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Original Article

The increased expression of β2ARs depresses airway remodeling in children with bronchial asthma

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Abstract: Recent studies have shown that beta-2 adrenergic receptors (β2-ARs) are associated with the severity of asthma in children and are involved in the occurrence and development of airway remodeling in asthma. We collected the blood and clinical data of 33 children with bronchial asthma accompanied by airway remodeling and analyzed their changes before and after treatment. After treatment, the expression of β2-ARs in the bronchoalveolar lavage fluid (BALF) of asthmatic children was found to increase, and similar trends also occurred in the ratio of forced expiratory volume (FEV1), peak expiratory flow (PEF) and FEV1 to forced vital capacity (FVC) increased significantly in one second, and the reticular basement membrane thickness (RBMT) and fibroblasts count (FsC). However, declining trends also occur in the serum levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). This study suggests that β2-ARs are highly likely to be associated with asthma. Their reduced concentration may lead to airway remodeling.

Keywords: Children asthma, airway remodeling, inflammation, treatment, β2AR smRNA

Introduction

Bronchial asthma (asthma) is the most common chronic inflammatory disease of the respiratory tract in childhood. It is estimated to have an impact similar to other major chronic diseases such as diabetes or Alzheimer disease [1]. Notably, asthma is induced by both genetic and environmental factors and characterized by airway hyperresponsiveness (AHR) [2]. Chronic inflammation and repeated episodes of bronchial spasms for asthmatic patients cause incomplete airway repair and eventually lead to airway remodeling [2]. Although these characteristics of asthma have been recognized, the mechanism of airway remodeling and its drug therapy is not very clear. Recent studies have shown that β2ARs are involved in asthma severity in school children. Airway hyperresponsiveness in asthmatic patients is caused by a decrease in the density or expression of β2ARs on the airway smooth muscle cells [3]. It seems that β2ARs are concerned with airway remodeling in asthma. In this paper, we highlight the effect of β2ARs on airway remodeling in asthmatic children and reveal that their expression discrepancies before and after inhalation treatments with low-dose budesonide powder might provide a theoretical basis for asthmatic treatment and its reversal of airway remodeling.

Materials and methods

Subjects

We selected 33 children with bronchial asthma in the department of pediatrics from our hospital between March 2015 and March 2017. 19 of the children (57.5%) were newly diagnosed. All the children were treated with low-dose budesonide powder for a long time, and we performed bronchoscopy examinations to confirm airway remodeling. The bronchial asthma was confirmed in line with the diagnostic criteria for pediatric bronchial asthma revised by the subspecialty group of respiratory diseases, the Society of Pediatrics, Chinese Medical Association, in 2008 [4], including recurrences of

respiratory symptoms such as paroxysmal coughing, dyspnea, wheezing and/or AHR, or either reversible or spontaneous airflow obstruction. The exclusion criteria for asthmatic children: insufficiency of the heart, liver, kidneys, or psychosis; also, those who had not used beta 2 receptor agonists. All the participants had no history of autoimmune disease or malignant tumors. This study was approved by the ethics committee of our institutes and received the informed consent of the parents from all the research subjects.

Treatment and examination

All the children were required to have been prescribed budesonide (so as to reflect actual real-world patient usage and not protocoled use) [5], and were treated with a regular inhalation of budesonide for 12 months (200 μg twice daily) and we performed all the operations both before treatment and on the 360th day after treatment. The airway remodeling was confirmed via bronchoscopy examination. The protein expression of β2ARs was detected by western blot analysis. The expressions of β2Ars mRNA in BALF from the asthmatic children were measured using reverse a transcriptase-polymerase chain reaction (RT-PCR).

The total RNAs in BALF were extracted by the Trizol method, and then transcribed to cDNA. After the participants fasted for 12 hours, venous blood was collected from each and placed in two tubes. One was applied to the analysis of the erythrocyte sedimentation rate (ESR) and the peripheral blood eosinophil count, which was used in a PUC-2068A dynamic ESR analyzer (Prang Company, China) and an XE2100D automated hematology analyzer (Sysmex, Japan). The other was centrifuged at a speed of 3000 rotations for 10 min, and the separated serum was stored at -80°C for the determination of the concentrations of IL-6 and TNF-α. These processes used enzyme-linked immunosorbent assay kits according to the manufacturers' instructions (Shanghai Jing Kang Biological Engineering Co., Ltd., China). The total protein immunoglobulin E (IgE) in BALF was measured using a BN-II special protein analyzer (Siemens, Germany) with the immune nephelometry method in accordance with the kit's instructions (Siemens, Germany). The hematoxylin and eosin stain was applied to the analysis of RBMT and FCs in the respiratory tract. The pulmonary function was assessed using the Power Cube (Germany) pulmonary function instrument, including these parameters such as forced expiratory volume in 1 sec (FEV1), the ratio of FEV1 to forced vital capacity (FVC) and peak expiratory flow (PEF).

