Original Paper

MicroRNA-485 Modulates the TGF- β /Smads Signaling Pathway in Chronic Asthmatic Mice by Targeting Smurf2

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Key Words

MicroRNA-485 • Smurf2 • Chronic asthma • TGF- β /Smads signaling pathway • Proliferation • Apoptosis

Abstract

Background/Aims: Chronic asthma pathogenesis continue to plague millions of people worldwide. We aimed to elucidate the detailed mechanisms of microRNA-485 (miR-485) in airway smooth muscle cells (ASMCs) proliferation and apoptosis in chronic asthmatic mice.

Methods: A mouse model of chronic asthma was established. Ovalbumin was used to induce chronic asthma in the mice. The levels of transforming growth factor β (TGF- β), interleukin (IL)-4, IL-5, IL-13 and IL-17 in bronchoalveolar lavage fluid in mice were measured by enzyme-linked immunosorbent assays (ELISAs). ASMCs were transfected with miR-485 mimic, miR-485 inhibitor and scrambled miRNA. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were applied to detect the mRNA and protein levels of Smurf2, α -SMA, TGF- β 1 and decapentaplegic homolog (Smads). The MTT assay was utilized for cell proliferation, while flow cytometry was conducted to assess cell cycle distribution and apoptosis.

Results: Lower expression of miR-485 and higher expression levels of TGF- β 1, IL-4, IL-5, IL-13 and IL-17 were detected in mice with chronic asthma. Smurf2 was identified as the target gene of miR-485. Upregulation of miR-485 mimic and downregulation of Smurf2 increased expression levels of Smurf2, α -SMA, TGF- β 1 and Smad3, inhibited cell proliferation and increased apoptosis, while contrary results were observed in ASMCs transfected with miR-485 inhibitor. **Conclusion:** Overexpressed miR-485 inhibits cell proliferation and promotes apoptosis of ASMCs through the Smurf2-mediated TGF- β /Smads signaling pathway in mice with chronic asthma.

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Introduction

Asthma is complex disorder characterized by wheezing, coughing, dyspnoea, chest tightness, variable airflow, airflow obstruction, airway wall remodeling, airway chronic inflammation, and airway hyperresponsiveness [1, 2]. People suffering from asthma generally display a faster rate of lung dysfunction than that of normal healthy individuals, which is largely manifested by progressive remodeling of airway walls [3]. The classifications of asthma severity based on both current and previous guidelines range from intermittent to severe persistent asthma [4]. *Mycoplasma pneumoniae* and *Chlamydomphila pneumoniae* are typical bacteria observed in asthmatic airways and have been associated with clinical characteristics, such as exacerbation risks, symptom control, and steroid sensitivity [5]. The objective of asthma treatment is to preclude symptoms via decreasing airway hyperresponsivity and inflammation [6]. Inflammation has various potential triggers, which include reactions to lung infection [7]. International principles suggest that the dose of inhaled corticosteroids (ICSs) used for the asthma treatment should be based on the symptoms, spirometry, pulmonary-function tests, and bronchodilator requirements [8]. In recent decades, progressive studies of microRNAs (miRs) have provided promising therapeutic strategies for complex multifactorial human diseases, such as asthma [9]. Recently, studies have shown altered expression of miRs in the pathophysiological processes involving inflammatory diseases, such as the progression of atherosclerosis and non-small-cell lung cancer [10, 11]. However, the detailed effects of miRs continue to elude the scientific community, and little is known about their roles in immunologically driven and inflammatory disorders [12].

MiRs are non-coding RNA fragments comprising 22 between 21 and 23 nucleotides and are related to the transcription process [13]. Research on extracellular miRs has shown promise regarding new and improved therapeutic strategies as well as potential novel noninvasive biomarkers for asthma and other diseases [14]. MiRs have been reported to play essential roles in the pathogenesis of asthma, and miR-20b is believed to suppress airway inflammation in mice with asthma [15]. Likewise, miR-485 has been highlighted as another tumor suppressor agent in inhibiting proliferation and migration in breast cancer [16]. Furthermore, miR-485-3p and miR-485-5p were all found to modulate cell migration, mitochondrial respiration, and cell invasion in breast cancer cells via regulatory and inhibitory effects on the expression levels of the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1 α [17]. Smad ubiquitin regulatory factor 2 (Smurf2) is an E3 ubiquitin ligase that controls the transforming growth factor β (TGF- β)/decapentaplegic (Smads) signaling pathway. Smurf2 is connected to various kinds of cellular responses; however, the precise mechanisms regulating Smurf2 abundance remain largely unknown [18]. The TGF- β signaling pathway is related to tumorigenesis through various processes, including metastasis and angiogenesis [19]. Recently, the TGF- β /Smads signaling pathway has been reported to be involved in the airway remodeling in asthma [20]. However, few studies have been able to clarify the correlation among miR-485, Smurf2 and chronic asthma. Thus, in this study, we aimed to explore the effect of miR-485 on proliferation and apoptosis of airway smooth muscle cells (ASMCs) through the TGF- β /Smads signaling pathway by binding to Smurf2 in mice with chronic asthma.

Materials and Methods

Ethics statements

This protocol of this study was approved by the Animal Ethics Committee. All animal experiments were consistent with the Guide for the Care and Use of Laboratory Animal by international committees.

Experimental animals

A total of 30 specific-pathogen-free (SPF) female BALB/c mice (aged 6~8 weeks), weighing approximately 20 ± 2 g, were purchased from the Laboratory Animal Center of Medical University of Chongqing (Chongqing, China). The mice were fed in an animal house at 21–27°C with a relative humidity of 40~60% and alternation of light and dark for 12 h. Additionally, the mice had free access to food and water (aseptic feed and drinking water in accordance with room pressure standard). After mice were fed for 1 weeks under the aforementioned conditions, the experiment was conducted.

Animal grouping and model establishment of mice with chronic asthma

A total of 30 female BALB/c mice were assigned to the saline (n = 15) and ovalbumin (n = 15) groups with a random number table. Ten mice were selected from each group for noninvasive pulmonary function detection, and the selected mice were numbered to prepare lung tissue specimens and bronchoalveolar lavage fluid (BALF). The animal model was established as described in a relevant literature protocol [1]. The mice in the ovalbumin group were sensitized by intraperitoneal injection of 0.2 ml sensitizing liquid containing 100 μ g ovalbumin and 400 μ g Aluminum Hydroxide Gel (Sigma-Aldrich Chemical Company, St Louis MO, USA) on the 1st and 14th day. The mice in the saline group received intraperitoneal injection of 0.2 ml of normal saline solution. As the mice were in phase excitation from the 1st day, the mice in the ovalbumin group were put into a 0.5 m³ atomized inhalation chamber (Sussex 99SL, Boehringer Ingelheim, UI) and inhaled 5% ovalbumin after it was atomized through a nebulization generator (VMD, Shanghai Yuyan Instrument Inc., Shanghai, China). Mice in the saline group inhaled normal saline for 8 weeks (3 times a week, 30 min each). Mice with chronic asthma developed the following symptoms after successful model establishment: shortness of breath, irregular breathing rhythm, visibly trembling limbs, irritability, cough, hunched back, and cyanosis after active stimulation. When the mice were stimulated by using higher doses of ovalbumin, the lips and noses of the mice displayed more obvious cyanotic features.

