Journal of Asthma and Allergy Dovepress

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The Expression Levels of Transforming Growth Factor β1 and Tumor Necrosis Factor Receptor Associated Factor 6 in Allergic Rhinitis Patients and Their Potential Relationship with Epithelial - Mesenchymal Transition: A Pilot Prospective Observational Study

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Objective: To study the role of transforming growth factor beta 1 (TGF-β1) and tumor necrosis factor receptor related factor 6 (TRAF6) in the progression of epithelial mesenchymal transformation (EMT) in allergic rhinitis (AR).

Methods: A total of 30 patients underwent nasal endoscopic surgery at our Hospital were selected for 15 patients in each group based on their allergy status. Inferior turbinate mucosa tissue was obtained and analyzed using immunohistochemical (IHC) tests, real-time quantitative PCR (qRT-PCR) detection, and Western blotting (WB) tests to measure TGF-β1, TRAF6, E-cadherin, Vimentin, and α-Smooth Muscle Actin (α-SMA) expression levels.

Results: The expression levels of TGF-β1, TRAF6, Vimentin, and α-SMA were significantly higher in the AR group compared to the control group as shown by IHC, qRT-PCR, and WB ($P < 0.05$). E-cadherin expression was significantly lower group than in the control group ($P < 0.05$). Protein expression of TGF-β1 showed significantly positive correlations with TRAF6 ($r = 0.8188$, $P = 0.0002$), α-SMA ($r = 0.8076$, $P = 0.0003$), and Vimentin ($r = 0.6917$, $P = 0.0043$). There was a significantly negative correlation between protein expression of TGF-β1 and E-cadherin ($r = -0.8032$, $P = 0.0003$). Protein expression of TRAF6 showed a significantly negative correlation with E-cadherin ($r = -0.6405$, $P = 0.0101$) but positive correlations with α-SMA ($r = 0.5809$, $P = 0.0231$) and Vimentin ($r = 0.555$, $P = 0.0318$).

Conclusion: TGF-β1, TRAF6, and EMT-related markers (Vimentin, α-SMA) were highly expressed in the nasal mucosa of AR patients. TGF-β1 and TRAF6 may be involved in the epithelial-mesenchymal transition in allergic rhinitis.

Keywords: allergic rhinitis, transforming growth factor β1, tumor necrosis factor receptor associated factor 6, epithelial-mesenchymal transition, immunohistochemical

Introduction

Allergic rhinitis (AR) is a common inflammatory disease of the nasal mucosa, which is characterized by sneezing, runny nose, nasal congestion, and itching.[1] In the AR-related innate immune mechanisms, nasal mucosa epithelial cells play a crucial role.[2] With T cells, mast cells, inflammatory cells, and eosinophils migrating to the nasal mucosa, the normal nasal mucosa tissue would divide and rebuild.[3] Epithelial-Mesenchymal transition (EMT) refers to the pathological

Received: 18 April 2024
Accepted: 14 October 2024
Published: 1 November 2024

progress, during which the epithelial cells can be phenotypically transformed into mesenchymal cells, and can further transfer into fibroblasts secreting extracellular matrix (ECM) and causing the thickening of the extracellular matrix. Previous studies have suggested that EMT can induce tissue remodeling which is a kind of repair process that occurs after tissue is damaged.[4] The tissue remodeling of AR is characterized by the decomposition of nasal mucosal epithelial structure, the increase and accumulation of subepithelial ECM,[5] the increased number of goblet cells, and the inflammatory infiltration.[6] Recent studies have found that EMT was observed in asthma, with the decreased expression of epithelial markers such as E-cadherin and β-catenin, and with the increased expression of mesenchymal markers such as N-cadherin, α-Smooth Muscle Actin (α-SMA), Vimentin, and fibronectin.[5] Asthma and AR have certain pathological similarities,[7] so the airway tissue remodeling of asthma has reference value for AR research. Transforming growth factor β (TGF-β) has pro-fibrotic and anti-inflammatory functions, it is an important regulator of respiratory diseases,[8] so in the induction process of EMT, TGF-β is the most concerned and the most critical. At present, it has been suggested that the expression level of TGF-β1 in the nasal mucosa of AR rats is significantly increased.[6] In addition, tumor necrosis factor receptor associated factor 6 (TRAF6) is a class of adaptor proteins and E3 ubiquitin ligases belonging to the TRAF family.[9] It has been found that TRAF6 is involved in immune response and associated with the progression of a variety of diseases, and it is one of the downstream participants of the TGF-β receptor, Toll-like receptor family, T cell receptor, and tumor necrosis factor receptor (TNFR) superfamily members.[10–13] Based on the regulatory mechanism of EMT, further exploration of these important targets involved in the regulation process is of great significance for finding new effective ways to prevent AR and improve its prognosis. However, there are few reports on the expression levels of TGF-β1, TRAF6, and EMT in AR patients. In this study, immunohistochemistry (IHC), real-time quantitative PCR (qRT-PCR), and Western blotting (WB) were used to detect the expression of TGF-β1, TRAF6, and EMT markers (E-cadherin, Vimentin, α-SMA) in the nasal mucosa of AR patients, and to investigate their roles in the progress of epithelial-mesenchymal transformation in AR patients.

