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Original Article

Wuling San and Xiao Chaihu Decoction affect airway inflammatory response and airway smooth muscle cell proliferation in mice with allergic asthma via miR-486-5p/AQP5 axis

Xirui Xue, Xiaoping Liu, Suzhen Wei, Xin Wang, Yanxia Yang

Northwest Minzu University, Lanzhou 730000, Gansu Province, China

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Abstract: Objective: This study aimed to investigate the effects of Wuling San and Xiao Chaihu Decoction on allergic asthma, and elucidate the potential mechanism of Wuling San and Xiao Chaihu Decoction for ameliorating allergic asthma. Methods: BALB/c mice were intraperitoneally injected with ovalbumin (OVA) to establish animal model of allergic asthma. Transforming growth factor beta 1 (TGF-β1) was used to induce the proliferation of airway smooth muscle cells (ASMCs) in order to establish the cell model. Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to quantify the expression levels of miR-486-5p and aquaporin-5 (AQP5) in cells and tissues. Dual-luciferase reporter assay was used to verify the targeting relationship between miR-486-5p and AQP5. MTT assay and flow cytometry were carried out to evaluate cell proliferation and apoptosis, respectively. Enzyme-linked immunosorbent assay (ELISA) was conducted to measure the levels of interleukin-4 (IL-4), IL-5 and IL-13 in the bronchoalveolar lavage fluid (BALF). Hematoxylin and eosin (HE) staining and Masson staining were used to detect the recruitment of eosinophils and collagen deposition. Results: In both *in vivo* and *in vitro* experiments, Wuling San and Xiao Chaihu Decoction significantly reduced the number of eosinophils, the levels of inflammatory factors in the BALF of asthmatic mice, and the deposition of collagen in lung tissues, and they also significantly inhibited the proliferation of ASMCs and accelerated their apoptosis (all $P<0.05$). Wuling San and Xiao Chaihu Decoction significantly upregulated the expression of AQP5 while inhibited the expression of miR-486-5p; additionally, miR-486-5p negatively regulated the expression of AQP5 (all $P<0.05$). Overexpression of miR-486-5p or silencing AQP5 can partially reverse the therapeutic effect of Wuling San and Xiao Chaihu Decoction on allergic asthma in mice and the inhibitory effect on the abnormal proliferation of ASMCs (all $P<0.05$). Conclusion: Wuling San and Xiao Chaihu Decoction can influence the proliferation and apoptosis of ASMCs and the expression of inflammatory factors in mice with allergic asthma through inhibiting the expression of miR-486-5p and upregulating the expression of AQP5.

Keywords: Wuling San and Xiao Chaihu Decoction, miR-486-5p, aquaporin-5, inflammatory response, airway smooth muscle cells

Introduction

Asthma is a chronic respiratory disease, and the number of patients is increasing due to environmental factors [1]. The current treatment of asthma mainly relies on the inhalation of corticosteroids, supplemented by inhalation of long-acting β2 agonists, montelukast or theophylline [2]. However, significant heterogeneity exists among asthma patients, which compromises the clinical outcomes of these therapeutic methods [3]. Therefore, a comprehensive investigation of the mechanism underlying the initiation and progression of asthma is not only necessary, but also essential for the development of effective medications in the future.

Asthma is characterized by continuous airway inflammation, airway hyperresponsiveness, and airway remodeling [4]. Airway inflammation is considered to be the culprit of asthma. Therefore, controlling airway inflammation is an important way to treat asthma [5]. Airway remodeling is described as pathological remodeling process of cellular and molecular components in the airway walls, including airway wall

thickening, epithelial rupture, subepithelial fibrosis, extracellular matrix deposition, and abnormal proliferation of airway smooth muscle cells (ASMCs) [6]. The abnormal proliferation of ASMCs decreases the area of airway lumen, leading to an increase in airway resistance [7]. Therefore, inhibition of abnormal proliferation of ASMCs and airway inflammation has become a promising strategy for the treatment of asthma.

Wuling San and Xiao Chaihu Decoction are widely used in traditional Chinese medicine to treat acute nephritis, nephrotic syndrome, gastroenteritis and Meniere's disease [8]. Xiao Chaihu Decoction and Wuling San are both described in "*Shanhan Lun*" by Zhongjing Zhang in the Han Dynasty. In recent years, Wuling San and Xiao Chaihu Decoction have been applied in the treatment of exopathy, asthma, chronic obstructive pulmonary disease, and spleen and stomach diseases. Modern pharmacological studies have reported that Wuling San and Ramulus cinnamomi (Guizhi) can reduce myocardial oxygen consumption, increase oxygen delivery capacity, and improve lung ventilation and blood gas indicators; Poria cocos (Fuling) can regulate the water and salt metabolism; Polyporus umbellatus (Zhuling) and Alisma orientalis (Zexie) can inhibit the reabsorption of water and electrolytes by renal tubules to increase the glomerular filtration rate, which exerts a diuretic effect [9]. Xiao Chaihu Decoction has the effects of regulating immunity, anti-allergy, and anti-inflammation. Previous application of "liver and spleen Zhongjiao theory in the treatment of asthma" in clinical practice has revealed that the application of Wuling San and Xiao Chaihu Decoction has achieved good clinical outcomes in treating asthma [10]. Therefore, it is reasonable to explore the effect and mechanism of Wuling San and Xiao Chaihu Decoction on the initiation and development of allergic asthma.

Aquaporins (AQPs) refer to a family of conserved transmembrane proteins that are expressed in various organs and systems [11]. There are 13 AQPs (AQP0 to AQP12) in humans, of which 4 (AQP1, 3, 4, and 5) are expressed in the respiratory system [12]. Studies have demonstrated that Salvia miltiorrhiza (Danshen) ameliorates airway inflammation and pulmonary edema in mice with OVA-induced allergic asthma by upregulating AQP5 [13]. Thus, AQP5 is selected as the study target and microRNAs (miRNAs) that may regulate its expression are also investigated. By using the target prediction database, AQP5 and miR-486-5p have been confirmed to have specific binding sites to each other. Previous studies have elucidated that the expression of miR-486 is upregulated in asthma, however, it is limited in the investigation of the relationship between miR-486 expression level and asthma, whereas the function of miR-486 and the detailed mechanism are exclusive [14]. Therefore, miR-486-5p was selected in our study to investigate whether Wuling San and Xiao Chaihu Decoction may regulate the progression of allergic asthma via miR-486-5p/AQP5 axis.