Noninvasive pulmonary function detection

After 24 h following the last procedure, 10 mice from the saline and ovalbumin groups were selected for noninvasive pulmonary function detection using a Buxco noninvasive lung function instrument. After all parts of the Buxco noninvasive lung function instrument were connected and adjusted, the resting BALB/c mice were placed into a volume restrainer and then stimulated with methacholine at concentrations of 0, 6.25 g/l, 12.5 g/l, 25 g/l, 50 g/l, and 100 g/l. Each concentration inhalation lasted for 2 min, and the data were recorded for a 3 min period. Penh values were determined, and the differences between the two groups were subsequently compared.

Preparation for lung tissues, leukocytes and BALF

After 24 h following the last procedure, 10 mice from each group were anaesthetized by intraperitoneal injection of 0.6 ml 3% pentobarbital sodium. Next, the mouse tracheas were exposed, a 22 G intravenous needle was inserted into the trachea and the needle cores were then pulled out and fixed accordingly. The double lungs were lavaged with 0.6 ml normal saline solution with intratracheal injection and pump sucking (3 times for each operation). The lavage fluid was then retrieved, with a rate of recovery > 80%. The whole procedure was then repeated 3 times. BALF was then collected, and the total number of BALF cells was counted followed by classification of the leukocytes. The levels of the inflammatory factors TGF- β 1, interleukin (IL)-2, IL-4, IL-5, IL-13 and IL-17 in the lavage fluid were then detected after centrifugation for BALF. At last, the mice were sacrificed, and their chests were opened under aseptic conditions. The heart and lung tissues were then isolated completely. After ligation of the right lung bronchus, the right lung tissues were quickly extracted, placed in a freeze-thaw tube (liquid nitrogen), and stored in a refrigerator at -70°C.

Hematoxylin-eosin (HE) staining and Masson's trichrome staining

HE staining: after bronchoalveolar lavage, the left upper lobe lung tissues of mice were removed and immediately fixed in 4% paraformaldehyde (F0135, Beijing Leagene Biological Co., Ltd., Beijing, China) for 24 h. The fixed tissues were washed by running water, dehydrated, cleared, embedded in paraffin, and sliced into 5 μ m sections. Paraffin sections were dewaxed and stained in hematoxylin (G1120, Beijing Solarbio Biological Co., Ltd., Beijing, China). Finally, a Zeiss fluorescence microscope (PrimoStar iLED, Beijing Atobo Technologies Inc, Beijing, China) was applied to photograph and observe the pathological changes in the airway of the mice in each group.

Masson's trichrome staining: paraffin sections were dewaxed into distilled water and fixed in 4% paraformaldehyde for 24 h. The sections were then washed in the water for 30 s, stained in hematoxylin for 1~2 min, dehydrated in hydrochloric ethanol for 2~3 s, and returned to warm running water for 1~2 min. Distilled water and/or deionized water was used to wash sections for 1 min. Next, the sections were stained by ponceau-acid fuchsin solution for 5~8 min, rinsed with 1% acetic acid solution for 1 min, differentiated by 1% molybdophosphoric acid aqueous solution for 3~5 min, and finally rinsed again by 1% acetic acid solution for 1 min. The staining conditions were observed under a microscope, and the sections were directly stained by 1% aniline blue or green light liquid for 5 min, rinsed by 1% acetic acid solution for 1 min, and differentiated by 1% glacial acetic acid aqueous solution for 5 s. This was followed by an observation for coloring conditions under a microscope. Then, the sections were quickly dehydrated in alcohol, anhydrous alcohol I for 5~10 s, and anhydrous ethanol II and III for 5~10 s. The sections were cleared by xylene (I-III) for 1~2 min and sealed in neutral balsam. Both the inflammatory conditions of the lung tissue and collagen deposition in the airway wall were observed using a microscope. The results indicated that the collagen fiber was stained blue, muscle-fiber cytoplasm stained red, and muscle stained blue-brown. After the Masson staining of the lung tissues, ten bronchial cross-sections of 100~200 μ m in diameter were randomly selected in each section under a light microscope ($\times 200$). Images obtained by an image acquisition system. In addition, image analysis software Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) was employed to test the perimeter of the basement membrane (Pbm, μ m²), total wall area (Wat, μ m²), area of the inside wall (Wai, μ m²), smooth muscle area (Wsm, μ m²), and thickness of collagen deposition under the basement membrane (Wcol, μ m²).

Immunohistochemistry

Immunohistochemical staining was conducted by using SP reagent kit (SP-9001, Beijing Nobleryder Technologies Inc., Beijing, China). Lung tissue sections of mice from each group were sliced into 4 μ m, reserved at room temperature for 30 min, and fixed in acetone at -20°C for 10 min. In addition, the sections were also dewaxed in water, rinsed 3 times by phosphate buffered saline (PBS) (5 min for each), and washed by 3% H₂O₂ for 5~10 min to inhibit the endogenous peroxidase activity. Next, the distilled water was used to rinse the sections, and the sections were soaked in PBS (5 min for each). The sections were then sealed in 5~10% normal goat serum with polyvinyl alcohol (PVA, Applygen Technologies Inc., Beijing, China) and incubated at 37°C for 10~15 min. Moreover, sections were incubated with rabbit anti-Smurf2 antibodies (ab38543, Abcam Inc., Cambridge, MA, USA) at 4°C overnight, and then, the sections were placed at room temperature for 30 min and rinsed 3 times with PBS (5 min for each). Moreover, the sections were incubated with corresponding biotinylated goat anti-rabbit immunoglobulin G (IgG) (fluorescein isothiocyanate (FITC), 1: 1000, ab7086, Abcam Inc., Cambridge, MA, USA), namely, secondary antibody working solution, at 37°C for 1 h. After rinsing 3 times using PBS (5 min for each), the sections were then mixed with horseradish peroxidase (HRP) (3,3'-diaminobenzidine tetrahydrochloride (DAB), Imunbio Inc., Beijing, China)-labeled streptavidin working fluid, incubated at 37°C for 1 h, and rinsed 3 times using PBS (5 min for each). The diaminobenzidine (DAB) staining method was used (3,3'-diaminobenzidine tetrahydrochloride (DAB), Guangzhou Whiga Technologies Inc., Guangdong, China) for 3~10 min, and after this, the reaction was terminated. The sections were washed thoroughly using double distilled water and re-stained by hematoxylin (Shanghfs Inc., Shanghai, China), followed by conventional dehydration, cleaning, and mounting. In this experiment, the PBS acted as the negative control (NC) replacing the primary antibody. The positive staining showed a tan color. A total of 10 high power fields of vision were randomly selected in each section, and 5 images in each field of vision were selected ($\times 400$). Furthermore, the image analysis software Image-Pro Plus 6.0 (Media Cybernetics Inc., MD, USA) was utilized to detect the number of positive cells in each photograph as well as the percentage of positive cells.

Enzyme-linked immunosorbent assay (ELISA)

According to the instructions provided by the ELISA reagent kit (YQ, Imunbio Inc., Beijing, China), the levels of the inflammatory factors TGF- β 1, IL-4, IL-5, IL-13, and IL-17 in the collected mouse alveolar lavage fluid of the mice in the saline and ovalbumin groups were detected accordingly. The blank, NC and positive control wells were used as controls. The optical density (OD) of each well was then detected using a microplate reader (BS-1101, Detie Inc., Jiangsu, China) at 450 nm.