Methods and Materials

Patients, Grouping and Sample Obtain

From March 2021 to December 2021, a pilot prospective observational study was carried out on patients who underwent endoscopic sinus surgery at the First Affiliated Hospital of Nanchang University. According to the random number table method, the AR patients who underwent endoscopic vidian neurectomy were randomly selected into the AR group, and the healthy patients who underwent nasal septum correction for nasal septum deviation with inferior turbinate hypertrophy were included in the control group, with 15 patients in each group. The inferior turbinate mucosa tissues of the two groups were collected. There were 8 males and 7 females in the AR group, ranging in age from 13 to 63 years, and there were 7 males and 8 females in the control group, with age ranging from 15 to 60 years. All patients included in the AR group were definitely diagnosed by comprehensive evaluation of historic medical characteristics, clinical manifestations, and examination results (including skin prick test and serum-specific IgE antibody detection).[14] Patients in the control group had no clinical symptoms of the nose and no similar diseases such as chronic rhinosinusitis and with a negative skin-prick test for allergens. Patients who had taken oral corticosteroids 3 months before or had used glucocorticoid nasal spray within 1 month were excluded. Additionally, Patients with fungal sinusitis, sinonasal tumors, gastroesophageal reflux disease, aspirin triad, bronchial asthma, choanal polyps, autoimmune diseases, cystic fibrosis, primary ciliary motor dysfunction, and other diseases were excluded.

All laboratory tests were performed for 3 times.

Immunohistochemistry (IHC) Detection Process

Inferior turbinate mucosal tissues obtained from both groups were trimmed, fixed, embedded, and sectioned. The samples were dewaxed, hydrated, antigen-recovered, and inactivated. Then, the samples were incubated with primary and secondary antibodies (details about the primary and secondary antibodies can be found in the Supplementary information 1), followed by staining with Diaminobenzidin (DAB). After dehydration and air drying, neutral gica was added to seal the slide, and the slides were then observed and photographed under a microscope at 400x magnification.

Semi-quantitative methods were used to judge the results of IHC:[15] (1) A score of 0–4 was used to assess the intensity of cell staining: a score of 0 indicated no staining, a score of 1 for pale yellow cells, 2 for brown cells, and 3 for sepia cells. (2) Five high-magnification fields (400x) were randomly selected to calculate the percentage of positive staining cells in the total number of cells: 0 score indicates the percentage < 10%, 1 score indicates the percentage of 10%-25%, 2 score for 25%-50%, 3 score for 50%-75%, and 4 score for > 75%. Then the above two scores were multiplied to obtain the final score: 0–4 score for negative IHC result and 5–12 score for positive IHC result.

Real-Time Quantitative PCR (qRT-PCR) Detecting Process

Tissue RNA was obtained from the inferior turbinate mucosa of the two groups using the kit method according to the specification operating process of the manufacturer and the Trizol method was used. The concentration and purity of the presented RNA samples were checked by UV spectrophotometer. Subsequently, cDNA was prepared by reverse transcription, and 2μL of the transcript product was extracted for PCR reaction with 40 amplification cycles. The reaction conditions were strictly controlled according to the manufacturer's instructions. Finally, the fluorescent expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as the reference to quantity the expression of different markers. The probes of different markers were listed in the Supplementary information 1.

Western Blotting (WB) Detection Process

Protein samples were extracted and the concentration was determined by Bicinchoninic acid (BCA) method. Protein samples were prepared by adding 5×loading buffer and the ratio of volume to protein was 1:4. An electrophoresis gel was papered and the initial voltage was 90V, the first time of electrophoretic duration was approximately 30min. Then, the voltage was adjusted to 150 V for another hour of electrophoretic. The membrane transfer was performed under a constant flow of 220mA for a duration of about 100 minutes. Then, the membrane was blocked using 5% skim milk solution and subsequently placed on a shaker at room temperature with constant shaking for 60 minutes. Primary antibodies were administered and then the sample was placed in a 4°C refrigerator and was incubated overnight with slow shaking. Secondary antibodies were solubilized in 1% skim milk and the sample was then incubated for 60 minutes at 25°C with shaking. Details about the primary and secondary antibodies can be found in the Supplementary information 2. At last, luminous drops were used to exposure and the sample was observed by instrument.