This study hypothesized that Wuling San and Xiao Chaihu Decoction could inhibit airway inflammation and abnormal proliferation of ASMCs in mice with allergic asthma through miR-486-5p/AQP5 axis, which could provide alternative strategies for the clinical treatment of allergic asthma.

Materials and methods

Agentia preparation

Wuling San and Xiao Chaihu Decoction extracts were homemade, including 90 g of Polyporus umbellatus (Zhuling, peeled), 90 g of Poria cocos (Fuling), 90 g of Atractylodis macrocephalae rhizoma (Baizhu), 150 g of Alisma orientalis (Zexie), 60 g of Ramulus cinnamomi (Guizhi, peeled), 125 g of Bupleurum (Chaihu), 45 g of Scutellaria baicalensis (Huangqin), 45 g of Codonopsis pilosula (Franch.) of Nannf. (Dangshen), 65 g of Rhizoma pinelliae (Banxia), 45 g of Licorice (Gancao, roasted), 45 g of Zingiber officinale Roscoe (Shengjiang), and 60 g of Ziziphus jujuba (Dazao). All medicinal materials were soaked in 7000 mL of water for 2 hours, simmered for 2 hours, and filtered. After the above steps were repeated once, the filtrates were combined and cooled, from which the supernatant was collected and concentrated to 2 g/mL under a reduced pressure.

Animal model

Ovalbumin (OVA, YT0233, Beijing Yita Biotech Co., Ltd., China) was used to induce allergic asthma using BALB/c mice (Guangdong Medical Experimental Animal Center, China). The mice were randomly selected as control group (blank group), OVA group, and OVA+ agentia group (OVA+Wuling San and Xiao

Chaihu Decoction extract), with 6 mice per group. Mice were injected intraperitoneally with 20 mg of OVA (0219122401, Beijing Zhijie Fangyuan Technology Co., Ltd., China), once every 14 days for a total of 42 days. From 21 to 42 days after induction, 1% OVA was used to stimulate asthma for 30 minutes, 3 times a week. Mice of OVA+agentia group were orally gavaged with 8 mL/kg Wuling San and Xiao Chaihu Decoction 1 hour before OVA stimulation. Twenty-four hours after the last stimulation, mice were intraperitoneally injected with 40 mg/kg of sodium barbiturate, followed by CO_2 euthanasia method, after which blood samples, bronchoalveolar lavage fluid (BALF), and lung tissues were collected for subsequent analyses. All mice were housed at room temperature. All animal experiments were conducted in accordance with the *Guidelines for Animal Protection* and approved by the animal ethics committee of our hospital (Approval No. 2019(01):21).

Cell culture

The isolated ASMCs were cultured in DMEM medium (31600, Beijing Solarbio Biotechnology Co., Ltd., China) containing 10% fetal bovine serum (FBS, Wuhan Procell Life Technology Co., Ltd., China), streptomycin (100 μ/mL, Shanghai Beyotime Biotechnology Co., Ltd., China), and penicillin (100 μ/mL, Shanghai Beyotime Biotechnology Co., Ltd., China). The cells were cultured in an incubator with 5% CO_2 at 37°C.

ASMCs proliferation model

ASMCs isolated from BALB/c mice were incubated with 5 ng/mL TGF-β1 (RPA124Mu01, Wuhan Cloud-Clone Technology Co., Ltd., China) for 24 hours to establish the model of abnormal proliferation of ASMCs. In addition, ASMCs were incubated with 5 ng/mL TGF-β1 and 10 mM Wuling San and Xiao Chaihu Decoction for 24 hours as the treatment group. The cells were then collected for subsequent analyses.

Transfection and experimental groups

miR-486-5p mimic and negative control (miR-NC) recombinant lentiviral vectors, small interfering RNA recombinant lentiviral vector (shRNA), and negative control (sh-NC) of AQP5 were all purchased from Shanghai Jima Pharmaceutical Technology Co., Ltd. The mice were intravenously injected with miR-486-5p recombinant lentivirus with a concentration of 5×10^7 TU/mL (200 μL/mouse) and AQP5 lentiviral solution (100 μL/mouse). After treatment with Wuling San and Xiao Chaihu Decoction, asthmatic mice or ASMCs stimulated by TGF-β1 were transfected with miR-486-5p mimic/NC or sh-AQP5/NC for 48 hours for subsequent experiments. The sequences for transfection were listed as follows: miR-486-5p mimic: AGGGGCUGGCUUUCCUCUGGUC; miR-NC: UUCUCCGAACGUGUCACGUTT; sh-AQP5: GACCAGAGGAAAGCCAGCCCCU; sh-NC: CAGUACUUUUGUGUAGUACAA.

The cells were grouped as blank group (untreated ASMCs), TGF-β1 group (ASMCs+TGF-β1), TGF-β1+agentia group (ASMCs+TGF-β1+Wuling San and Xiao Chaihu Decoction), TGF-β1+agentia+sh-AQP5 group (ASMCs+TGF-β1+Wuling San and Xiao Chaihu Decoction+sh-AQP5), TGF-β1+agentia+sh-NC group (ASMCs+TGF-β1+Wuling San and Xiao Chaihu Decoction+sh-AQP5 control), TGF-β1+agentia+miR-486-5p mimic group (ASMCs+TGF-β1+Wuling San and Xiao Chaihu Decoction+miR-486-5p mimic), and TGF-β1+agentia+miR-NC Group (ASMCs+TGF-β1+Wuling San and Xiao Chaihu Decoction+miR-486-5p control).