Isolation, purification and identification of primary ASMCs

The trachea and lung tissues of the mice in the saline and ovalbumin groups (5 mice in each group) were rinsed in D-Hanks/PBS solution and stripped of their outer membrane and connective tissues. The tissues were cut into sections at 3 mm. The sections were washed by D-Hank's liquid (HMK0011, Beijing Huamaike Biotechnology Inc., Beijing, China) with the supernatant discarded, incubated with Dulbecco modified Eagle's medium (DMEM) (SH30021, Beijing North TZ-Biotech Develop Inc., Beijing, China) low-glucose medium containing 0.15% collagenase, and then transferred to sterile centrifuge tubes. In addition, the sections were digested in a constant-temperature electromagnetic stirrer (1008011909, Tianjin Shidanda Trade Inc., Tianjin, China) at 37°C for 30 min, centrifuged at 88 g for 5 min with the supernatant discarded. Next, sections were incubated with an appropriate DMEM low-glucose medium containing 10% fetal bovine serum (FBS), followed by centrifugation at 88 g with the precipitate obtained and combined with the medium to prepare a cell suspension. After shaking and mixing, the cell suspension was transferred into a disposable petri dish, and the precipitate was uniformly distributed among the petri dish. The cells were then washed using D-Hank's liquid, treated with 0.25% trypsin containing 0.02% EDTA in a 5% CO₂ incubator (XBQ-3H, Taicang HLD Inc., Jiangsu, China) at 37°C for 7 ~ 9 min, and incubated in medium containing 20% FBS for terminating the digestion. The cells were centrifuged at 88 g for 5 min with the supernatant discarded, resuspended by DMEM culture medium containing 20% FBS, seeded into a new culture dish and cultured in a 5% CO₂ incubator at 37°C, with the medium changed once every two to three days. When cells reached 80% confluence, they were treated with trypsin and observed under the microscope. The cells were purified using a differential adherence method. The purity of these cells was consequently 100% after repeated purification and subculture. ASMCs in the 5~8 generation were used for the subsequent experiments. The primary ASMCs were identified using immunocytochemistry staining methods.

Cell grouping and transfection

The ASMCs of mice in the saline and ovalbumin groups were divided into six subgroups: blank group (without any sequence transfection), scrambled group (transfected with scrambled miRNA), miR-485 mimic group (transfected with miR-485 mimic sequence), miR-485 inhibitor group (transfected with miR-485 inhibitor sequence), siRNA-Smurf2 group (transfected with siRNA-Smurf2 sequence) and miR-485 inhibitor + siRNA-Smurf2 group (transfected with miR-485 inhibitor + siRNA-Smurf2). All these sequences were obtained from Shanghai GenScript Co. Ltd. (Shanghai, China). The cells were inoculated in a six-well plate 24 h before transfection, then transfected with the ASMCs immediately via mediation of liposome Lipofectamine2000 (11668, Invitrogen Inc., Carlsbad, CA, USA) when cell confluence reached approximately 50%. Furthermore, 250 μ l serum-free Opti-MEM culture medium (31985-070, Shanghai Boyaobio Biomart Inc., Shanghai, China) was used to dilute 100 pmol of miR-485 mimic, miR-485 inhibitor, siRNA-Smurf2, miR-485 inhibitor + siRNA-Smurf2 and NC (final concentration of 50 nM), mixed and incubated at room temperature for 5 min. Meanwhile, another 250 μ l serum-free Opti-MEM culture medium was used to dilute 5 μ l of Lipofectamine2000, mixed and incubated at room temperature for 5 min. The above two samples were mixed together, incubated at room temperature for 20 min and then added to cell culture wells at 37°C with 5% CO₂. After incubation for 6~8 h, the complete medium was changed, and the subsequent experiments were continued for cell culture for 24~48 h.

Dual-luciferase reporter gene assay

The biological prediction websites microRNA.org (<http://www.microrna.org/microrna/getGeneForm.do>) and mirdb.org (<http://mirdb.org/>) were used to analyze the target gene of miR-485 and verify whether Smurf2 was a direct target gene of miR-485. Dual-luciferase reporter gene assays were performed to verify whether Smurf2 was a direct target gene of miR-485. The artificial synthesized Smurf2 3'UTR gene segment was introduced into the pMIR-reporter (VECT76543, Beijing Huayueyang Biotechnology Inc., Beijing, China) via the restriction sites SpeI and Hind III. The mutated complementary sequences of the seed sequence were designed in wild-type (WT) Smurf2, and the target segment was inserted into the pMIR-reporter reporter vector via T4 DNA ligase through restriction endonuclease digestion. Then, the luciferase reporter plasmids with WT and mutation type (MUT) sequences shown to be correct with sequencing were co-transfected with miR-485 separately into HEK-293T cells (K1727, Shanghai Bienuobio Inc., Shanghai, China). Moreover, after transfection for 48 h, the cells were collected and split, and the luciferase activity was tested by a firefly luciferase assay kit (K801-200, Biovision, CA, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

When cell confluence reached 80% after transfection, the cells were washed twice using PBS, treated with 0.25% trypsin and made into a single cell suspension. A total of $3 \times 10^3 \sim 6 \times 10^3$ cells were inoculated in each well of the 96-well plate (0.2 ml/well), and 6 replicates were used. Next, the cells were placed in an incubator; followed by removal of the plate at 24 h, 48 h, and 72 h. The cells were then cultured with 100 μ l MTT liquid (5 g/l) (GD-Y1317, Shguduo Biomart Inc., Shanghai, China) for another 4 h with the supernatant discarded. Each well was mixed with 100 μ l methyl sulfoxide (DMSO) (D5879-100ML, Sigma-Aldrich Chemical Company, St Louis MO, USA), gently oscillated and mixed for 10 min to dissolve the formazan crystals produced by living cells. A microplate reader (BS-1101, Detie Inc., Jiangsu, China) was used to detect the values at 590 nm of each well. Each experiment was repeated 3 times, and a cell viability curve was drawn with the time point as the X-axis and the OD value as Y-axis.

Flow cytometry

A propidium iodide (PI) single staining method was performed to detect the cell cycle distribution: cells were collected 48 h after transfection, washed 3 times with cold PBS, and centrifuged with supernatant discarded. The cells were re-suspended with PBS again, with the cell concentration adjusted to approximately 1×10^5 cells/ml, fixed with 70% pre-cooling ice ethanol at -20°C , and incubated at -20°C overnight. The cells were centrifuged (800 g) at 4°C with the supernatant discarded and resuspended with PBS containing 1% FBS 2 times. Furthermore, cells were resuspended by 400 μ l binding buffer, followed by adding 50 μ l RNase A (R4875, Shanghai Via-genepbio Technologies Inc., Shanghai, China) and incubating at 37°C for 30 min. Then, cells were mixed with 50 μ l PI (50 mg/l) (GK360, Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) in dark condition and incubated at room temperature for 30 min, with the cell cycle detected by flow cytometry. The method of judging the distribution of cells in different phases was as follows: the PI staining in flow cytometry was used to detect the DNA content in cells, and the phases of the cell cycle were G1/G0, S and G2/M phases. Cells in the G1/G0 phase had the DNA content of diploid cells, cells in the G2/M phase had the DNA content of tetraploid cell and cells in the S phase had DNA content between diploid and tetraploid. Each experiment was repeated 3 times.