Statistical Analysis

All the data collected in this study were analyzed using SPSS 26.0 software. Normally distributed measurement data were expressed as mean±standard deviation (SD), and the comparisons were examined by Student-*t* test and Mann–Whitney test (non parametric distribution). The categorical data were expressed as n(%), and the differences between the two groups were examined by chi-square analysis or Fisher's exact test. Correlation analysis was performed by Pearson correlation analysis. $P<0.05$ was considered statistically significant.

Results

Immunohistochemical (IHC) Detection of the Expression of TGF-β1, TRAF6, and EMT Markers (E-cadherin, Vimentin, α-SMA)

TGF-β1 and E-cadherin proteins were mainly localized on the cell membrane, showing membrane staining. TRAF6 and α-SMA proteins were expressed in the cytoplasm and nucleus, showing cytoplasmic and nuclear staining. Vimentin protein was expressed on the cell membrane and cytoplasm, showing membrane and cytoplasmic staining (Figure 1).

As shown in Table 1, the positive expression rates of TGF-β1, TRAF6, Vimentin, and α-SMA in AR group were 93.33%, 80.00%, 73.33%, and 86.67%, respectively, which were higher than those in control group (20.00%, 26.67%, 26.67%, and 33.33%) with statistically significant differences, $P < 0.05$. However, the positive expression rate of E-cadherin in AR group was 26.67%, which was lower than that in control group (66.67%), $P < 0.05$.

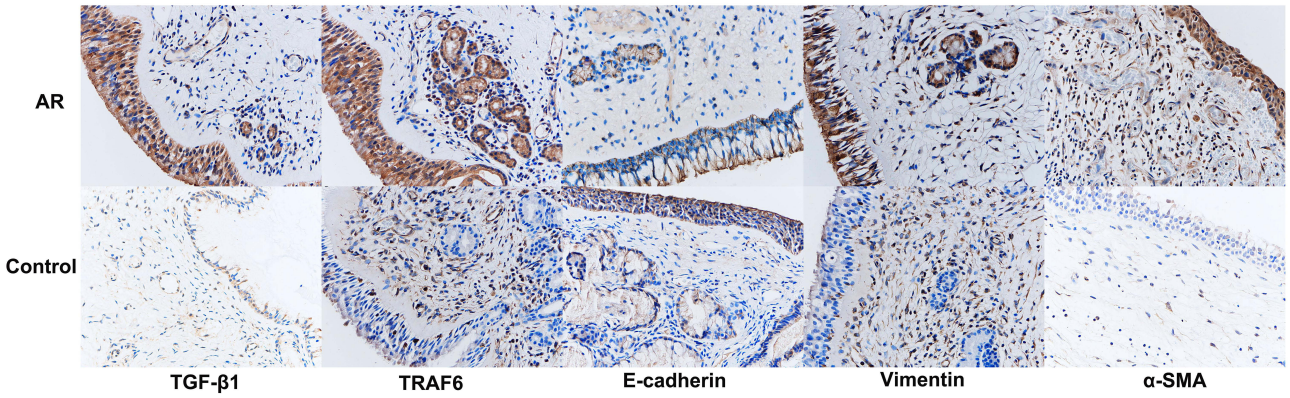

Figure 1 The immunohistochemistry (IHC) comparisons of the expressions about transforming growth factor β1 (TGF-β1), tumor necrosis factor receptor associated factor 6 (TRAF6), E-cadherin, Vimentin, and alpha Smooth Muscle Actin (α-SMA) between the Allergic rhinitis (AR) group and the control group at 400x magnification.

qRT-PCR Detection the mRNA Expression Levels of TGF-β1, TRAF6, and EMT Markers (E-Cadherin, Vimentin, α-SMA)

The mRNA expression levels of TGF-β1, TRAF6, Vimentin, and α-SMA were higher in AR group than those in control group, $P < 0.05$, but the mRNA expression of E-cadherin was lower than that of the control group, $P < 0.05$ (Table 2 and Figure 2).

Western Blotting Detection the Protein Expression of TGF-β1, TRAF6, and EMT Markers (E-cadherin, Vimentin, α-SMA)

The protein expression levels of TGF-β1, TRAF6, Vimentin, and α-SMA in the AR group were higher than those in control group, $P < 0.05$. While, the expression of E-cadherin protein was lower than that of control group, $P < 0.05$ (Table 3 and Figure 3).

The Correlation Analysis of TGF-β1, TRAF6, and EMT Markers (E-Cadherin, Vimentin, α-SMA)

The protein expression level of TGF-β1 was positively correlated with TRAF6 ($r = 0.8188$, $P = 0.0002$), with α-SMA ($r = 0.8076$, $P = 0.0003$), and with Vimentin ($r = 0.6917$, $P = 0.0043$). There was a negative correlation between the protein expression of TGF-β1 and the E-cadherin ($r = -0.8032$, $P = 0.0003$).