Bronchoscopy

All the participants were treated with fiberoptic bronchoscopy in a remission period of asthma (more than a week from the last attack). The fiberoptic bronchoscopy (Olympus, Tokyo, Japan) was performed with preoperative intravenous atropine and preoperative local anesthesia in the upper airway for 30 minutes. Specimens of 3 mucous segments from each patient's right main bronchus, middle lobe bronchus, and lower lobe bronchus were collected for observation using hematoxylin-eosin staining (HE staining). Bronchoalveolar lavage fluid (BALF) was centrifuged at a speed of 3000 turns for 10 minutes at 4°C, and the severed supernatant was stored at -70°C. HE staining was used in the mucous membrane specimens. The reticular basement membrane thickness was obtained from the average thickness of 3 mucous membranes from each patient, which was measured with a micrometer under an optical microscope with a magnification of 1000×, combining a range from the basement of the bronchial epithelium to its outer edge of the reticular layer. Next, the number of upper subcutaneous fibroblasts from air tubes for asthmatic children was counted in 5 views of an oil immersion microscope. The reticular basement membrane thickness and the number of upper subcutaneous fibroblasts were treated as airway remodeling parameters.

RT-PCR

RT-PCR was carried out according to the TaqMall RNA Reverse Transcripts Kit's operation manual. β-actin was used as an internal control, and the designed primers were applied using Primer5 software. The primer sequences for β2AR mRNA were as follows: justice chain, 5'-CCTCCTTCTTGCCTATCCA-3', antisense chain, 5'-TAGGTTTTCGAAGAAGACCG-3'. The length of the amplified product was 120 bp. The primer sequences for β-αctin were as follows: justice chain, 5'-GGCACTGGGGCTTCATCTGAC-3', antisense chain, 5'-GCCTTCCATCCCTTTGCTTAG-3'. The length was 115 bp. The PCR reaction systems were as follows: 10 μL of Taqman PCR

Table 1. Comparisons of clinical and laboratory characteristics between the pre-treatment and post-treatment groups ($\bar{x}$ ± SD)

Items	*Pre-treatment group*	*Post-treatment group*	*P-value*
Age (year, mean ± SD)	8.7 ± 1.2	8.9 ± 1.0	0.563
Male gender	16 (48.5%)	17 (51.5%)	0.976
Duration of asthma (years)	2.3 ± 1.2	2.4 ± 1.3	0.863
Allergic history	17 (51.5%)	15 (45.4)	0.772
Total IgE (IU/ml)	323.8 ± 136.9	179.0 ± 79.3	< 0.001
ESR (mm/h)	17.3 ± 8.7	15.9 ± 7.1	0.067
Peripheral blood eosinophil (10^9/L)	0.32 ± 0.15	0.15 ± 0.07	0.010

IgE: immunoglobulin E; ESR: erythrocyte sedimentation rate.