Annexin V-FITC/PI two-parameter staining was conducted for detection of cell apoptosis: after 48 h of transfection, cells were treated with trypsin with ethylenediamine tetraacetic acid (EDTA), collected in flow tubes, and centrifuged with supernatant discarded. Furthermore, the cells were washed 3 times with cold PBS and centrifuged with supernatant discarded. Next, Annexin-V-FITC/PI was prepared by mixing 5 μ l of FITC and 5 μ l of PI, which was incubated at room temperature for 15 min away from light. The cells were inoculated in 96-well cell culture plates (2×10^5 cells for each), while the blank, scrambled and experiment groups were transfected with miR-485 (100 nmol/l) were transfected. According to the instructions of the cell apoptosis assay kit (Annexin V-FITC Apoptosis Detection Kit-I, 556547, Becton, Dickinson and Company, NJ, USA), the cells were suspended again by 500 μ l binding buffer, and flow cytometry was used to detect cell apoptosis. Each experiment was repeated 3 times.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (T06ES60, Yeasen Biotechnology Co., Ltd., Shanghai, China) was used to extract the total RNA from cells (ASM cells in each group after transfection) and tissues (the lower lobe of right lung), and the concentration and purity of RNA were determined. According to the instructions of the reverse transcription kit (DT-101, TaKaRa Biotechnology Ltd., Dalian, Liaoning, China), the sample RNA was reverse transcribed into cDNA with a total system of 10 μ l. The obtained cDNA was diluted by 65 μ l diethypyrocarbonate (DEPC) and fully mixed. The real-time PCR reaction system included 5 μ l SsoFast EvaGreen Supermix (1708882, BioLabs, Beverly, MA, USA), 0.5 μ l Forward primer (10 μ M), 0.5 μ l Reverse primer (10 μ M), and 4 μ l cDNA. The amplification condition for RT-qPCR was as follow: pre-denaturation at 95°C for 10 s and 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s and extension at 60°C for 30 s. Primers were synthesized by Shanghai Biological Engineering Co., Ltd. (Shanghai, China) (Table 1). A 2 μ g total RNA sample was used as the template, and U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal references. The $2^{-\Delta\Delta\text{Ct}}$ method was performed to calculate the relative mRNA expression of target genes (miR-485, Smurf2, TGF- β and Smads), and the formula was as follows: $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{experimental group}} - \Delta\text{Ct}_{\text{normal group}}$, $\Delta\text{Ct} = \text{Ct}_{\text{(target gene)}} - \text{Ct}_{\text{(internal reference)}}$, the mRNA transcription expression of target genes = $2^{-\Delta\Delta\text{Ct}}$ [22]. The total RNA of the ASM cells of mice in each group after transfection was collected and incubated for 48 h, and the total RNA of the cells was extracted.

Mice with chronic asthma have increased BALF, eosinophils, monocytes and lymphocytes as well as increased Penh values

The results of the total number of BALF cells and the differential white blood cell count showed that in the saline group, the BALF consisted mostly of monocytes. In the ovalbumin group, the number of BALF cells, eosinophils, monocytes and lymphocytes were higher than that in the saline group ($p < 0.05$) (Fig. 1A). The mice were stimulated with various concentrations of methacholine. The changes in the Penh values of the mice in the saline and ovalbumin groups are shown in Fig. 1B. Compared with the saline group, the airway reactivity of the ovalbumin group significantly increased ($p < 0.05$). These results indicated that chronic asthmatic mice showed more BALF and leucocytes with increased Penh values.

Mice with chronic asthma show severe lesions in airways and more deposited airway collagen fiber

HE and Masson staining methods were used to assess conventional lung tissue sections, and a light microscope was used for observation purposes. The results of HE staining are illustrated in Fig. 2A, indicating that the mouse airways of the saline group did not display any significant changes or notable inflammatory cell infiltration. However, the airway wall and airway smooth muscle of the mice in the ovalbumin group showed remarkable thickening, broadened strata submucosum, mucosal epithelial hyperplasia, and increased mucus secretion ($p < 0.05$).

The results of the Masson staining showed that the collagen fiber was stained blue, muscle-fiber cytoplasm was red, and the nuclei were blue-brown. After Masson staining of the lung tissues, image analysis software was used to analyze various factors, and the measured values were standardized by using the perimeter of the basement membrane (Pbm). These values are represented as the density of the airway wall thickness (Wat/Pbm, $\mu\text{m}^2/\mu\text{m}$), smooth muscle layer (Wam/Pbm, $\mu\text{m}^2/\mu\text{m}$), thickness of the inside airway wall (Wai/Pbm, $\mu\text{m}^2/\mu\text{m}$) and thickness of collagen deposition under the basement membrane (Wcol/Pbm, $\mu\text{m}^2/\mu\text{m}$). The results of Masson staining are further shown in Fig. 2B, revealing that compared with mice in the saline group, the mice in the ovalbumin group exhibited significantly increased deposition of collagen fiber ($p < 0.05$). Compared with the mice in the saline group, Wat/Pbm, Wam/Pbm, Wai/Pbm and Wcol/Pbm were significantly elevated in mice in the ovalbumin group ($p < 0.05$) (Fig. 2C). The findings above suggested that chronic asthmatic mice showed pathological changes in airways and increased deposition of airway collagen fiber.

Chronic asthmatic mice show higher positive expression rates of Smurf2 in ASMCs

Immunohistochemistry was conducted to examine the expression of Smurf2 in ASMCs of mice in the saline group and the ovalbumin group. The sections were stained and then observed. The results indicated that Smurf2 displayed weakened positive expression

Fig. 1. Increased numbers of BALF cells and white blood cells and decreased lung function are found in chronic asthmatic mice. A: the total number of BALF cells and the differential white blood cell count; B: airway reactivity curves of mice in the saline and ovalbumin groups. *, $p < 0.05$ compared with the saline group (N = 10). BALF, bronchoalveolar lavage fluid.

Fig. 2. Mice with chronic asthma show severe lesions in airways and increased airway collagen fiber. A: HE staining ($\times 200$) for pathological features of lung tissue of mice in the saline and ovalbumin groups; B: Masson staining ($\times 100$) for pathological features of lung tissue of mice in the saline and ovalbumin groups; C: Masson staining for detecting the conditions of Wat/Pbm, Wam/Pbm, and Wcol/Pbm; *, $p < 0.05$ compared with the saline group, ($N = 10$); bronchial wall thickness (Wat/Pbm, $\mu\text{m}^2/\mu\text{m}$), smooth muscle thickness (Wam/Pbm, $\mu\text{m}^2/\mu\text{m}$), area of the inside wall (Wcol/Pbm, $\mu\text{m}^2/\mu\text{m}$), and thickness of collagen deposition under the basement membrane (Wcol/Pbm, $\mu\text{m}^2/\mu\text{m}$); hematoxylin-eosin.

Fig. 3. Higher positive expression rates of Smurf2 are found in ASM cells of chronic asthmatic mice. A: microscopic examination of immunohistochemistry ($\times 100$); B: positive expression rate of Smurf2; *, $p < 0.05$ compared with the saline group ($N = 10$). Smurf2, Smad ubiquitin regulatory factor 2.

lighter expression in the ASM cells of mice in the saline group. However, the mice in the ovalbumin group had high positive expression of Smurf2 with brown granules in the ASM cells. These results indicated that the positive expression of the Smurf2 in ASM cells was obviously higher in the ovalbumin group than the saline group ($p < 0.05$) (Fig. 3).