Table 1 The Count Number of the Expressions About Transforming Growth Factor β1 (TGF-β1), Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6), E-Cadherin, Vimentin, and Alpha Smooth Muscle Actin (α-SMA) in the Allergic Rhinitis (AR) Group and the Control Group Under Immunohistochemistry (IHC) at 400x Magnification

Groups	TGF-β1, n(%)	TRAF6, n(%)	E-cadherin, n(%)	Vimentin, n(%)	α-SMA, n(%)
AR group (n=15)	14 (93.33%)	12 (80.00%)	4 (26.67%)	11 (73.33%)	13 (86.67%)
Control group (n=15)	3 (20.00%)	4 (26.67%)	10 (66.67%)	4 (26.67%)	5 (33.33%)
χ^2	16.4310	8.5711	4.8211	6.5331	8.8891
***P* values**	<0.0001	0.0034	0.0281	0.0106	0.0029

Table 2 Relative mRNA Expression Levels of TGF-β1, TRAF6, E-Cadherin, Vimentin, and α-SMA in Allergic Rhinitis (AR) Group and Control Group

Groups	TGF-β1	TRAF6	E-cadherin	Vimentin	α-SMA
AR group (n=15)	1.280±0.096	1.130±0.059	0.718±0.056	1.275±0.087	1.605±0.054
Control group (n=15)	0.766±0.064	0.694±0.071	1.530±0.087	0.733±0.069	0.835±0.053

Note: Data were expressed as mean ± SD (standard deviation).
Abbreviations: TGF-β1, transforming growth factor beta 1; TRAF6, tumor necrosis factor receptor associated factor 6; α-SMA, α-Smooth Muscle Actin.