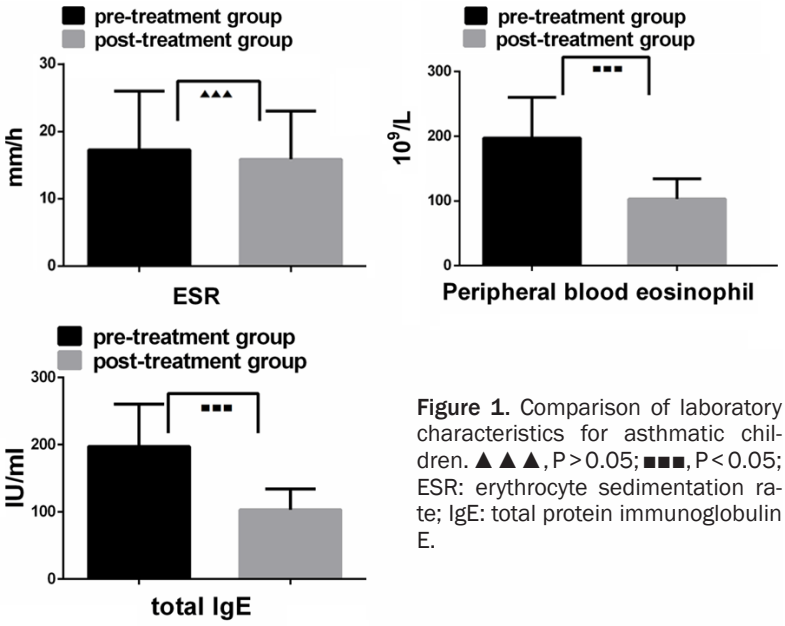

Figure 1. Comparison of laboratory characteristics for asthmatic children. ▲▲▲, P > 0.05; ■■■, P < 0.05; ESR: erythrocyte sedimentation rate; IgE: total protein immunoglobulin E.

premix products, 1.33 μL of cDNA, 7.67 μL of water. The reaction conditions were as follows: degeneration at 95°C for 10 min, annealing at 60°C for 1 min, and the extension at 60°C for 1 min. The above operation was repeated for 50 cycles and terminated at 4°C. The amplified products were subjected to electrophoresis, following further analysis and calculation through an image processing system to obtain the optical density of β2Ars mRNA and β-actin. With the optical density of β-actin as a reference, the change values (ΔCT) in the optical density of β2-Ars mRNA were gained, and their relative expressions were gained by $2^{-\Delta ct}$.

Western blot

The proteins from the β2ARs (30 μg) were separated on 10% SDS polyacrylamide gels (SDS-PAGE). After electrophoresis, the proteins were transferred to PVDF membrane filters (Millipore Biotechnology, Billerica, MA, USA). The membranes were incubated overnight at 4°C with the appropriate primary antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies in a blocking solution for 1 h at room temperature, and the immunoreactive bands were visualized with a chemiluminescence reagent (ECL, Millipore Biotechnology, Billerica, MA, USA) and quantified using a Bio-Rad imaging system (Bio-Rad Laboratories, Inc, Hertfordshire, UK).

Statistical analysis

The data was analyzed using GraphPad Prism (version 6.01; GraphPad Software, Inc.) and expressed with ($\bar{x}$ ± SD). The expression of β2-AR mRNA, the concentrations of the inflammatory factors, the parameters of lung function and airway remodeling, age, duration of asthma, and ESR were compared using a *t* test, and the gender and allergy history were compared using an x^2 test. The Pearson method was applied to the correlation analysis. The clinical significance of β2AR mRNA was evaluated by the participants' work characteristic curve (ROC curve). A *P* value < 0.05 was considered significant.

Results

Clinical characteristics

A total of 33 children with bronchial asthma were selected. All the children were treated with low-dose budesonide powder for a long time and then divided into two groups: the pre-treatment group and the post-treatment group, as shown in **Table 1** and **Figure 1**. These obser-

Table 2. Comparisons of the parameters of pulmonary function and airway remodeling between the pre-treatment and post-treatment groups ($\bar{x}$ ± SD)

Items	*Pre-treatment group*	*Post-treatment group*	*P-value*
FEV1 (L)	1.63 ± 0.35	2.03 ± 0.57	0.039
FEV1/FVC (%)	53.67 ± 5.76	65.19 ± 6.65	0.017
PEF (L/min)	54.39 ± 5.86	66.77 ± 6.33	0.023
RBMT (μm)	6.29 ± 1.39	3.86 ± 0.97	< 0.001
FsC (n/5oil immersion microscope vision)	17.3 ± 3.5	10.6 ± 2.3	0.019

FEV1: forced expiratory volume in 1 sec; FVC: forced vital capacity; PEF: peak expiratory flow; RBMT: reticular basement membrane thickness; FsC: fibroblasts Count.

vations in age, sex, duration of asthma, ESR, or allergic history did not differ between the pre-treatment and post-treatment groups (all P > 0.05). Nevertheless, the significant differences in total IgE and peripheral blood eosinophil were determined between the two groups of asthmatic children (all P < 0.05).

Results of pulmonary function and airway remodeling

Pulmonary function in all subjects was evaluated, including FEV1, FEV1/FVC, and PEF. The outcomes in FEV1, FEV1/FVC, and PEF for children with bronchial asthma were shown to prominently increase after treatment compared with those before treatment (all P < 0.05). All subjects underwent fiberoptic bronchoscopies to assess their airway remodeling during asthma remission. The observations of these airway remodeling parameters showed that the results of RBMT and FsC after treatment were significantly lower than those before treatment (both P < 0.05), as shown in **Table 2** and **Figure 2**.