Chronic asthmatic mice show aggravated inflammation

The levels of inflammatory factors, namely, TGF- β 1, IL-4, IL-5, IL-13 and IL-17, in the BALF of mice in the saline and ovalbumin groups were detected by ELISAs. The results demonstrated that the levels of TGF- β 1, IL-4, IL-5, IL-13 and IL-17 in the ovalbumin group had significantly increased in comparison with the saline group ($p < 0.05$). Thus, the results indicated that chronic asthma promoted inflammation (Fig. 4).

Reduction of miR-485 expression and increased expression levels of Smurf2, α -SMA, TGF- β 1 and Smad3 are found in chronic asthma mice

The miR-485 expression and both the mRNA and protein expression levels of Smurf2, α -SMA, TGF- β 1 and Smad3 in airway tissues of mice in the saline and ovalbumin groups were detected by RT-qPCR and western blot analysis. Compared with the saline group, the mice in the ovalbumin group displayed remarkably reduced levels of miR-485 expression, while exhibiting significantly increased mRNA and protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 ($p < 0.05$) (Fig. 5). Therefore, chronic asthmatic mice showed lower expression of miR-485 but higher Smurf2, α -SMA, TGF- β 1 and Smad3 levels in airway tissues.

Smurf2 is verified as the target gene of the miR-485

According to the analysis of the online software microRNA.org and miRbase, there was a specific binding region between the Smurf2 sequence and miR-485. The specific binding region in the 3'UTR between the Smurf2 sequence and the miR-485 sequence is shown in Fig. 6A. Smurf2 was confirmed to be the target gene of miR-485 by dual luciferase reporter gene assays (Fig. 6B). Compared with the NC group, the luciferase activity of the 3'UTR of the Smurf2 WT was inhibited by miR-485 ($p < 0.05$), but this was not the case for the luciferase activity of the mutant 3'UTR, suggesting that miR-485 could bind specifically with the Smurf2-3'UTR.

Fig. 4. The levels of TGF- β 1, IL-4, IL-5, IL-13 and IL-17 are increased in mice with chronic asthma. *, $p < 0.05$ compared with the saline group (N = 10); TGF- β 1, transforming growth factor-beta-1; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; IL-17, interleukin-17; ELISA, enzyme-linked immunosorbent assay.

Fig. 5. The miR-485 expression is reduced, but the mRNA and protein levels of Smurf2, α -SMA, TGF- β 1 and Smad3 are increased in airway tissues of chronic asthma mice. A: the miR-485 expression and mRNA levels of Smurf2, α -SMA, TGF- β 1 and Smad3 of airway tissues in the saline and ovalbumin groups detected by the RT-qPCR; B: the protein levels of Smurf2, α -SMA, TGF- β 1 and Smad3 of airway tissues in the saline and ovalbumin groups detected by western blot analysis; C: the protein band images examined by western blot analysis; *, $p < 0.05$ compared with the saline group (N = 10); miR-485, microRNA-485; Smurf2: Smad ubiquitin regulatory factor 2; α -SMA, alpha smooth muscle actin; TGF- β 1, transforming growth factor-beta-1; RT-qPCR, reverse transcription quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Smads, mothers against decapentaplegic homolog.

Morphology and identification of ASMCs

The results showed that under an optical microscope (Fig. 7A), the ASMCs cultured *in vitro* were spindle-shaped, with 1~2 nuclei located in the center of the cell. Cell growth resulted in a bundle arrangement in a region, forming morphological features with a characteristic peak shape. The results of α -SMA immunohistochemical staining revealed that the brown yellow color observed was indicative of the positive α -SMA in cytoplasm. The blue color indicated positive staining of hematoxylin in the nucleus under an optical microscope (Fig. 7B). The blue cytoplasm of all the nuclei indicated positive staining for α -SMA, which was largely suggestive of the presence of ASMCs.

miR-485 overexpression and siRNA-Smurf2 inhibit mouse ASMC proliferation *in vitro*

MTT assays were used to detect the effects of different groups, and the effect of time on cell proliferation ability (Fig. 8). In the ASMCs of mice in the normal and asthmatic mice, when compared with the blank group, cell proliferation after transfection for 48 h and 72 h was clearly inhibited in the miR-485 mimic and siRNA-Smurf2 groups (all $p < 0.05$), while the miR-485 inhibitor group

Fig. 6. Smurf2 is the target gene of miR-485. A: the predicted binding site of Smurf2 3'UTR in the miR-485; B: luciferase activity detection by dual-luciferase reporter gene assays; *, $p < 0.05$ compared with the NC group; the experiment was repeated 3 times. NC, negative control; miR-485 mimic, microRNA-485 mimic; WT, wild-type Smurf2; MUT, mutant Smurf2, Smad ubiquitin regulatory factor 2.

Fig. 7. Morphology and identification of ASMCs under an optical microscope. A: ASMCs cultured in vitro under an optical microscope ($\times 200$). B: ASMC immunohistochemical staining under an optical microscope ($\times 200$). ASMCs, airway smooth muscle cells.

Fig. 8. MiR-485 overexpression and siRNA-Smurf2 inhibit mouse ASMC proliferation in vitro. A: cell proliferation of ASMCs of normal mice in each group; B: cell proliferation of ASMCs of asthmatic mice in each group; *, $p < 0.05$ compared with the blank group; #, $p < 0.05$ compared with the siRNA-Smurf2 group; the experiment was repeated 3 times. ASMCs, airway smooth muscle cells; Smurf2, Smad ubiquitin regulatory factor 2.

showed increased cell proliferation ($p < 0.05$). There was no statistical significance of cell proliferation in the scrambled and miR-485 inhibitor + siRNA-Smurf2 groups ($p > 0.05$). Compared with the siRNA-Smurf2 group, the miR-485 inhibitor + siRNA-Smurf2 group showed increased cell proliferation ($p < 0.05$). Overall, the inhibition of miR-485 expression promoted the cell proliferation of mouse ASMCs *in vitro*, and miR-485 overexpression and siRNA-Smurf2 inhibited mouse ASMC proliferation *in vitro*.

miR-485 overexpression and siRNA-Smurf2 arrest cells at G1 phase and promote apoptosis of ASMCs in mice

PI single staining was used to detect the cell cycle distribution of ASMCs of the normal and asthmatic mice after transfection. The results are shown in Fig. 9. In the ASMCs of normal and asthmic mice, compared with the control group, cells in the G1 phase were significantly reduced, while cells in the S phase clearly increased in the blank, miR-485 mimic, miR-485 inhibitor, siRNA-Smurf2 and miR-485 inhibitor + siRNA-Smurf2 group ($p < 0.05$). Compared with the blank group, the cell growth was arrested in the blank, and

Fig. 9. Overexpression of miR-485 arrests cells at G1 phase of ASMCs in mice. A, B: the cell cycle proportion of ASMCs of normal mice in each group; C, D: the cell cycle proportion of ASMCs of asthmatic mice in each group; *, $p < 0.05$ compared with the blank group; #, $p < 0.05$ compared with the siRNA-Smurf2 group. ASMCs, airway smooth muscle cells; Smurf2, Smad ubiquitin regulatory factor 2.

increased cells were in the G1 phase in the miR-485 mimic and the siRNA-Smurf2 groups; furthermore, a reduction of cells in the S phase and inhibited cell proliferation of ASMCs were observed in the miR-485 mimic as well as the siRNA-Smurf2 groups ($p < 0.05$); the cells in the G1 phase decreased but those in the S phase increased in the miR-485 inhibitor group (all $p < 0.05$), and the scrambled and miR-485 inhibitor + siRNA-Smurf2 groups showed no significant differences ($p > 0.05$). No obvious difference was found at G2 phase in each group ($p > 0.05$). Compared with the siRNA-Smurf2 group, the miR-485 inhibitor + siRNA-Smurf2 group showed a decrease in the cells at the G1 phase, while in contrast, the cells in the S phase had increased (all $p < 0.05$).