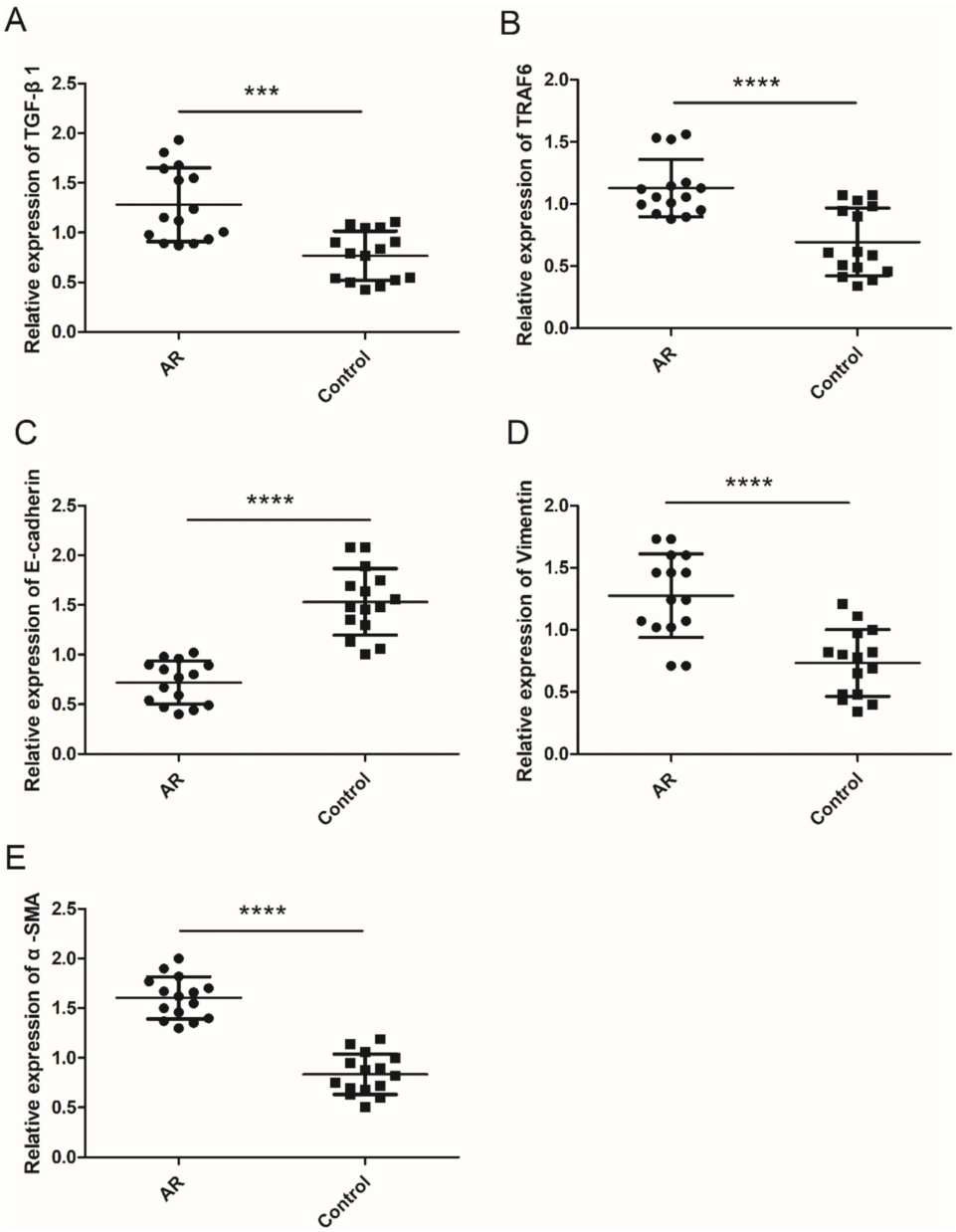

Figure 2 The relative expression of mRNA about (**A**) transforming growth factor β1, TGF-β1; (**B**) tumor necrosis factor receptor associated factor 6, TRAF6; (**C**) E-cadherin; (**D**) Vimentin and (**E**) alpha Smooth Muscle Actin, α-SMA between the Allergic rhinitis (AR) group and the control group. Data are presented as scatter plots, including medians and interquartile ranges. Student-*t* test was used to compare between the two groups, *** < 0.001, **** < 0.0001.

There was a negative correlation between the protein expression of TRAF6 and E-cadherin (r = −0.6405, P = 0.0101). The protein expression of TRAF6 was positively correlated with α-SMA (r = 0.5809, P = 0.0231) and Vimentin (r = 0.5550, P = 0.0318) (Figure 4).

Table 3 Relative Protein Expression Levels of TGF-β1, TRAF6, E-Cadherin, Vimentin, and α-SMA in Allergic Rhinitis (AR) Group and Control Group

Groups	TGF-β1	TRAF6	E-cadherin	Vimentin	α-SMA
AR group (n=15)	0.637±0.025	0.771±0.065	0.267±0.025	0.795±0.037	1.030±0.055
Control group (n=15)	0.395±0.023	0.530±0.060	0.539±0.023	0.648±0.038	0.801±0.056

Note: Data were expressed as mean ± SD (standard deviation).
Abbreviations: TGF-β1, transforming growth factor beta 1; TRAF6, tumor necrosis factor receptor associated factor 6; α-SMA, α-Smooth Muscle Actin.