Results of β2ARs and inflammatory factors

The expressions of mRNA and protein for β2ARs in the BALF of asthmatic children were detected by RT-PCR and Western blot, respectively. The serum concentrations of IL-6 and TNF-α were determined by ELISA, as shown in **Table 3**, **Figures 1**, **3**. The augmented expressions of mRNA and protein for the β2-ARs were observed for the asthmatic children after treatment (both P < 0.05). However, these lower serum concentrations of IL-6 and TNF-α were revealed in the post-treatment group (both P < 0.05).

Correlation analysis

Pearson's method was applied to the correlative analysis of the research data, as shown in **Figure 4**. The expression of β2Ars mRNA was shown to be negatively correlated with IL-6, TNF-α, RBMT, and FsC in asthmatic children (P < 0.05).

ROC curve analysis

The data in the pre-treatment group and the post-treatment group served as the dependent variable. The area under the curve (AUC) of the β2Ars mRNA expression was 0.823 (95% CI:0.682~0.936, P < 0.05). The sensitivity and specificity of diagnosing asthma at the best critical value were 87.9% and 89.2%. These findings suggested that β2ARs is useful in assessing the remission of asthma. The ROC curves of β2Ars mRNA expression are shown in **Figure 5**.

Discussion

In this study, the expression of mRNA and protein for β2ARs in BALF prominently increased after treatment with budesonide in children with asthma. However, these decreased outcomes of RBMT, FsC, IL-6, as well as TNF-α were also observed in the post-treatment group. These negative correlations between the expression of β2Ars mRNA and the concentrations of IL-6 and TNF-α, RBMT, and FsC, respectively, were observed both before and after treating the asthmatic children. We also demonstrated the increased results of FEV1, PEF, and FVC in children with bronchial asthma after treatment. Moreover, information from the ROC curve confirmed the positive effect of β2ARs on assessing the remission of bronchial asthma.

Increasing evidence suggests that the receptor theory could respond intensely to the pathogenesis of asthma. The surface receptors found in airway epithelial cells, such as toll-like receptors and other pattern recognition receptors, could distinguish specific patterns on

