL3 gene inhibits the activating JNK1/2-MMP-9 signaling pathway in asthma

JNK1/2-MMP-9 pathway is a key pathway for airway

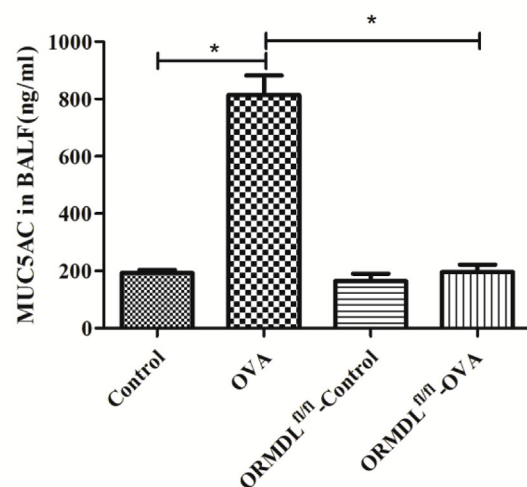
A



B



C



D

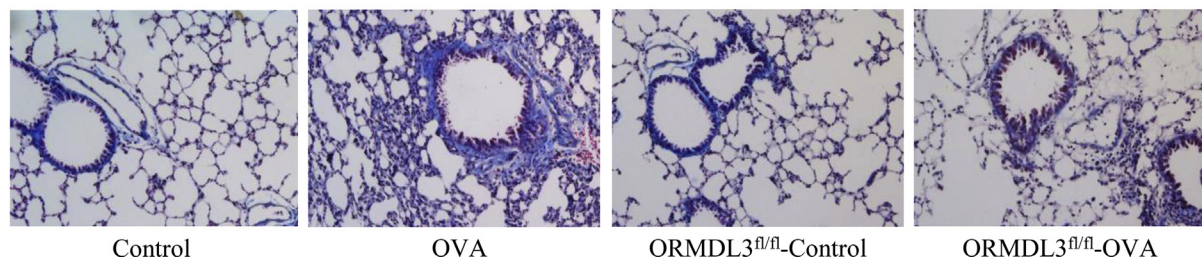


Fig. 3. ORMDL3 knockout mice have reduced mucus secretion and reduced collagen deposition around the lungs. (A) Observation of airway mucus secretion in mouse lung tissue by PAS glycogen staining. (B) Detection of MUC5AC mRNA expression level in mouse lung tissue by qPCR (*: ORMDL3^{fl/fl}-OVA vs OVA; $n = 6$ in each group). (C) Detection of MUC5AC protein expression concentration in mouse BALF by ELISA (*: ORMDL3^{fl/fl}-OVA vs OVA; $n = 6$ in each group). (D) Masson staining was used to detect collagen deposition around the bronchi of mouse lung tissue (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammation and airway remodeling in asthma. We extracted mouse lung tissue protein for Western blot and examined whether ORMDL3 gene has an effect on JNK1/2-MMP-9 pathway. The results showed that the JNK1/2-MMP-9 pathway was activated in the OVA group compared with control group, while the activation of the JNK1/2-MMP-9 pathway was significantly inhibited in the ORMDL3^{fl/fl}-OVA group compared with OVA group ($P < 0.05$, Fig. 4D).

4. Discussion

Asthma is a common respiratory disease. The main clinical manifestations are cough, wheezing, chest tightness and shortness of breath. It is characterized by recurrent airway obstruction and bronchospasm. The causes of asthma are complicated, mainly caused by genetic and environmental factors [3]. At present, anti-inflammatory, bronchial spasm and diastolic smooth muscle are often used in the treatment of asthma. Although the regimen can effectively control asthma symptoms, due to contraindications and side effects after treatment, most asthma patients still have asthma symptoms and poor quality of life after treatment [14]. With the advancement of modern medicine, the treatment of asthma has been perfected, but the pathogenesis of asthma is still unclear. Therefore, it is important to look for new therapeutic targets and potential drugs for asthma. The pathogenesis of asthma is associated with genetic factors, especially key genes associated with asthma, which may play an important role in the pathogenesis of asthma [15]. The study found that ORMDL family genes, including ORMDL1, ORMDL 2 and ORMDL 3, were significantly up-regulated