The mice were grouped as control group (blank group), OVA group (OVA induction), OVA+agentia group (OVA+Wuling San and Xiao Chaihu Decoction), OVA+agentia+sh-AQP5 group (OVA+wuling San and Xiao Chaihu Decoction+sh-AQP5), OVA+agentia+sh-NC group (OVA+Wuling San and Xiao Chaihu Decoction+AQP5 control), OVA+agentia+miR-486-5p mimic group (OVA+Wuling San and Xiao Chaihu Decoction+miR-486-5p mimic) and OVA+agentia+miR-NC group (OVA+Wuling San and Xiao Chaihu Decoction+miR-486-5p control).

MTT assay

ASMCs were incubated in DMEM medium containing 0.5 mg/mL MTT (YT1466, Beijing Yita Biotech Co., Ltd., China) at 37°C for 4 hours. A microplate reader (Shanghai Yuanye Biotechnology Co., Ltd., China) was used to measure optical density (OD) values at 570 nm to evaluate cell viability.

Flow cytometry

ASMCs were collected by centrifugation at 800 g. The apoptosis was detected using Annexin V-FITC apoptosis detection kit (C1062S, Shang-

Table 1. qRT-PCR sequences

Name	Sequence (5'-3')
miR-486-5p	
Forward	CAGTCCTGTACTGAGCTGC
Reverse	GTGCAGGGTCCGAGGT
AQP5	
Forward	CGGGCTTTCTTCTACGTGG
Reverse	GCTGGAAGGTCAGAATCAGCTC
GAPDH	
Forward	ACCACAGTC CATGCCATCAC
Reverse	TCCACCACCCT GTT GCTGTA
U6	
Forward	GCUUCGGCAGCACAUAUACUAAA
Reverse	CGCUUCACGAAUUUGCGUGUCAU

Note: qRT-PCR: Quantitative real-time polymerase chain reaction; AQP5: aquaporin-5.

hai Biyuntian Biotechnology Co., Ltd., China) and a flow cytometer (Accuri C6, BD, USA).

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's instructions, the levels of IL-4, IL-5, and IL-13 in serum and BALF were measured using the corresponding ELISA kits (Multi-Sciences, China).

Hematoxylin and eosin (HE) staining

BALF samples were stained via HE staining. Briefly, one milliliter of BALF was centrifuged at 3500 rpm at 4°C for 15 minutes, then 0.1 mL of the precipitate was incubated with Bouin solution (4% formaldehyde; Beijing Haide Biotech, China). After samples were paraffin embedded, sectioned, and stained, the average number of eosinophils in each group was counted under a light microscope at 200× magnification.

Masson staining

Masson staining was performed on lung tissues to detect the amount of collagen accumulation. First, the tissues were incubated with hematoxylin solution (Shanghai Maokang Biotechnology Co., Ltd., China) for 6 minutes, with carmine (Shanghai Maikelin Biochemical Technology Co., Ltd., China) and acid fuchsin solution (Nanjing reagent) for 1 minute, and finally with phosphomolybdic acid solution (Nanjing SenBeiJia Biotechnology Co., Ltd., China) for 5 minutes. After incubation with aniline blue solution (Shanghai Hongshun Biological Technology Co., Ltd., China) for 5 minutes, the images were taken under a microscope at 200× magnification.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol® reagent (Thermo Fisher Scientific, Inc. USA). A total of 5 μg of extracted RNA was reverse transcribed into complementary DNA (cDNA) with M-MLV reverse transcriptase (Thermo Fisher Scientific, Inc. USA). qRT-PCR was performed according to the ABI 7300 system (Thermo Fisher Scientific, Inc. USA) using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc. USA). The thermal cycling conditions included: initial denaturation at 95°C for 15 seconds, denaturation at 94°C for 30 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 40 seconds. GAPDH and U6 were used as internal controls of AQP5 and miR-486-5p, respectively. The gene expression was analyzed via $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed in **Table 1**.

Dual-luciferase report assay

The wild-type (WT) and mutant AQP5 sequences were sub-cloned into the pmirGLO vector (E1330, Beijing Promega Biotechnology Co., Ltd., China). Subsequently, Lipofectamine® 3000 (Thermo Fisher Scientific, Inc. USA) was used to co-transfect the vectors with miR-486-5p mimic, inhibitor, and NC control into 293T cells (2×10^5 cells/well). After 48 hours transfection, the luciferase activity was measured using the dual-luciferase reporter system (Beijing Promega Biotechnology Co., Ltd., China).

Statistical analysis

SPSS 21.0 software was used to analyze the data in this paper. The quantitative data were analyzed by Shapiro test and Levene test to determine whether they were in line with normal distribution and equal variation. If normal distributed with equal variation, data were presented as mean ± standard deviation. The comparison between the two groups was done by t-test, while the comparison between multiple groups was conducted by the analysis of variance, within which the comparison between