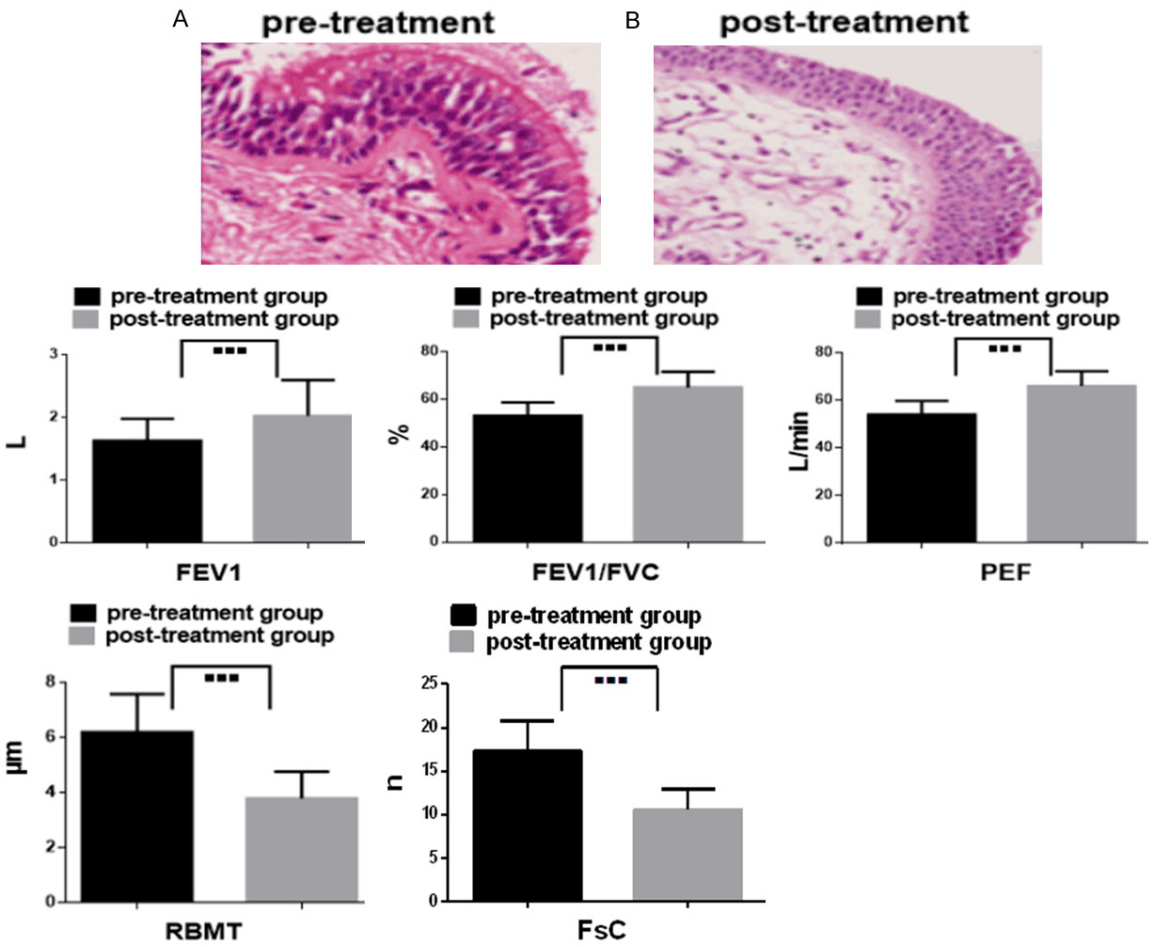

Figure 2. Comparison of the parameters of pulmonary function and airway remodeling. ■■■, P < 0.05; (A) shows the thickened basement membrane in pre-treatment group (The black arrows indicates this); (B) shows the thinned basement membrane in the post-treatment group (The black arrows indicate this). Furthermore, these reduced outcomes of FEV1, FEV1/FVC and PEF in the post treatment group compared with those in the pre-treatment group (P < 0.05). Nevertheless, RBMT and FsC in the same condition give an enhanced result (P < 0.05). FEV1: forced expiratory volume in 1 sec; FVC: forced vital capacity; PEF: peak expiratory flow; RBMT: reticulation basement membrane thickness; FC: fibroblast count.

Table 3. Comparison of the expressions of mRNA and protein for β2ARs, and inflammatory factors ($\bar{x}$ ± SD)

Items	*Pre-treatment group*	*Post-treatment group*	*P-value*
β2ARs mRNA	0.43 ± 0.07	0.86 ± 0.23	< 0.001
β2ARs protein	0.51 ± 0.09	0.95 ± 0.27	< 0.001
IL-6 (ng/L)	197.23 ± 63.70	103.29 ± 31.67	< 0.001
TNF-α (ng/L)	127.60 ± 33.22	67.58 ± 19.26	< 0.001

β2ARs: beta 2 adrenergic receptors; IL-6: Interleukin-4; TNF-α: tumor necrosis factor-α.

pathogen molecules (pathogen-associated molecular patterns) and lead to increased inflammation, Th2 cell activation, and alternative macrophage activation for asthma patients. These and alternative macrophages were considered to regulate the production of growth factors, including TGF-β and the vascular endothelial growth factor, which could lead to airway remodeling [6]. In addition, data from the IL-4 receptors suggest that the expressions of the IL-4 receptors may impair the inhibition of Treg cells from the lungs and participate in respiratory syncytial virus infections in asthmatic patients and induce Th2-like effector phenotypes in Treg cells [7].

The exogenous catecholamines, such as the β2-adrenergic agonists inhaled in asthma treatment, can cause airway dilation by acting on membrane-bound β2ARs on airway epithelial and smooth muscle cells [8]. This may be