Annexin V/PI double staining was used to detect the apoptosis of ASMCs of normal and asthmatic mice after transfection. The results are shown in Fig. 10. In the ASMCs of normal and asthmatic mice, compared with the blank group, the cell apoptosis rate in the miR-485 inhibitor group was reduced significantly (all $p < 0.05$). The cell apoptosis rate in the miR-485 mimic and siRNA-Smurf2 groups increased remarkably ($p < 0.05$), while the

Fig. 10. Overexpression of miR-485 promotes apoptosis of ASMCs in mice. A, B:: the cell apoptosis of ASMCs of normal mice in each group; C, D: the cell apoptosis of ASMCs of asthmatic mice in each group; *, $p < 0.05$ compared with the blank group; #, $p < 0.05$ compared with the siRNA-Smurf2 group. ASMCs, airway smooth muscle cells; Smurf2, Smad ubiquitin regulatory factor 2.

scrambled and miR-485 inhibitor + siRNA-Smurf2 groups showed no significant differences in the apoptosis rates ($p > 0.05$). Compared with the siRNA-Smurf2 group, the miR-485 inhibitor + siRNA-Smurf2 group showed decreased cell apoptosis ($p < 0.05$). The results showed that inhibition of miR-485 expression could in turn inhibit apoptosis of ASMCs in mice. Additionally, overexpression of miR-485 and siRNA-Smurf2 could promote apoptosis of ASMCs in mice.

The mRNA and protein expression levels of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs are decreased by overexpressed miR-485

RT-qPCR was used to detect the miR-485 expression and mRNA expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in each group after ASMC transfection. The results (Fig. 11A, B) that in the ASMCs of both the normal and asthmatic mice, compared with the

Fig. 11. Overexpressed miR-485 decreases the mRNA and protein levels of Smurf2, α -SMA, TGF- β 1 and Smad3 in the ASMCs. A: the miR-485 expression and mRNA levels of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of normal mice in each group; B: the miR-485 expression and mRNA levels of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of asthmatic mice in each group; C: protein bands of each factor in ASMCs of normal mice in each group detected by western blot analysis; D: protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of normal mice in each group detected by western blot analysis; E: protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of asthmatic mice in each group detected by western blot analysis; F: the protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of asthmatic mice in each group.

group. *, $p < 0.05$ compared with the blank group; #, $p < 0.05$ compared with the siRNA-Smurf2 group. The experiment was repeated 3 times. RT-qPCR, reverse transcription quantitative polymerase chain reaction; ASMCs, airway smooth muscle cells; Smurf2, Smad ubiquitin regulatory factor 2; miR-485, microRNA-485.

blank group, miR-485 expression was significantly lower, while the mRNA expression of Smurf2, α -SMA, TGF- β 1 and Smad3 were all higher in the miR-485 inhibitor group (all $p < 0.05$). The miR-485 expression was significantly higher, while the mRNA expression of Smurf2, α -SMA, TGF- β 1 and Smad3 were all lower in the miR-485 mimic and siRNA-Smurf2 groups compared with the blank group (all $p < 0.05$). Compared with the siRNA-Smurf2 group, miR-485 expression was significantly lower, while the mRNA expression of Smurf2, α -SMA, TGF- β 1 and Smad3 were higher in the miR-485 inhibitor + siRNA-Smurf2 group (all $p < 0.05$). These results revealed that overexpressed miR-485 decreased the mRNA expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of mice.

Western blot analysis was used to detect the protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in each group after ASMC transfection. The results suggested (Fig. 1F) that in the ASMCs of both the normal and asthmatic mice, compared with the blank group, the protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 were all higher in the miR-485 inhibitor group (all $p < 0.05$). The protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 were all lower in the miR-485 mimic and siRNA-Smurf2 groups compared with the blank group (all $p < 0.05$). Compared with the siRNA-Smurf2 group, the protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 were higher in the miR-485 inhibitor + siRNA-Smurf2 group (all $p < 0.05$). These results revealed that overexpressed miR-485 decreased the protein expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in ASMCs of mice.

Discussion

Asthma is a long-term respiratory condition characterized by bronchoconstriction, which actively acts to damage the airways over time [23]. Bronchial asthma usually remains uncontrolled even with treatment with long-acting beta (2)-agonists or both, and additional treatments are ultimately required [24]. Although tremendous efforts have been made to improve asthma therapy, due to the heterogeneity and complexity of asthma, effectively controlling asthma is difficult [25]. These findings suggest that more effective treatments are urgently needed. Previous studies have revealed miRNAs to be associated with chronic asthma and other human diseases, such as colonic Crohn's Disease [26-29]. Thus, the central objective of this study was to elucidate the mechanisms involved in miR-285 regulation of chronic asthma, and we found that overexpressed miR-485 could inhibit the proliferation and promote apoptosis of ASMCs in mice with chronic asthma through suppression of the Smurf2-dependent TGF- β /Smads signaling pathway.

Initial observations indicated significant increases in the expression levels of TGF- β 1, IL-4, IL-5, IL-13 and IL-17 in mice with chronic asthma. It has been widely demonstrated that the pathogenic features of asthma are comprised of airway remodeling, intense eosinophilic infiltration, and airway hyperresponsiveness mediated via Th2 cytokines, including IL-4, IL-5, and IL-13, from inflammatory mediators in the activated mast cells [30]. Furthermore, TGF- β 1, IL-4 and IL-13 may also share a correlation with the development and susceptibility of asthma within the Chinese population [31]. Furthermore, increased serum IL-17 acts as an independent risk factor in cases of severe asthma [32]. A novel E3 ubiquitin ligase, as shown by light interference microscopy, was found to directly bind to Smurf2, thus promoting TGF- β expression in osteosarcoma (U2OS) cells [33]. Based on the aforementioned information discussed, we have ultimately deduced that the high levels of TGF- β 1, IL-4, IL-5, IL-13 and IL-17 indicate a successfully established ovalbumin group.