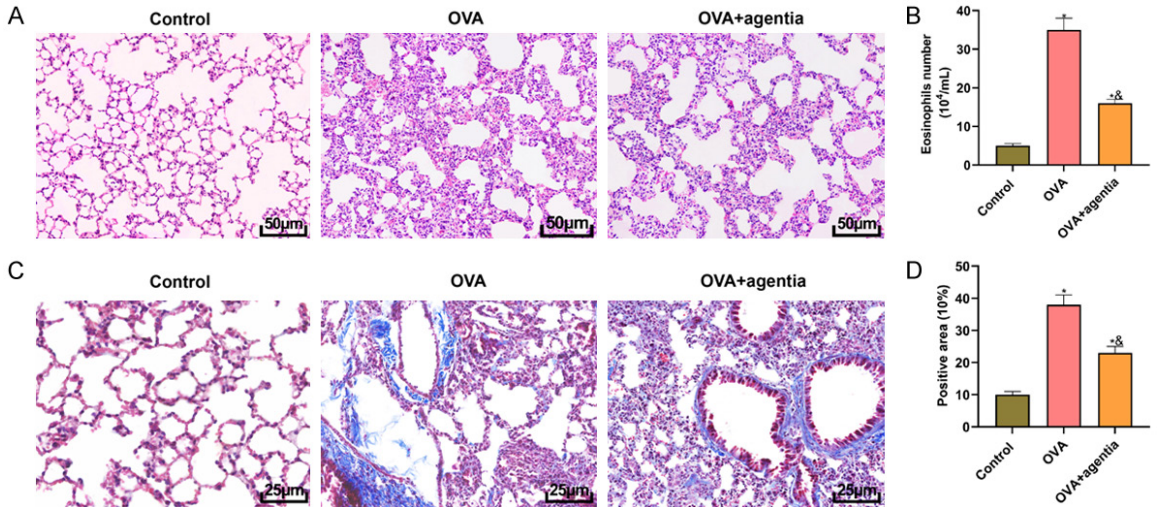

Figure 1. Wuling San and Xiao Chaihu Decoction can inhibit the recruitment of inflammatory cells and collagen deposition in allergic asthma mice (n=6). A, B: HE staining of BALF; C, D: Masson staining of lung tissues. Compared with the control group, *P<0.05; Compared with the OVA group, &P<0.05. OVA: ovalbumin; HE: Hematoxylin and eosin; BALF: bronchoalveolar lavage fluid.

two groups was performed using SNK-q. If not normally distributed or with unequal variance, data were presented as median (lower quartile, upper quartile) (M (P25, P75)). In this case, the comparison between the two groups was carried out by Wilcoxon signe-rank test or Kruskal-Wallis test, while the comparison among multiple groups was done by Friedman test. P<0.05 indicated that the difference was statistically significant.

Results

Wuling San and Xiao Chaihu Decoction inhibited the recruitment of inflammatory cells and collagen deposition in allergic asthma mice

First, BALF samples were collected from each group of mice for HE staining to observe the changes regarding the number of eosinophils. **Figure 1A** and **1B** demonstrated that the number of eosinophils in the OVA group was significantly higher than that in the control group. Masson staining was performed on the lung tissues of each group of mice to observe the changes of collagen distribution. The results showed that the collagen deposition in the lung tissues of the OVA group was significantly more than that of the control group (P<0.05; **Figure 1C** and **1D**), indicating the successful establishment of allergic asthma mice model. After treatment with Wuling San and Xiao Chaihu Decoction, the number of eosinophils and collagen deposition in asthmatic mice were significantly reduced (both P<0.05; **Figure 1**). The data revealed that Wuling San and Xiao Chaihu Decoction had a significant protective effect on lung tissues and a certain inhibitory effect on the progression of allergic asthma.

Wuling San and Xiao Chaihu Decoction can decrease the levels of inflammatory factors and inhibit the proliferation of ASMCs in allergic asthma mice

ELISA was used to measure the levels of IL-4, IL-5, and IL-13 in BALF samples of each group. The results showed that the levels of inflammatory factors induced by OVA were significantly reduced by Wuling San and Xiao Chaihu Decoction (P<0.05; **Figure 2A-C**). To further explore the effects of Wuling San and Xiao Chaihu Decoction on allergic asthma, ASMCs were used to further explore its possible mechanism. The results of cell proliferation by MTT assay demonstrated that compared with the control group, TGF-β1 significantly increased the proliferation of ASMCs, while Wuling San and Xiao Chaihu Decoction significantly inhibited the effect of TGF-β1 (P<0.05; **Figure 2D**). The flow cytometry data showed that the apoptosis decreased by TGF-β1 was significantly increased by Wuling San and Xiao Chaihu Decoction (P<0.05; **Figure 2E** and **2F**). The above results indicated that Wuling San and Xiao Chaihu Decoction had anti-inflammatory

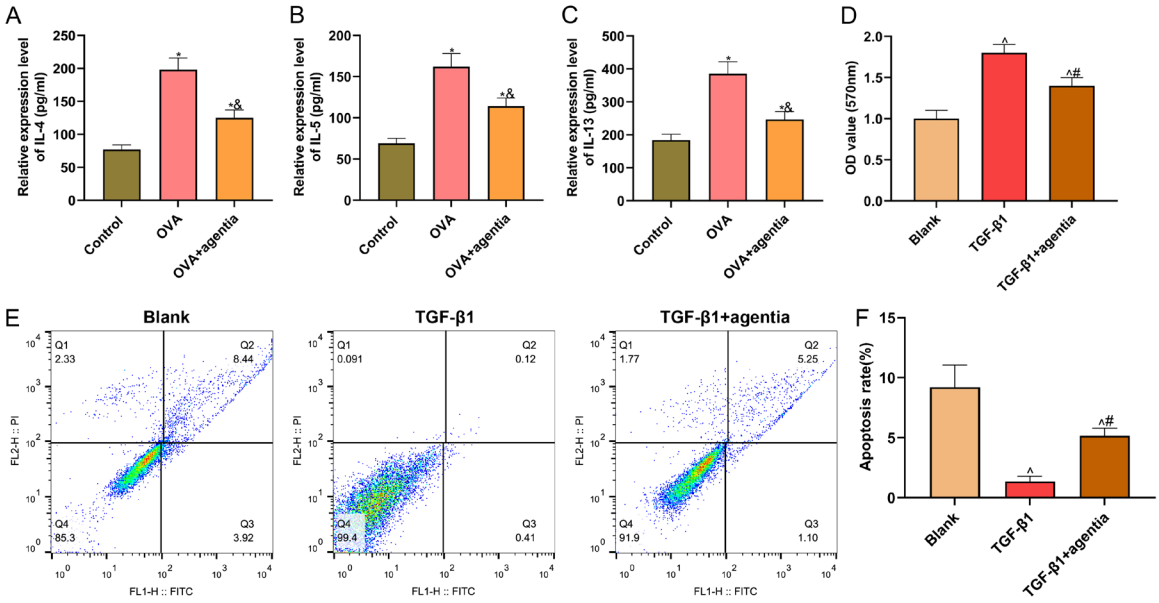

Figure 2. Wuling San and Xiao Chaihu Decoction can inhibit the levels of inflammatory factors and the proliferation of ASMCs in allergic asthma mice. A: Detection of IL-4 in BALF by ELISA; B: Detection of IL-5 in BALF by ELISA; C: Detection of IL-13 in BALF by ELISA; D: Cell viability evaluated by MTT; E, F: Results of apoptosis. Compared with the control group, *P<0.05; compared with the OVA group, &P<0.05; compared with the blank group, ^P<0.05; compared with the TGF-β1 group, #P<0.05. OVA: ovalbumin; BALF: bronchoalveolar lavage fluid; ELISA: enzyme-linked immunosorbent assay; IL-4: levels of interleukin-4; TGF-β1: transforming growth factor beta 1.

effects on allergic asthma, which can effectively inhibit the proliferation of ASMCs and induce cell apoptosis.