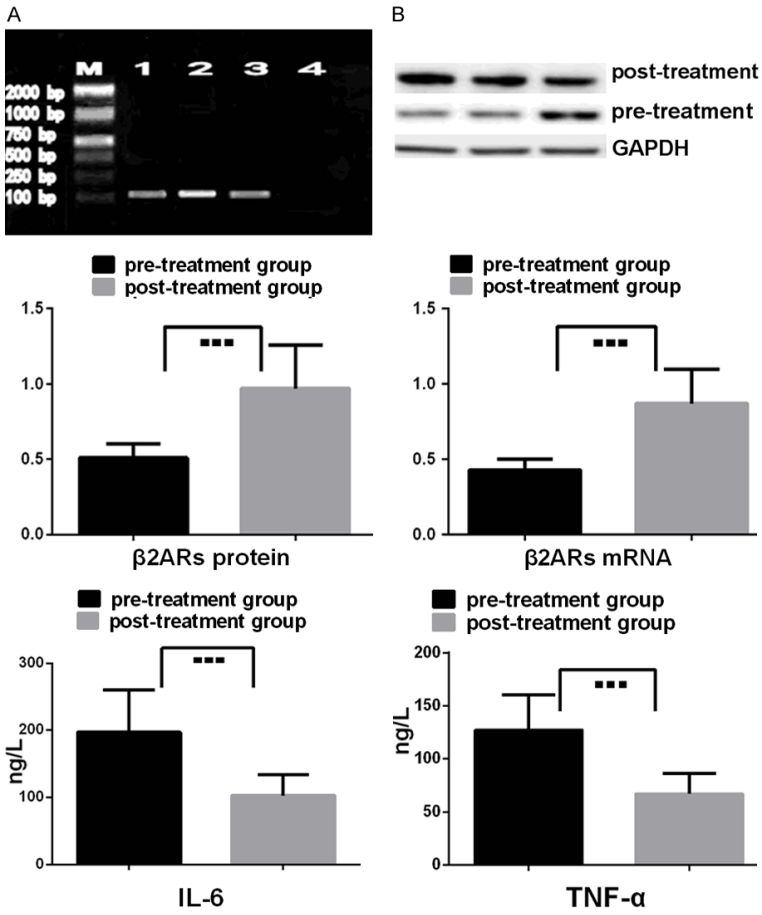

Figure 3. Comparison of the expression of mRNA and protein for β2ARs in BALF and the concentrations of the inflammatory factors in serum for asthmatic children. ■■■, $P < 0.05$; (A, B) show the expression of mRNA and protein for β2ARs in BALF on the basis of the electrophoretic analysis of PCR amplification products and the gray level analysis of Western blot, respectively. In Figure a, M shows the expression of the marker (DL 2000), and 1, 2, 3 and 4 show the expression of β2ARs mRNA in the pre-treatment group, the post- treatment group, the β-actin group, and the negative control group, respectively. (B) shows the protein expression before and after treatment for β2ARs. These histograms describe the upregulation of β2ARs mRNA as well as the downregulation of IL-6 and TNF-α in the post treatment group compared with the upregulation and downregulation in the pre-treatment group ($P < 0.05$). β2ARs: beta 2-adrenergic receptors; BALF: bronchoalveolar lavage fluid; IL-6: Interleukin-6; TNF-α: tumor necrosis factor-α.

related to the properties, density and conformations of the molecular structure of β2ARs on the cell surface. Several single nucleotide polymorphisms (SNP) within the promoter affect the expression/regulation of β2ARs coding 39UTR domains [9-11]. Highly resistant β2AR+79*G (Glu27) may contribute to agonist-stimulated receptor downregulation [12]. Clinical studies have confirmed that genetic variation might result in a differential clinical response to mild asthma. Furthermore, bronchiectasis is more likely to be caused by salbutamol responding to β2AR+46 (a homozygote) compared to homozygous individuals with the G allele [13, 14]. Data from larger pharmacogenetic studies reveals that mild or moderate asthma disease almost exclusively involved β2ARs polymorphisms, but severe asthma remained unclear [15-18]. We confirmed that β2Ars mRNA expression in BALF was significantly upregulated after treatment with budesonide for asthmatic children, suggesting that β2ARs might be related to the pathological process of asthma, especially in the treatment surveillance of asthma.

Increasing evidence has revealed inflammation involving asthma. It has been suggested that T regulatory (TREG; Foxp3+CD4+CD25+) cells can prevent chronic inflammatory and autoimmune diseases due to the homeostasis of cellular immune responses [19]. Previous studies revealed that increased TREG suppressive function was responsible for TREG cells from aerobically exercised mice in Th: TREG cell co-cultures, and both effectively relieved chronic airway inflammation and increased lung function for an ovalbumin-driven asthmatic murine [20, 21].

TNF-α was confirmed to have a very important effect on asthma pathogenesis. It was a multifunctional proinflammatory cytokine in response to inflammation, infection, macrophages, eosinophils, epithelial cells, and neutrophils [22]. In addition, an increased TNF-α release in monocytes can be stimulated through β2-ARs downregulation induced by endogenous catecholamines in septic shock [23]. This presented sufficiently the correlation between TNF-α and β2-ARs, in spite of this not arising in asthmatic patients.