in smooth muscle cells of asthma patients. However, single nucleotide polymorphism analysis found that ORMDL 3 was the most important factor [16]. Previous studies have found a strong association between ORMDL3 gene mutations and childhood asthma [11]. Meta-analysis found that the ORMDL3 gene rs7216389 polymorphism is closely related to the incidence of asthma in Chinese children and adults [10,11]. Since the first report, ORMDL3 has been used extensively in the study of childhood asthma, and ORMDL3 can also be expressed in fetal and adult tissues. Studies have found that overexpression of ORMDL3 in a mouse asthma model increases pathological remodeling of asthmatic airways, including increased smooth muscle cell proliferation, subepithelial fibrosis, and increased mucus secretion [17]. Numerous studies have shown that ORMDL3 transgenic mice can increase important pathological features of asthma such as airway remodeling and airway response, which may be closely related to microRNA (miRNA) regulation. Previous studies have shown that miRNA can participate in the regulation of airway and smooth muscle function in asthma animal models through self-level changes [18]. miR-10a can promote ASM proliferation by regulating its target gene PI3KCA, and miR-133a regulates IL-13 through regulation, thereby affecting the expression of Ras homolog gene family member A (RhoA), and increased expression of RhoA can participate in contraction regulation of smooth muscle [19]. Studies have shown that ORMDL3 gene is involved in the pathogenesis of asthma. It is speculated that ORMDL3 can bind to the sarcoplasmic reticulum, inhibit its adenosine triphosphatase (ATP) activity, thereby reducing the ATP concentration in the endoplasmic reticulum and promoting the activation of transcription factor 6 in unfolded proteins. The