Silencing AQP5 inhibited the therapeutic effect of Wuling San and Xiao Chaihu Decoction on allergic asthma

To further explore the mechanism of Wuling San and Xiao Chaihu Decoction's effect on allergic asthma, firstly, qRT-PCR was conducted to quantify AQP5 expression in lung tissues and ASMCs before and after treatment with Wuling San and Xiao Chaihu Decoction. The results showed that compared with the control group, OVA and TGF-β1 significantly reduced the expression of AQP5, while Wuling San and Xiao Chaihu Decoction significantly increased the expression of AQP5 (P<0.05; **Figure 3A** and **3B**). Next, AQP5 was silenced to explore the mechanism by which Wuling San and Xiao Chaihu Decoction and AQP5 affected allergic asthma. **Figure 3C** and **3D** showed that AQP5 was successfully knocked down (P<0.05). ELISA data showed that Wuling San and Xiao Chaihu Decoction significantly reduced the levels of inflammatory factors that were upregulated by OVA, while silencing AQP5 significantly inhibited the effect of Wuling San and Xiao Chaihu Decoction (P<0.05; **Figure 3E-G**). For abnormal proliferation of ASMCs, treatment with Wuling San and Xiao Chaihu Decoction significantly inhibited cell proliferation and induced apoptosis compared with the control group, whereas the downregulation of AQP5 partially reversed this result (P<0.05; **Figure 3H-J**). The above data revealed that Wuling San and Xiao Chaihu Decoction upregulated the expression of AQP5 in allergic asthma, and silencing AQP5 inhibited the therapeutic effect of Wuling San and Xiao Chaihu Decoction on allergic asthma.

AQP5 was the target of miR-486-5p

AQP5 was used as the target to find miRNAs that may regulate its expression, and the target prediction databases including miRDB, miRWALK, and RNA22 were applied to predict the upstream miRNAs that may regulate AQP5, by which the results were intersected (**Figure 4A**). The results demonstrated that has-miR-486-5p and has-miR-874-5p were intersected miRNAs. Since we expected to find a miRNA functioning in the opposite way as AQP5 in allergic asthma and has-miR-874-5p could play a protective role in allergic asthma [15], has-miR-486-5p was selected in this study (**Figure 4B**). First, dual-luciferase report assay was carried out to verify the interaction between these two molecules, which showed that miR-486-5p can