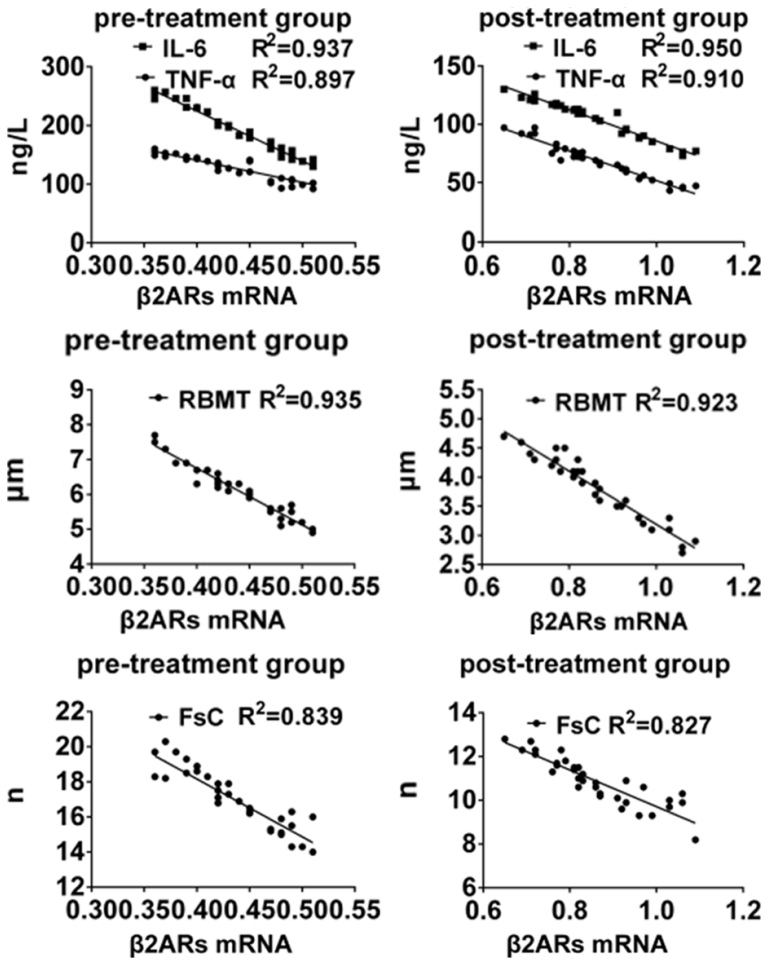

Figure 4. Evidence of correlation analysis. These negative correlations between β2ARs mRNA and IL-6, TNF-α, RBMT, and FsC are indicated ($P < 0.05$). β2ARs: beta 2-adrenergic receptors; IL-6: Interleukin-6; TNF-α: tumor necrosis factor α; RBMT: reticulation basement membrane thickness; FsC: fibroblasts count.

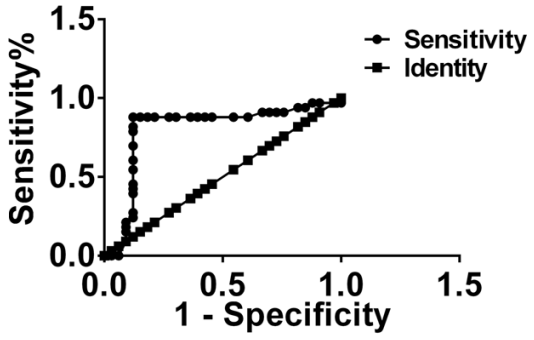

Figure 5. Evidence of ROC curve analysis. The AUC of β2ARs mRNA expression is 0.823 (95% CI: 0.682~0.936, $P < 0.05$). The sensitivity and specificity at the best critical value in the evaluation of the therapeutic effect on asthma are 87.9% and 89.2%, respectively.

IL-6 has been described as being involved in systemic inflammation and metabolic dysfunction, and a lower lung function was found in high-IL6-level asthma patients with frequent aggravating attacks [24]. The effect of salmeterol as a long-acting β2-agonist decreases the concentration of proinflammatory cytokines such as IL-6 and TNF-α in a model of allergen-challenged mice [25], which presents a potent bronchodilating effect on moderate treatment for severe asthma [26]. In this process, the activation of β2-ARs were induced by salmeterol that can go through the lipid bilayer on cell membranes where β2-ARs exist [27].

Notably, the enhanced plasma concentrations of TNF-α were revealed to respond to the development of childhood asthma [28]. Interestingly, the analysis of inflammatory cytokines displayed that the severity of airway diseases such as asthma is associated with plasma concentrations of IL-4, IL-8, IL-10, and TNF-α [2]. Meanwhile, β2-AR agonists have been disclosed to significantly enhance the production of bronchial epithelial cells, smooth muscle cells, and fibroblasts. In this process, an increased β2-ARs release can be presented [29]. In the present study, a similar point of view is presented. The concentrations of IL-6 and TNF-α in serum presented discrepant changes before and after treatment in asthmatic children, which were apparently correlated with the expression of β2-ARs in BALF. These observations provided evidence of β2-ARs participating in the pathological mechanism of asthma in children as a monitoring effect on asthmatic treatment.