Compared with the blank and NC groups, the miR-485 inhibitor group exhibited decreased levels of miR-485 expression as well as enhanced mRNA and protein levels of Smurf2, α -SMA, TGF- β 1 and Smad3, while contrary results were observed in the miR-485 mimic and siRNA-Smurf2 groups, indicating miR-485 suppressed the expression of Smurf2, α -SMA, TGF- β 1 and Smad3 in chronic asthma. Smurf2 is comprised of multiple WW domains that could interact with the Smad7 molecule and regulate TGF- β cytokine signaling [34]. A previous study demonstrated that Smurf2 could be inhibited by miRNA [18]. Additionally,

the α -SMA expression in asthma was evidently increased by a Nuclear Factor- κ B blocker, which also promoted apoptosis and coordinated cell proliferation of the airway smooth muscle in mice with asthma [35]. Additionally, TGF- β 1 is one of several candidate loci in the pathogenesis of asthma in addition to being highly polymorphic. The connection between the T869C and C-509T polymorphisms of the TGF- β 1 gene may be a risk factor of asthma [36]. TGF- β 1 expression displayed increased levels in mice with asthma, while a correlation was detected between the higher TGF- β 1 ratio in the chronic group and higher collagen levels [37]. Importantly, a former study revealed that TGF β 1-induced cell proliferation was impeded by up-regulated miR-29 [38]. Smad3, one of the susceptibility genes in asthma, is as a single pathway for asthma vulnerability that influences the airway epithelial sensitivity for TGF- β [39]. Furthermore, the Smad protein plays an important role in regulating intracellular mediators for TGF- β 1 signaling. Smurf2, an ubiquitin ligase for Smad, also plays a vital role in the adjustment of TGF- β 1/Smad signaling [40]. Smad3 was found to be negatively regulated by overexpression of miRNA [41]. As reflected in this study, miR-485 overexpression effectively inhibited the expression levels of Smurf2, α -SMA, p-Smad1 and Smad3.

Furthermore, our study clarified that compared with the control group, the miR-485 inhibitor group showed significantly increased cell proliferation, while the apoptosis rates showed drastically reduced levels. The results indicated that miR-485 inhibits cell proliferation and promotes the rate of cell apoptosis. Moreover, including miR-485-3p, mapped to the 14q32.31 locus have been reported to modulate migration, proliferation, and apoptosis, as well as the invasion of metastatic prostate cancer cells [42]. MiR-485-5p has been shown to negatively regulate the progression of gastric cancer through modulating flotillin-1 [43]. Meanwhile, it also was found to suppress cell invasion and proliferation in hepatocellular carcinomas through regulation of calmodulin 2 [44]. Moreover, miR-29b was reported to have a suppressive effect on myofibroblastic stellate cell activation, including crosstalk with TGF- β 1/Smad3 [45]. The inhibitory role of miR-485 in cell proliferation was formerly reported [46]. A previous study revealed that miR-485 regulates host and influenza virus transcripts to control viral replication and target antiviral immunity [47]. In addition, the dual-luciferase reporter assay in our study also confirmed Smurf2 as the target gene of miR-485. Similarly, miR-485 was found to suppress ASMC proliferation through inhibiting the GF β R2/p-Smad3 pathway [48]. Thus, overexpressed miR-485 suppresses cell proliferation and promotes cell apoptosis of ASMCs in mice with chronic asthma.

Conclusion

In conclusion, the present study investigated whether miR-485 was down-regulated specifically in chronic asthma and whether it participated in the regulation of cell apoptosis and proliferation. We determined that this molecule was closely linked with the development of chronic asthma. Our study demonstrated that miR-485 suppressed cell proliferation and promoted cell apoptosis by targeting Smurf2 through the TGF- β /Smads signaling pathway in mice with chronic asthma, suggesting that miR-485 could be a new underlying target for the treatment of chronic asthma. However, there were still some limitations in this study as a result of limited data and the experimental environment. Future experiments and deeper analysis will be conducted to further substantiate the key findings of this study.

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Disclosure Statement

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References

- 1 Yu M, Eckart MR, Morgan AA, Mukai K, Butte AJ, Tsai M, Galli SJ: Identification of an IFN-gamma/mast axis in a mouse model of chronic asthma. *J Clin Invest* 2011;121:3133-3143.
- 2 Cates C: Chronic asthma. *BMJ* 2001;323:976-979.
- 3 Garbacki N, Di Valentin E, Huynh-Thu VA, Geurts P, Irrthum A, Crahay C, Arnould T, Derom C, Fardoux J, Cataldo D, Colige A: MicroRNAs profiling in murine models of acute and chronic asthma: identification of miR-181 as a novel miRNAs targets. *PLoS One* 2011;6:e16509.
- 4 Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, Jr DAR, Castro M, Curran-Evelyn D, Fitzpatrick AM: Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010;181:315-323.
- 5 Carr TF, Kraft M: Chronic Infection and Severe Asthma. *Immunology Reviews* 2016;36:483-502.
- 6 Falk NP, Hughes SW, Rodgers BC: Medications for Chronic Asthma. *Curr Fam Pract* 2016;94:454-462.
- 7 Kew KM, Undela K, Kotorts I, Ferrara G: Macrolides for chronic asthma. *Cochrane Database Syst Rev* 2015;10.1002/14651858.CD002997.pub4CD002997.
- 8 Smith AD, Cowan JO, Brassett KP, Herbison GP, Taylor TH: Use of exhaled carbon dioxide measurements to guide treatment in chronic asthma. *N Engl J Med* 2005;353:2163-2173.
- 9 Wang JW, Li K, Hellermann G, Lockey RF, Mohapatra S, Mohapatra S: Regulating the Regulators: microRNA and Asthma. *World Allergy Organ J* 2011;4:94-103.
- 10 Canfran-Duque A, Rotllan N, Zhang X, Fernandez-Fernandez R, Ramirez-Hidalgo C, Araldi E, Daimiel L, Busto R, Fernandez-Hernando C, Suarez-Villa LA: miR-21 promotes apoptosis, plaque necrosis, and vascular inflammation during atherosclerosis. *EMBO Mol Med* 2017;9:1244-1262.
- 11 Chen LJ, Li XY, Zhao YQ, Liu W, Wu L, Li Z, Wu HB: Down-regulated microRNA-375 expression as a predictive biomarker in non-small cell lung cancer brain metastasis and its prognostic significance. *Pathol Res Pract* 2017;213:882.
- 12 Collison A, Herbert C, Simpson JS, Mattes JP, Oster PS, Kumar RK: Altered expression of microRNA in the airway wall in chronic asthma: miR-126 as a potential therapeutic target. *BMC Pulm Med* 2011;11:29.
- 13 Pietrusinska M, Paialek A, Chojak P, Szemraj J, Gozdzińska-Nielepkowicz A, Pietras T: Preliminary studies: differential microRNA expression in asthma and chronic obstructive pulmonary disease. *Postepy Dermatol Alergol* 2017;37:276-280.
- 14 Pua HH, Insel PA: MicroRNA regulation of allergic inflammation and asthma. *Curr Opin Immunol* 2017;80:101-106.
- 15 Ma H, Zhou H, Luo Y, Guo S, Song C: Mir-20b-Induced Increase in Myeloid-Derived Suppressor Cells in the Lungs of Mice with Chronic Asthma. *Ann Clin Lab Sci* 2017;47:76-82.
- 16 Bayarritz A, Bandala C, Perezsantos JL: miR-485 acts as a tumor suppressor by inhibiting cell growth and invasion in breast carcinoma T47D cells. *Asian Pac J Cancer Prev* 2013;14:3757-3760.
- 17 Lou C, Xiao M, Cheng S, Lu X, Jia S, Ren Y, Li Z: MiR-485-3p and miR-485-5p suppress breast cancer cell metastasis by inhibiting PGC-1alpha expression. *Cell Death Dis* 2016;7:e2159.
- 18 Cao S, Xiao L, Rao JN, Zou T, Liu L, Zhang D, Turner DJ, Gorospe M, Wang JY: Inhibition of Smurf2 translation by miR-322/503 modulates TGF-beta/Smad2 signaling and intestinal epithelial homeostasis. *Mol Biol Cell* 2014;25:1234-1243.
- 19 Slattery ML, Lundgreen A, Herrick JS, Wolff RK, Caan BJ: Genetic variation in the transforming growth factor-beta signaling pathway and survival after diagnosis with colon and rectal cancer. *Cancer* 2011;117:4175-4183.
- 20 Qu ZH, Yang ZC, Chen L, Lv ZD, Yi MJ, Ran N: Inhibition airway remodeling and transforming growth factor-beta1/Smad signaling pathway by astragalus extract in asthmatic mice. *Int J Mol Med* 2012;29:564-568.