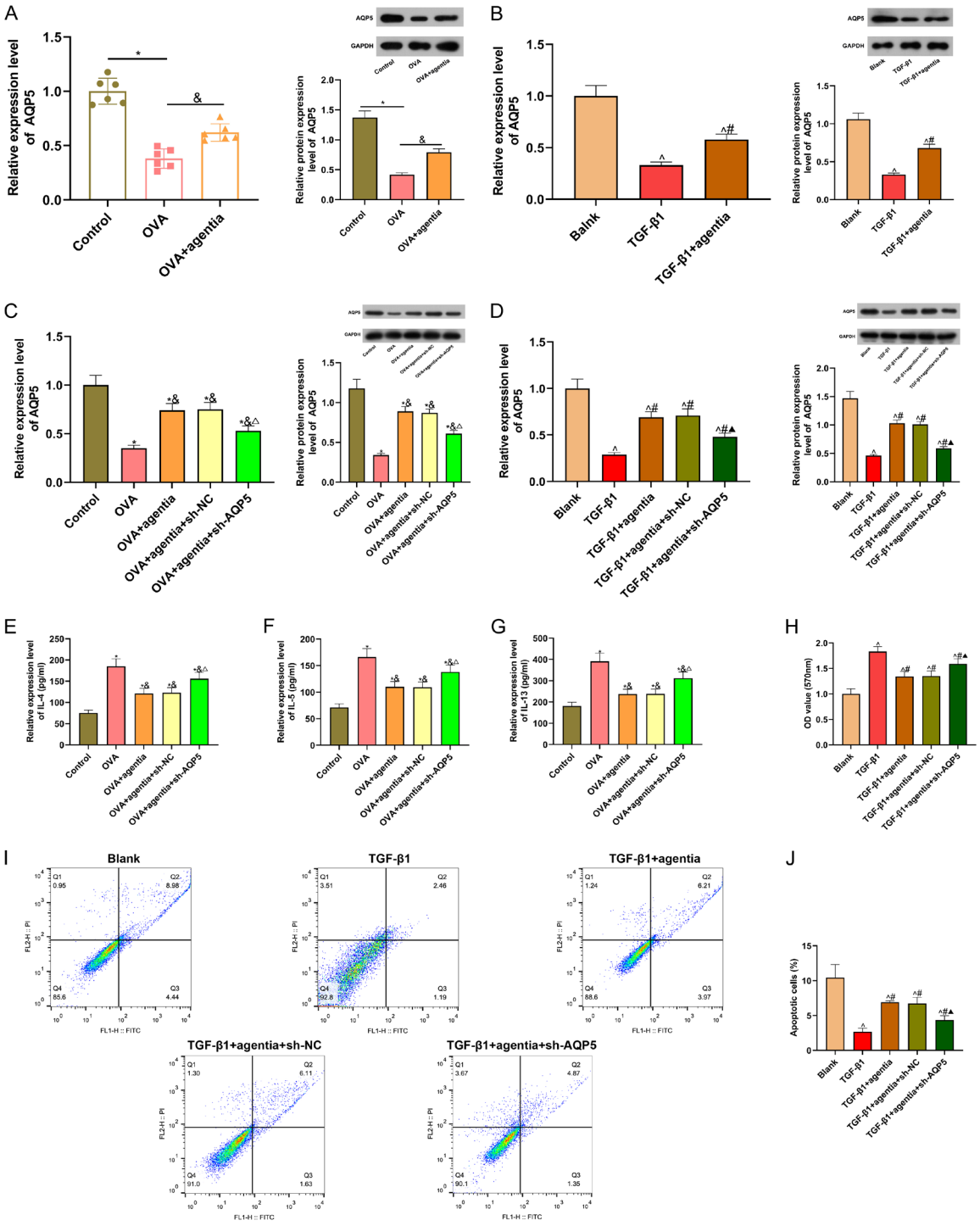

Figure 3. Silencing AQP5 can inhibit the therapeutic effect of Wuling San and Xiao Chaihu Decoction on allergic asthma. A: mRNA and protein expression of AQP5 in BALF (n=6); B: mRNA and protein expression of AQP5 in ASMCs; C, D: Knockdown efficiency of AQP5 measured by qRT-PCR; E: Detection of IL-4 level in BALF by ELISA; F: Detection of IL-5 level in BALF by ELISA; G: Detection of IL-13 level in BALF by ELISA; H: Cell viability evaluated by MTT; I, J: Cell apoptosis results. Compared with the control group, $^{*}P<0.05$; compared with the OVA group, $^{\&}P<0.05$; compared with the OVA+agentia+sh-NC group, $^{\Delta}P<0.05$; compared with the blank group, $^{\wedge}P<0.05$; compared with the TGF-β1 group, $^{\#}P<0.05$; compared with the TGF-β1+agentia+sh-NC group, $^{\blacktriangle}P<0.05$. qRT-PCR: quantitative real-time polymerase chain reaction; AQP5: aquaporin-5; OVA: ovalbumin; BALF: bronchoalveolar lavage fluid; ELISA: enzyme-linked immunosorbent assay; IL-4: levels of interleukin-4; ASMCs: airway smooth muscle cells; sh-NC: negative control; TGF-β1: transforming growth factor beta 1.

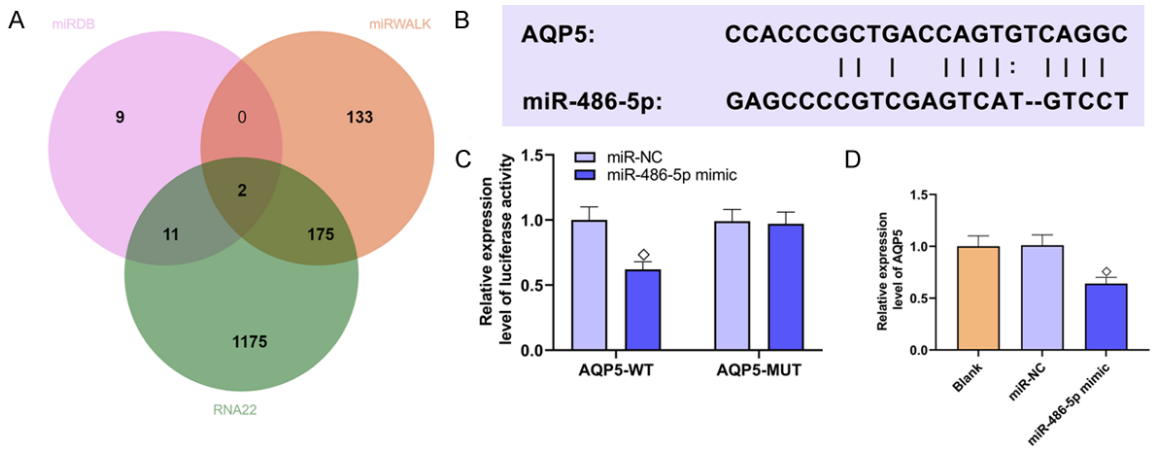

Figure 4. AQP5 is the target of miR-486-5p. A: Venn diagram showing the intersection of miRNAs regulatingAQP5 via miRDB, miRWALK, and RNA22 databases; B: Specific binding sites of AQP5 and miR-486-5p; C: Dual-luciferase report assay results; D: Impact of miR-486-5p on AQP5 expression. Compared with miR-NC group, ◇P<0.05. AQP5: aquaporin-5.

significantly reduce the fluorescent activity of AQP5-WT, but it had almost no effect on the fluorescent activity of AQP5-MUT (P<0.05; **Figure 4C**). In addition, miR-486-5p mimic and NC were transfected into ASMCs to observe the changes of AQP5 expression. qRT-PCR data revealed that compared with the control group, overexpression of miR-486-5p can significantly reduce AQP5 Level, which suggested that miR-486-5p can negatively regulate the expression of AQP5 in ASMCs (P<0.05; **Figure 4D**).

Overexpression of miR-486-5p inhibited the therapeutic effect of Wuling San and Xiao Chaihu Decoction on allergic asthma

qRT-PCR data showed that the expression of miR-486-5p was significantly upregulated after OVA stimulation compared with the control group, while Wuling San and Xiao Chaihu Decoction significantly downregulated the expression of miR-486-5p (P<0.05; **Figure 5A** and **5B**). The mice that were treated with Wuling San and Xiao Chaihu Decoction were overexpressed with miR-486-5p to explore the possible role of miR-486-5p in allergic asthma. **Figure 5C** and **5D** demonstrated the transfection efficiency by miR-486-5p mimic (P<0.05). The results of **Figure 5E-G** showed that overexpression of miR-486-5p significantly increased the levels of IL-4, IL-5 and IL-13 that were downregulated by Wuling San and Xiao Chaihu Decoction (P<0.05). The data of cell proliferation and apoptosis revealed that cell viability was significantly reduced while the rate of apoptosis was significantly increased in the treatment group by Wuling San and Xiao Chaihu Decoction in comparison to the TGF-β1 group, which, however, were partially reversed by miR-486-5p mimic (P<0.05; **Figure 5H-J**).