Airway remodeling is defined as a variation of airway construction, including subepithelial fibrosis, smooth muscle hyperplasia, and goblet cell hyperplasia. However, little is known

about the development mechanisms of airway remodeling [30]. A previous analysis proposed that airway remodeling for patients with asthma might be inspired by chronic airway inflammation such as inflammatory mediators and cytokines [31]. Nevertheless, with the inhibition of airway remodeling via prophylaxis for the inflammation of the airway [32], a novel therapy that controlled airway remodeling for asthmatic patients required a further understanding of its mechanism.

Most review papers indicate that airway remodeling for asthmatics might be associated with repeated episodes of asthma accompanied by recurrent bronchoconstriction [2]. β2AR agonists in response to β2ARs on airway epithelial and smooth muscle cells contribute to cAMP-elevating agents to result in elevated cAMP expression that presents a potent inhibiting effect on bronchial constriction. This mechanism has not yet been fully elucidated [33, 34].Traditionally, the activation of cAMP brings about a relaxation of the airway's smooth muscle [35] due to this process of mediating activation of protein kinase A(PKA) that participates in the phosphorylation process of multiple proteins, including myosin light chain kinase and potassium channels. These processes result in a changed microstructure for airway smooth muscle cells, further resulting in a sarcomere extension to attain the rapidly reversed bronchoconstrictor effect [36-38]. Other studies have shown that PKA acts independently on β2ARs mediating the relaxation of tracheal smooth muscle in a guinea pig model. Nevertheless, the mechanism behind this is unclear [39].

It has been observed that airway smooth muscle cell proliferation and migration are restrained by cAMP elevators such as β2ARs agonists that induce the activation of β2-ARs, especially in chronic asthma where an increase in airway smooth muscle mass is caused by cell proliferation which is more likely to lead to airway remodeling [40]. The expansion of the blocked airway by using drugs that activate β2-ARs can relieve asthma attacks. This evidence reveals the positive effects of β2-ARs in response to the relaxation of airway smooth muscle and suggests that a decrease in the number of β2-ARs or hypofunction can lead to the onset of asthma. An increased frequency of asthma attacks further advances airway remodeling.

In the present study, these declines of RBMT and FsC in airway tissue were observed in the post-treatment children with asthma. A similar condition also occurred with inflammatory cytokines in serum. Moreover, lung function after treatment for children with bronchial asthma was also found to be clearly improved. Meanwhile, these parameters of airway remodeling and inflammatory cytokines negatively correlated with the expression of β2Ars mRNA in BALF. Furthermore, information from the ROC curve confirmed the nice AUC of β2Ars mRNA expression between the two groups of asthma sufferers and the higher sensitivity and specificity assessing the remission of bronchial asthma. This suggested that active treatment by regulating the number of β2ARs could relieved airway remodeling for asthmatic children, following the improvement of symptoms and the remission of inflammatory reactions. It further established that β2ARs can be an important marker of assessing airway remodeling for bronchial asthma.

Conclusions

This clinical study confirmed that these observations in age, sex, duration of asthma, ESR, or allergic history did not differ between the pretreatment group and the post-treatment group. Nevertheless, these significant differences in total IgE and peripheral blood eosinophil were revealed between the two groups of asthma sufferers. Moreover, the expression of β2-ARs mRNA correlated with these parameters of inflammation and airway remodeling in asthmatic children, suggesting that β2-AR had an influence on the pathogenesis of asthma, especially on the treatment of asthma, even though the details of the underlying mechanisms remained to be elucidated.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

RT-PCR, reverse transcriptase-polymerase chain reaction; β2ARs, beta 2-adrenergic recep-

tors; BALF, bronchoalveolar lavage fluid; IL-6, Interleukin-6; TNF-α, tumor necrosis factor α; FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity; PEF, peak expiratory flow; RBMT, reticular basement membrane thickness; FsC, fibroblasts count; ROC curve, participant's work characteristic curve; AUC, area under the ROC curve; AHR, airway hyperresponsiveness; ESR, erythrocyte sedimentation rate; IgE, total protein immunoglobulin E; SD, standard deviation.

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