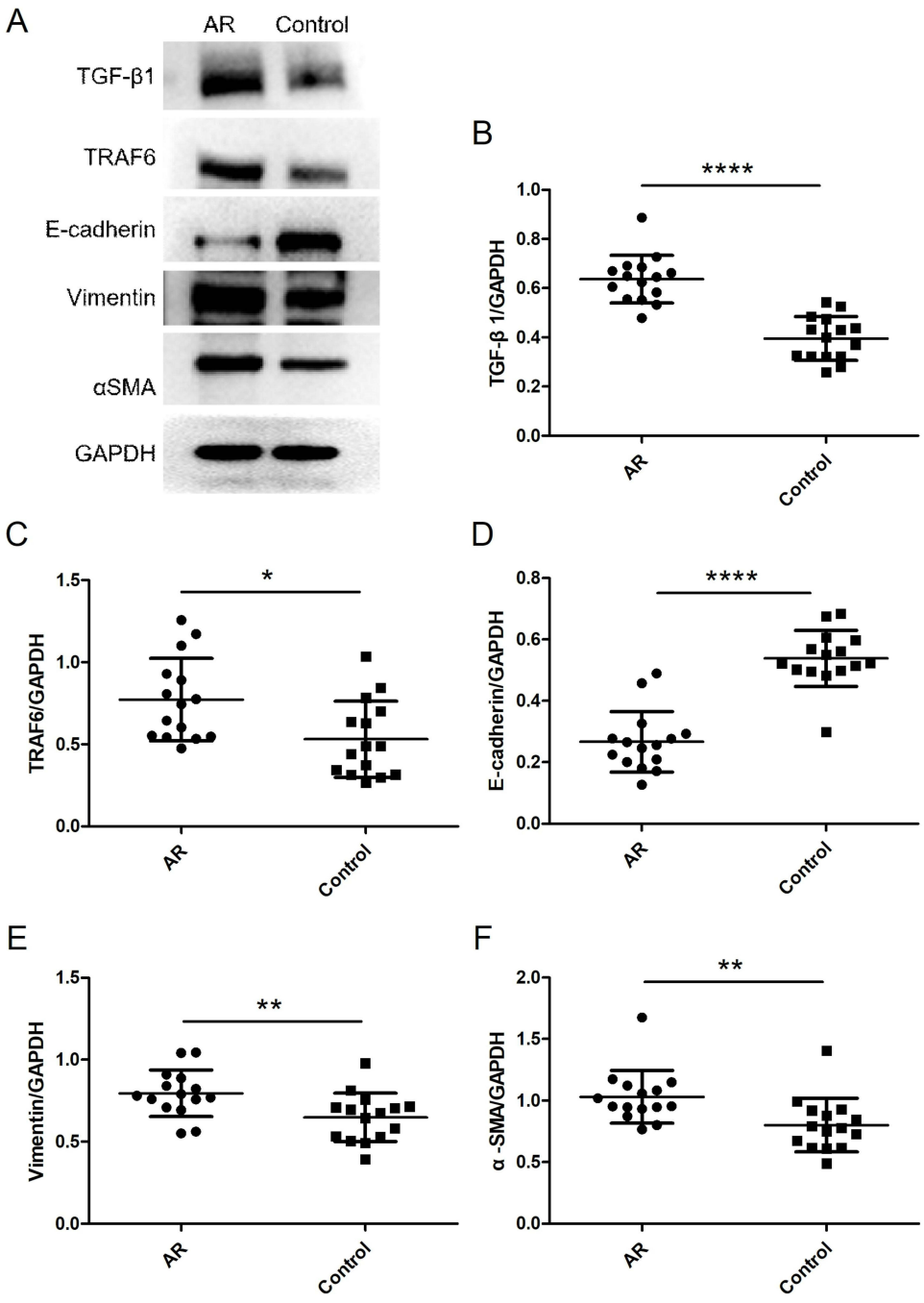

Figure 3 The original graph of Western blotting (**A**) is illustrated and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as the loading control. The protein expression of (**B**) transforming growth factor β1, TGF-β1; (**C**) tumor necrosis factor receptor associated factor 6, TRAF6; (**D**) E-cadherin; (**E**) Vimentin and (**F**) alpha Smooth Muscle Actin, α-SMA are compared between the Allergic rhinitis (AR) group and the control group. Data are presented as scatter plots, including medians and interquartile ranges. Student-*t* test was used to compare between the two groups, * < 0.05, ** < 0.01, **** < 0.0001.

Discussion

This study found that TGF-β1, TRAF6, Vimentin, and α-SMA were at higher levels in patients with AR than those in healthy patients. However, the E-cadherin expression was reduced in AR patients. These findings were consistent in the immunohistochemical detection, the qRT-PCR detection, and the Western blotting detection. Our findings suggest that EMT occurs in the nasal mucosa of AR patients. At the same time, our correlation analysis showed that the expression of TGF-β1 and TRAF6 had a positive correlation between them, which may be explained by that the TRAF6 is the