Discussion

Due to factors like smoking and air pollution, the incidence of asthma has been on the rise in recent years, and the treatment of asthma has also received more and more attention [16, 17]. This study demonstrates that Wuling San and Xiao Chaihu Decoction can inhibit the recruitment of inflammatory cells and collagen deposition and downregulate the levels of inflammatory factors in OVA-induced allergic asthma mice. In abnormal proliferated ASMCs via TGF-β1 stimulation, this medication can significantly inhibit the proliferation of ASMCs while induce their apoptosis. In addition, Wuling San and Xiao Chaihu Decoction can upregulate AQP5 expression and reduce the expression of miR-486-5p in allergic asthma, thereby ameliorating the airway inflammation and abnormal proliferation of ASMCs in allergic asthma.

As a combination of Xiao Chaihu and Wuling San, Wuling San and Xiao Chaihu Decoction is commonly used to treat chronic diseases, autoimmune diseases, tumors, etc. [18]. Studies have shown that Xiao Chaihu Decoction has anti-inflammatory effects in inflammatory response, such as hepatitis B and pancreatitis [19, 20]. Additionally, the application of Wuling San and Xiao Chaihu Decoction to treat asthma has achieved good clinical outcomes [10].

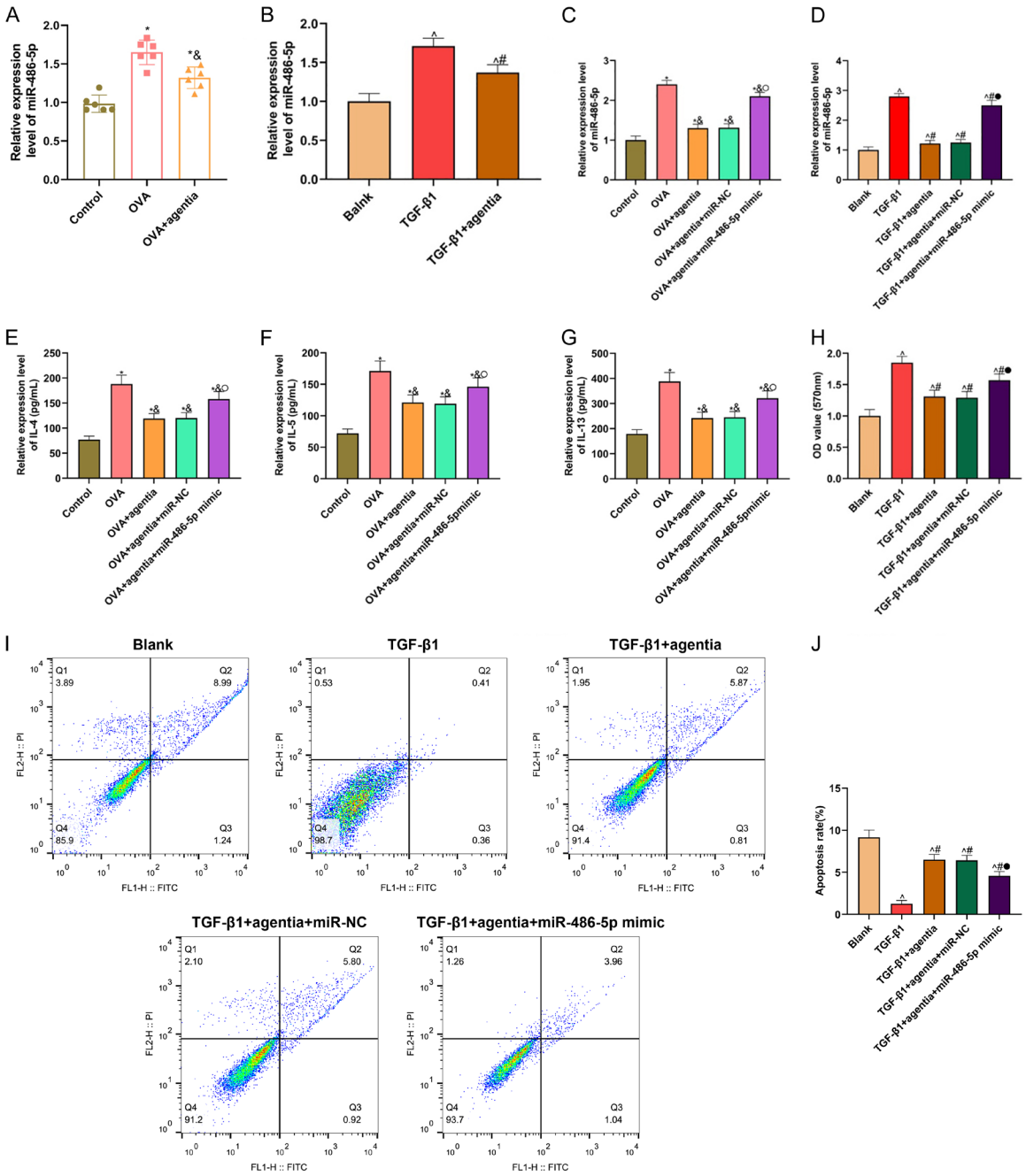

Figure 5. Overexpression of miR-486-5p can inhibit the therapeutic effect of Wuling San and Xiao Chaihu Decoction on allergic asthma. A: Expression of miR-486-5p in BALF (n=6); B: Expression of miR-486-5p in ASMCs; C, D: qRT-PCR to quantify the expression of miR-486-5p; E: Detect of IL-4 level in BALF by ELISA; F: Detect of IL-5 level in BALF by ELISA; G: Detect of IL-13 level in BALF by ELISA; H: MTT results to evaluate cell viability; I, J: Cell apoptosis results. Compared with control group, *P<0.05; compared with the OVA group, &P<0.05; compared with the OVA+agentia+miR-NC group, ○P<0.05; compared with the blank group, ^P<0.05; compared with the TGF-β1 group, #P<0.05; compared with the TGF-β1+agentia+miR-NC group, •P<0.05. miR-NC: miR-486-5p mimic, inhibitor, negative control; qRT-PCR: quantitative real-time polymerase chain reaction; AQP5: aquaporin-5; OVA: ovalbumin; BALF: bronchoalveolar lavage fluid; ELISA: enzyme-linked immunosorbent assay; IL-4: levels of interleukin-4; ASMCs: airway smooth muscle cells; TGF-β1: transforming growth factor beta 1.