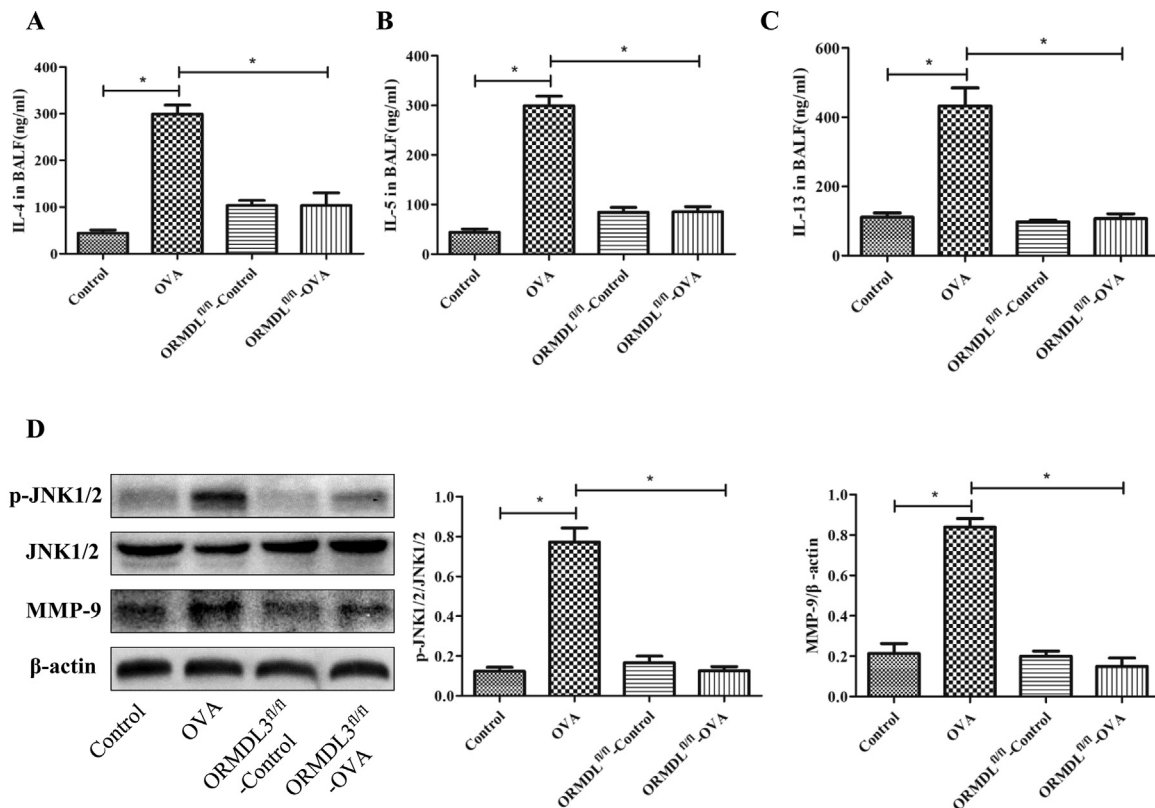


Fig. 4. Knockdown of ORMDL3 gene reduces asthma IL-4, IL-5 and IL-13, and inhibits the activation of JNK1/2-MMP9 pathway. (A) Detection of IL-4 in alveolar lavage fluid of mice by ELISA. (B) Detection of IL-5 in alveolar lavage fluid of mice by ELISA. (C) Detection of IL-13 in alveolar lavage fluid of mice by ELISA (*: ORMDL3^{fl/fl}-OVA vs OVA; $n = 6$ in each group). (D) Western blot analysis of activation of JNK1/2-MMP9 protein pathway in mouse lung tissue (*: ORMDL3^{fl/fl}-OVA vs OVA; $n = 6$ in each group).

transcription factor 6 (ATF6) pathway promotes the expression of the sarcoplasmic reticulum calcium pump, which promotes the inflammatory response, causes airway hyperresponsiveness, increases the susceptibility of children to asthma, and triggers asthma [20]. At present, the single nucleotide polymorphisms of the ORMDL3 gene, rs4794820 and rs7216389, have been extensively studied. Among them, rs4794820 is involved in the regulation of epithelial inflammatory response and airway responsiveness [21]. Studies have also shown that ORMDL3 plays an important role in the pathogenesis of inflammatory bowel disease and type I diabetes, suggesting that ORMDL3 may be involved in the immune system disorders [22]. Increased expression of ORMDL3 mRNA after stimulation of airway epithelial cells with allergens and cytokines (IL-4 or IL-13). The expression product of ORMDL3 inhibits Ca^{2+} -ATPase (SERCA) activity in the endoplasmic reticulum and leads to a decrease in Ca^{2+} concentration in the endoplasmic reticulum, while increasing the unfolded protein response (UPR) [23]. A large number of eosinophils were observed in HE staining sections of peripheral blood and lung tissues of asthmatic mice, and the expression of ORMDL3 mRNA in asthmatic mice was significantly higher than that of the control group [24]. In our study, we found that the ORMDL3 gene in asthmatic children and mice increased in mRNA and protein levels compared with the control group, and knocking down the ORMDL3 gene in the lung tissue of mice can alleviate airway hyperresponsiveness in asthmatic mice. Reduced airway inflammatory cell infiltration and inflammatory cytokine expression in mice, and reduced airway mucus hypersecretion in mice, consistent with most studies.

Matrix metalloproteinase is a calcium and zinc-dependent endopeptidase superfamily capable of degrading most extracellular matrices such as collagen and elastase. The main rate-limiting enzyme for degrading extracellular matrix is matrix metalloproteinase-9 (matrix metal-loproteinase, MMP-9) [5]. When asthmatic patients have an acute attack, not only inflammatory cells produce MMP-9, but also lung tissue cells and bronchial epithelial cells express MMP-9 in large amounts, which increases their expression levels [7]. Previous studies have found that the MMP-9 level and serum MMP-9 level of BALF in the asthma group are significantly higher than those in the control group, and the MMP-9 level and serum MMP-9 level of BALF in the moderate-to-severe group are significantly higher than those in the light-weight group [25,26]. The degree group suggests that MMP-9 may be involved in the onset and progression of asthma [27]. JNK1/2 is an upstream molecule that regulates MMP-9 protein, and studies have found that activation of JNK1/2 pathway exists in asthma. Therefore, in our study, we examined the activation of JNK1/2 pathway in knockout and wild-type asthma mice, and detected the expression of downstream MMP-9 protein. It was found that knockdown of ORMDL3 gene can inhibit activation of JNK1/2 pathway and inhibiting MMP-9 protein expression. Therefore, it is suggested that the ORMDL3 gene promotes the occurrence of asthma in part through activation of the JNK1/2-MMP9 signaling pathway.

In summary, this study was the first to verify the role of this gene in the pathogenesis of asthma in ORMDL3 knockout mice, and confirmed that knocking down the lung ORMDL3 gene can reduce airway hyperresponsiveness in asthmatic mice. Inflammation of the tract, high secretion of airway mucus, and collagen deposition, and the discovery that the mechanism by which the ORMDL3 gene promotes asthma may be partly through activation of the JNK1/2-MMP9 pathway in mice.

Conflicts of interest

There are no potential conflicts of interest to declare.

Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.06.122>.

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