- 21 Kearley J, Buckland KF, Mathie SA, Lloyd CM: Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* 2009;179:772-781.
- 22 Ayuk SM, Abrahamse H, Houreld NN: The role of photobiomodulation on gene expression of cell adhesion molecules in diabetic wounded fibroblasts *in vitro*. *J Photochem Photobiol B* 2016;161:368-374.
- 23 Chauhan BF, Ducharme FM: Anti-leukotriene agents compared to inhaled corticosteroids in the management of recurrent and/or chronic asthma in adults and children. *Cochrane Database Syst Rev* 2012;CD002314.
- 24 Virchow JC, Mehta A, Ljungblad L, Mitfessel H, group Ms: Add-on montelukast in inadequately controlled asthma patients in a 6-month open-label study: the MONTelukast In Chronic Asthma (MONICA) study. *Respir Med* 2010;104:644-651.
- 25 Blasi F, Bettoncelli G, Canonica GW, Centanni S, Crimi N, DiMaria G, Gasparini S, Gentile G, Gino M, Mereu C, Minghetti P, Nardini S, Paggiaro P, Papi A, Pistolesi M, Rossi A: The management of asthma in the phenotype and biomarker era: The proposal of a new diagnostic-therapeutic model. *Respir Med* 2016;53:665-667.
- 26 Wu F, Zhang S, Dassopoulos T, Harris ML, Bayless TM, Meltzer SJ, Brant SR, Kwon J: Identification of microRNAs associated with ileal and colonic Crohn's disease. *Inflamm Bowel Dis* 2015;21:1729-1738.
- 27 Ariel D, Upadhyay D: The role and regulation of microRNAs in asthma. *Respir Med* 2012;106:149-152.
- 28 Chiba Y, Misawa M: MicroRNAs and their therapeutic potential for human disease. MiR-133a and bronchial smooth muscle hyperresponsiveness in asthma. *J Pharmacol Exp Ther* 2010;334:264-268.
- 29 Jiang X: The emerging role of microRNAs in asthma. *Cell Physiol Biochem* 2013;33:35-40.
- 30 Nakajima H, Hirose K: Role of IL-23 and Th17 Cells in Airway Inflammation in Asthma. *Immune Netw* 2010;10:1-4.
- 31 Yang XX, Li FX, Wu YS, Wu D, Tan JY, Li M: Association of TGF- β 1, IL-4 and IL-13 gene polymorphisms with asthma in a Chinese population. *Asian Pac J Allergy Immunol* 2011;29:273-277.
- 32 Agache I, Ciobanu C, Agache C, Anghel M: Circulating serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010;104:1131-1133.
- 33 Huang Y, Yang Y, Gao R, Yang X, Wang J, Wang J, Yu L: RLIM interacts with Smurf2 and promotes TGF-beta induced U2OS cell migration. *Biochem Biophys Res Commun* 2011;414:181-185.
- 34 Ganji A, Roshan HM, Varasteh A, Mousavi SM, Sankian M: The effects of WW2/WW3 domains of Smurf2 molecule on TGF-beta signaling and anaplasia I gene expression. *Cell Biol Int* 2015;39:690-695.
- 35 Qiu C, Zhang J, Su M, Fan J: Nuclear factor-kappaB mediates the phenotype switching of airway smooth muscle cells in a murine model. *Int J Clin Exp Pathol* 2015;8:12115-12128.
- 36 Che Z, Zhu X, Yang Y, Chen L, Cao J, Liang C, Lu Y: The association between the C-509T and T869C polymorphisms of Smad3 gene and the risk of asthma: a meta-analysis. *Hum Immunol* 2014;75:141-150.
- 37 Sturges L, Halder M, Sandgraf RG, Camara NOS, Sogayar MC, Jancar S: Lung Remodeling in a Mouse Model of Asthma Involves a Balance between TGF- β 1 and BMP-7. *Plos One* 2014;9:e95959.
- 38 Wang Y, Chen J, Feng T, Guo Q: MiR-29 mediates TGFbeta 1-induced extracellular matrix synthesis through activation of Wnt/beta -catenin pathway in human pulmonary fibroblasts. *Technol Health Care* 2015;27:119-125.
- 39 Fauria Jellez G, Vandepoele K, Brouwer U, Koning H, Elderman RM, Hackett TL, Willemse BW, Holloway L, Van Roy F, Koppelman GH, Nawijn MC: Protocadherin-1 binds to SMAD3 and suppresses TGF-beta1-induced gene transcription. *Am J Physiol Lung Cell Mol Physiol* 2015;309:L725-735.
- 40 Zhang Z, Finnerty CC, He J, Herndon DN: Smad ubiquitination regulatory factor 2 expression is enhanced in hypertrophic scar fibroblasts from burned children. *Burns* 2012;38:236-246.
- 41 Zhao JL, Zhang L, Guo X, Wang JH, Zhou W, Liu M, Li X, Tang H: miR-212/132 downregulates SMAD2 expression to suppress the G1/S phase transition of the cell cycle and the epithelial to mesenchymal transition in cervical cancer cells. *IUBMB Life* 2015;67:380-394.
- 42 Formosa A, Markert EK, Lena AM, Italiano D, Finazzi-Agro E, Levine AJ, Bernardini S, Garabadgiu AV, Melino G, Candi E: MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32.31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells. *Oncogene* 2014;33:5173-5182.

- 43 Kang M, Ren MP, Zhao L, Li CP, Deng MM: miR-485-5p acts as a negative regulator in gastric cancer progression by targeting flotillin-1. *Am J Transl Res* 2015;7:2212-2222.
- 44 Guo GX, Li QY, Ma WL, Shi ZH, Ren XQ: MicroRNA-485-5p suppresses cell proliferation and invasion in hepatocellular carcinoma by targeting stanniocalcin 2. *Int J Clin Exp Pathol* 2015;8:12292-12299.
- 45 Liang C, Bu S, Fan X: Suppressive effect of microRNA-29b on hepatic stellate cell activation and its crosstalk with TGF-beta1/Smad3. *Cell Biochem Funct* 2016;34:326-333.
- 46 Anaya-Ruiz M, Bandala C, Perez-Santos JL: miR-485 acts as a tumor suppressor by inhibiting cell growth and migration in breast carcinoma T47D cells. *Asian Pac J Cancer Prev* 2013;14:3757-3760.
- 47 Ingle H, Kumar S, Raut AA, Mishra A, Kulkarni DD, Kameyama T, Takaoka A, Akira S, Kumar H: The microRNA miR-485 targets host and influenza virus transcripts to regulate antiviral immunity and restrict viral replication. *Sci Signal* 2015;8:ra126.
- 48 Chen M, Huang L, Zhang W, Shi J, Lin X, Lv Z, Zhang W, Liang R, Jiang S: MiR-23b controls TGF-beta1-induced airway smooth muscle cell proliferation via TGFbetaR2/p-Smad3 signals. *Mol Immunol* 2016;70:84-92.