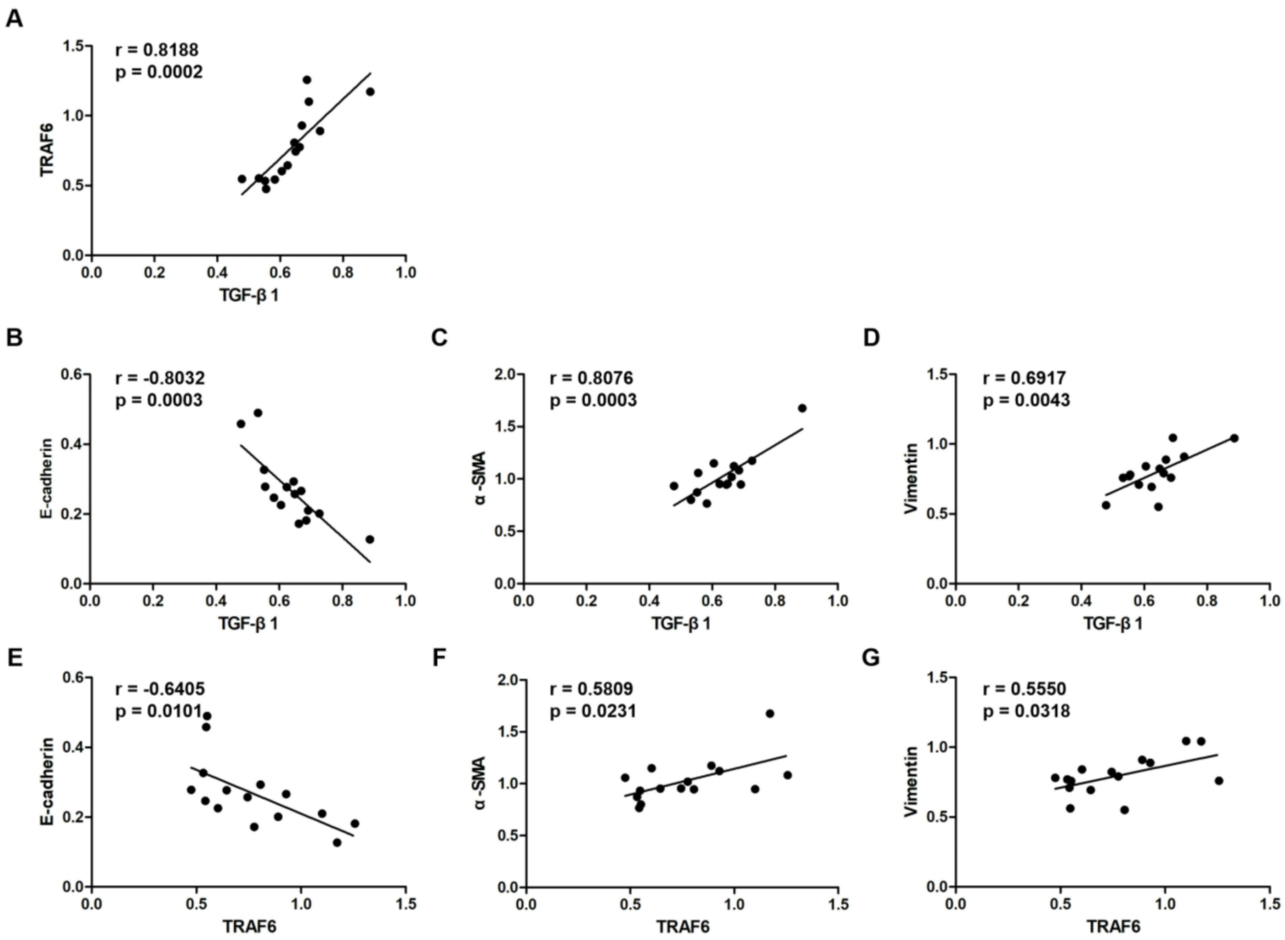

Figure 4 Pearson correlation analysis results of five protein expression in Allergic Rhinitis (AR) group. Between transforming growth factor beta 1 (TGF-β1) with (**A**) tumor necrosis factor receptor associated factor 6, TRAF6; (**B**) E-cadherin; (**C**) α-Smooth Muscle Actin, α-SMA; (**D**) Vimentin. And between TRAF6 with (**E**) E-cadherin, (**F**) α-SMA, (**G**) Vimentin.

downstream molecule of TGF-β. In addition, our correlation analysis showed that TGF-β1 and TRAF6 were negatively correlated with epithelial cell marker E-cadherin, but positively correlated with mesenchymal cell markers Vimentin and α-SMA. So TGF-β1 and TRAF6 may participate the EMT process in AR patients.

AR is an inflammatory disease of the nasal mucosa mediated by IgE.[16] The pathogenesis of AR is complex and diverse.[17] Epithelial cells are a natural physical barrier and are the source of the inflammatory response.[18] It has been found that immune imbalance can lead to the damage of the nasal mucosal epithelial barrier, and then promote the occurrence of chronic inflammation and tissue remodeling of the nasal mucosa, resulting in histological features such as inflammatory cell infiltration, basement membrane thickening, and interstitial edema.[17] It has been confirmed in the airways with similar epithelial cells and similar Th2-driven IgE dependence of inflammation that chronic inflammation may cause allergic respiratory symptoms.[19] In addition, tissue remodeling of the upper respiratory tract has also been reported in AR.[20] Under some pathological conditions, epithelial cells will change to cells with a mesenchymal phenotype, a process known as EMT.[21] EMT is involved in chronic inflammatory tissue remodeling in multiple organs and is involved in the pathophysiological process of airway inflammation, which has been reported in both chronic sinusitis[22] and asthma.[23] EMT plays a crucial role in chronic inflammation, tissue remodeling, embryonic development, cancer metastasis, and fibrosis of various tissues.[24,25] It is activated by hypoxia and a variety of inflammatory mediators such as TGF-β, epidermal growth factor (EFG) family, vascular endothelial growth factor (VEGF), Fibroblast Growth Factor (FGF), interleukin 6 (IL-6), etc.[24,25] It is mediated by the signaling pathways of receptor tyrosine kinase, Notch, Smad, Wnt, STAT3, and gp130-Src-YAP.[24,25] Among the pathways that can affect EMT, TGF-β may be one of the most critical pathways[26] under most inflammatory conditions in different