Based on these results, this study applies the OVA method to establish a mouse model of allergic asthma. Compared with the control group, the number of eosinophils was significantly increased in the BALF of the OVA group, the collagen deposition was significantly in-

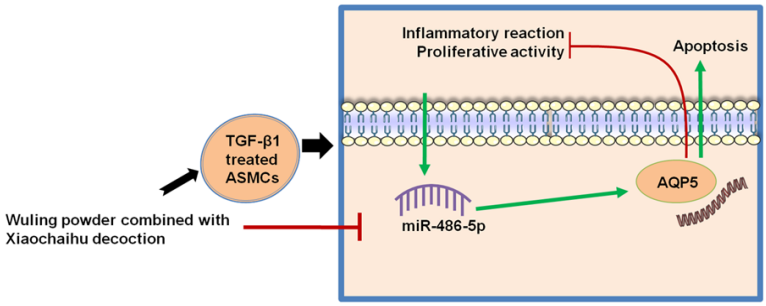

Figure 6. Wuling San and Xiao Chaihu Decoction can ameliorate the airway inflammation and AMSCs proliferation in allergic asthma by inhibiting miR-486-5p and upregulating AQP5. AQP5: aquaporin-5; ASMCs: airway smooth muscle cells; TGF-β1: transforming growth factor beta 1.

creased in the lung tissues, and the levels of inflammatory factors were also upregulated. After treatment with Wuling San and Xiao Chaihu Decoction, the recruitment of inflammatory cells and collagen deposition of asthmatic mice were significantly ameliorated. In addition, AMSCs were isolated and stimulated with TGF-β1 to establish an abnormally proliferating cell model. Our data revealed that the proliferation of ASMCs after TGF-β1 stimulation is significantly increased, whereas the apoptosis is decreased. After co-incubation with Wuling San and Xiao Chaihu Decoction, compared with the TGF-β1 group, the proliferation of ASMCs is significantly reduced, but the apoptosis is significantly increased. Both *in vivo* and *in vitro* experiments have indicated the protective effect of Wuling San and Xiao Chaihu Decoction on allergic asthma.

The role of AQP5 in allergic asthma has been preliminarily investigated. The expression of AQP5 in the lung tissues of OVA-sensitized mice is reduced. Curcumin can reverse the level of AQP5 in allergic lung tissue and play a therapeutic effect on OVA-induced allergic asthma, by which the expression of pro-inflammatory markers is downregulated, the lung dry-wet ratio is reduced, and goblet cell proliferation is decreased [21]. In line with previous results, we have found that the expression of AQP5 is downregulated in our allergic asthma mice. In addition, studies have found that Scutellaria baicalensis (Huangqin), the active ingredient in Wuling San and Xiao Chaihu Decoction, can reduce the inflammatory responses of OVA-induced asthmatic mice by inhibiting CCR7/CCL19/CCL21, which can also inhibit the expression of PDGF-induced phospho-p38, phospho-ERK1/2, and phospho-JNK to suppress the proliferation and migration of ASMCs [22, 23]. Therefore, we hypothesize that Wuling San and Xiao Chaihu Decoction may have a therapeutic effect on allergic asthma by regulating the expression of AQP5. Jujube seed can upregulate the expression of AQP5, leading to inhibition of the secretion of inflammatory factors and the suppression of the proliferation of goblet cells to alleviate allergic asthma [24]. In this study, we have tested asthmatic mice treated with Wuling San and Xiao Chaihu Decoction and found that the expression of AQP5 in the treatment group is significantly higher than that of the OVA group. ELISA data have shown that Wuling San and Xiao Chaihu Decoction could inhibit the secretion of inflammatory factors by upregulating AQP5. According to *in vitro* data, Wuling San and Xiao Chaihu Decoction suppress the proliferation of ASMCs by upregulating the expression of AQP5 and accelerating the cell apoptosis to ameliorate the allergic asthma. These results illustrate that Wuling San and Xiao Chaihu Decoction may exert the therapeutic effect on allergic asthma by upregulating AQP5.

To further investigate the mechanism of Wuling San and Xiao Chaihu Decoction in allergic asthma, this study has applied bioinformatics tools to screen and obtain the upstream target miRNA of AQP5, namely miR-486-5p. Studies have reported that the expression of miR-486 is upregulated in asthma, but the possible function and mechanism of miR-486 in the progression of asthma have not been elucidated [14]. This study found that miR-486-5p expression was upregulated in mice with OVA-induced allergic asthma via qRT-PCR, which was consistent with previous report. We also verified the interaction between AQP5 and miR-486-5p by using the dual-luciferase reporter assay, which shows that miR-486-5p can negatively regulate the expression of AQP5. Additionally, we have also shown that Wuling San and Xiao Chaihu Decoction could downregulate the expression level of miR-486-5p both *in vitro* and *in vivo*. Rescue experiment data have demonstrated that miR-486-5p can aggravate the malignant progression of allergic asthma. Wuling San and Xiao Chaihu Decoction

may ameliorate the airway inflammatory responses and abnormal proliferation of ASMCs in allergic asthma by downregulating the expression of miR-486-5p.

In summary, Wuling San and Xiao Chaihu Decoction can ameliorate the airway inflammation and AMSCs proliferation in allergic asthma by inhibiting miR-486-5p and upregulating AQP5, which is depicted in **Figure 6**. However, the sample size of this study is small, thus, future investigation with expanded sample size is warranted to validate our data. Our studies may provide a novel therapeutic target for the development of anti-asthma medications using natural compounds in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Yanxia Yang, Northwest Minzu University, No. 1 Northwest New Village, Chengguan District, Lanzhou 730000, Gansu Province, China. Tel: +86-17393145688; E-mail: yangyanxia202122@163.com

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