organs, such as the liver,[27] kidney,[8] and lung,[26] TGF-β1 is a major factor driving tissue fibrosis. TGF-β1 can activate the EMT program in some cells that experience phenotypic ectomesenchymal from the transition cells to form the fibroblast cells, secreting ECM components and causing fibrosis.[28] A Study has found that the total glucosides of paeony can improve oxidative stress, apoptosis, and inflammation in the AR through the Smad7/TGF-β pathway.[29] In addition, it reported that the level of TGF-β in the serum of patients with allergic rhinitis increased.[30] These findings above suggest that TGF-β may be involved in AR inflammation, but the exact mechanism remains unclear. TRAF6 belongs to the TRAF family and is a transducer of inflammatory signals.[31] TRAF6 regulates cell proliferation, differentiation, and apoptosis by activating NF-κB, JNK/p38, PI3K/AKT, and AP-1 pathways, and it also regulates innate and adaptive immunity, oxidative stress, and inflammation.[32] A Previous study has found that the mRNA level of TRAF6 is high in the trachea of obese male Wistar rats with asthma.[33] A recent report also showed that the TRAF6 can suppress Th17T cell differentiation by cutting the Smad signaling pathway.[34] A study has confirmed that miR-146a mimics can inhibit TLR4/TRAF6/NF-κB pathway to alleviate AR in mice.[35] These results suggest that TRAF6 plays an important role in AR inflammation. Similarly, our study suggests that the expression of TGF-β1 and TRAF6 are positively correlated in AR nasal mucosa and both are higher than those in healthy controls, suggesting that TRAF6 may be a downstream molecule of TGF-β, and TGF-β1 may participate in the EMT process of AR nasal mucosa through TRAF6.

In recent years, foreign scholars have proposed a noteworthy concept - allergic reactions.[36] It is interesting that allergic reactions are almost indistinguishable from hypersensitivity reactions in clinical practice. The main basis for determining whether it is an allergic reaction is the absence of serum specific IgE that can detect suspicious allergens. In fact, the essence of allergic reactions is non IgE mediated allergic reactions. Different studies have shown that the incidence of allergic reactions varies greatly, ranging from 0.1% to 75%, far higher than the traditional concept of allergic reactions. This reaction mainly affects the four key target organs of the skin, cardiovascular, respiratory, and gastrointestinal systems, leading to corresponding symptoms and pathological changes. The clinical manifestations can be diverse, ranging from annoying urticaria to angioedema syndrome, conjunctivitis, rhinitis, asthma, and even severe allergic reactions. Due to the fact that allergic reactions are not mediated by IgE, according to the current definition of allergic reactions, they cannot be simply classified as allergic reactions. But according to our updated concept, allergic reactions are actually nonIgE mediated allergic reactions. Some substances that trigger allergic reactions are neither antigens nor haptens. When they enter the human body, there is no incubation period or antigen antibody binding process, but they can quickly lead to clinical manifestations that are very similar to allergic reactions. In daily life, food, food additives, and drugs are the most common triggering factors for allergic reactions. The diagnosis of allergic reactions has its unique characteristics, such as a lack of specific IgE response to suspicious substances in the body, and the patient's serum IgE concentration will not increase. In terms of treatment, allergic reactions are similar to allergic diseases. However, it should be noted that unlike allergic reactions, allergen skin tests or specific IgE testing cannot provide effective indications for quasi allergic reactions. So, a detailed understanding of the medical history and conducting stimulation tests are particularly important. Common detection indicators include mast cell/eosinophil degranulation assay, measurement of active substances released by mast cells, total complement activity, and measurement of complement C3 and C5.

Limitations

First, the sample size of patients included in this study is still small, and further research is needed. Second, although TRAF6 has been identified as a potential downstream molecule of TGF-β, no specific interventions have been invested. Third, the expressions of TGF-β1, TRAF6, and EMT markers (E-cadherin, Vimentin, α-SMA) were not investigated in patients with different severity of AR.

Conclusions

TGF-β1, TRAF6, and EMT-related markers (Vimentin, α-SMA) were highly expressed in the nasal mucosa of AR patients. The expression levels of TGF-β1 and TRAF6 were positively correlated and they were all positively related with the EMT-related markers Vimentin, α-SMA). So TGF-β1 and TRAF6 may be involved in the epithelial-mesenchymal transition in allergic rhinitis, but should be verified in vivo study.

Abbreviations

TGF-β1, Transforming growth factor beta 1; TRAF6, Tumor necrosis factor receptor related factor 6; EMT, Epithelial mesenchymal transformation; AR, Allergic rhinitis; IHC, Immunohistochemical; qRT-PCR, Real-time Quantitative PCR; α-SMA:α-Smooth Muscle Actin; ECM, Extracellular matrix; TNFR, Tumor necrosis factor receptor; WB, Western blotting; DAB, Diaminobenzidin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; BCA, Bicinchoninic acid; EFG, Epidermal growth factor; VEGF, Vascular endothelial growth factor; FGF, Fibroblast Growth Factor; IL-6, Interleukin 6.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

The study followed the Declaration of Helsinki and was approved by the ethics committee of the First Affiliated Hospital of Nanchang University, and the informed consent forms were obtained from all patients.

Consent for Publication

Not applicable.

Acknowledgments

We are grateful to all staff professionals and participants.

Funding

This study was supported by Central Funds Guiding the Local Science and Technology Development (20221ZDG020066), National Key Research and Development Program of China (2023YFC2508005), and National Natural Science Foundation project (82260217).

Disclosure

The authors declare that they have no competing interests in this work.

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