Original Article

Expression of TGF-beta receptor 1 and Smads in the tissues of primary spontaneous pneumothorax

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Background: Primary spontaneous pneumothorax (PSP) is a common disease which is often caused by the rupture of bullae in the lungs. The underlying pathogenesis of PSP remains unclear. Some molecules may be involved in the development of PSP potentially. The aim of this study was to investigate the expression of TGF-beta receptor 1 (TβR1), Smad2, Smad3 and Smad4 in the resected bullae of patients with PSP.

Methods: From May 2015 to May 2016, 34 patients with PSP underwent video-assisted thoracoscopic surgery (VATS) bullectomy. Immunohistochemistry was performed to identify the expression of TβR1, Smad2, Smad3 and Smad4 in the resected pulmonary bullae tissues. The levels of these cytokines were calculated by immunoreactivity scoring system (IRS). Ten patients without pneumothorax associated disease were selected as the control group.

Results: The analysis showed that the expression levels of TβR1, Smad2 and Smad4 were significantly higher in bullae tissues of patients with PSP than that in normal lung tissues (P=0.012, 0.031, 0.000 respectively). There was no significant difference between the expression level of Smad3 in bullae tissue of PSP patients and that in normal lung tissues of the control group (P=0.140). However, the absolute quantity of Smad3 expression in PSP bullae tissues was (4.2529±1.7193), scored by the IRS, which is higher than that in the control lung tissues (3.2600±2.2132). Also, the expression of TβR1, Smad2, Smad3 and Smad4 were not showed correlation with the clinical characteristics of PSP patients, such as age, sex, body mass index (BMI), recurrence and side of pneumothorax.

Conclusions: TβR1, Smad2 and Smad4 highly expressed in bullae tissues of PSP patients. Our findings suggested that TβR1, Smad2 and Smad4 may be related to the development of PSP bullae.

Keywords: Primary spontaneous pneumothorax (PSP); bulla; TGF-beta receptor; Smad2 Protein; Smad3 protein; Smad4 protein

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Introduction

The incidence of primary spontaneous pneumothorax (PSP) was 7.4–18/100,000/year for males and 1.2–6/100,000/year for females according to the global statistical data (1,2). PSP usually presents as an acute onset and has high recurrence rate, so that brings a strong attack on the quality of patient's life and healthy economy of the community. Therefore, it is important to explore the pathogenesis of PSP. Unfortunately, no basal pulmonary disease is found in majority of the PSP cases though it is a popular disease especially in younger populations. So the causes and

pathogenesis of PSP have not yet been clarified till now. Some previous reports have shown that there is fibrosis in addition to the bullae in the apex of the lung in young PSP subjects (3,4). According to studies of histopathology, the histopathologic feature of PSP lesion is alveolar collapse accompanied by fibroelastosis. And these pathological findings are not secondary to pneumothorax but have existed before the episode of pneumothorax. One hypothesis based on the results of Kobayashi *et al.* is that as thickened pleura with fibroelastosis becomes less compliant, and pleural thickening is uneven, areas of thinner apical visceral pleura, which may occasionally be at the site of subpleural bullae, receive a higher negative pressure and thus become likely to rupture (5). The presence of inflammatory cells and occasional fibrosis in the lesion regions which altered the delicate structures of elastic fibers in the lung, suggested that inflammation or pulmonary fibrosis might play a role in the aberrant changes of PSP.

TGF-β1 and its downstream cytokines Smad families are related with pulmonary fibrosis according to previous literatures (6). Moreover, it is reported by Sibinska and some other researchers that activation of interstitial lung fibroblast and presence of extracellular matrix production are pathological features of pulmonary fibrosis, and the effect of TGF-β1 on the pathological changes is mainly mediated through the TGF-β/Smads signaling pathway. Moreover, the anti-fibrosis effect of heat shock protein HSP90 is related to TGF-β receptor destruction and inhibition of Smad2/3 activation (7,8). So, it is thought that the role of TGF-β and its downstream cytokines in PSP bullae might induce pulmonary fibrosis. In the TGF-β/Smads pathway, TβR is a receptor protein with high affinity for TGFβ on the surface of cell membrane. Smad2, Smad3 and Smad4 play an important role in conducting signal into the nucleus and regulating transcription. Our study aimed to these cytokines associated with pulmonary fibrosis. The expression of TGF-beta receptor 1 (TβR1), Smad2, Smad3 and Smad4 in either the bullae tissue or normal lung tissue were tested to explore whether there was correlation between fibrosis relevant factors and pulmonary bullae disease.

Methods

Participant samples

From May 2015 to May 2016, 34 consecutive PSP patients who underwent surgery treatment at the Department of Thoracic Surgery, Beijing Chao-Yang Hospital, and met our inclusion criteria were enrolled as the study group in this study. PSP was defined as spontaneous air accumulation in the pleural cavity without other evidence of clinical lung disease. The inclusion criteria were: (I) all cases were in accordance with PSP diagnostic criteria—the lungs of PSP patients were no other lesions, confirmed by chest imaging diagnosis; and (II) patient age between 15 and 40 years. The exclusion criteria were: (I) history of smoking; and (II) a history of chest trauma, rib fracture and pulmonary contusion; (III) a history of pulmonary surgery, including lobectomy, segmentectomy and wedge resection of the lung; and (IV) a history of lung diseases (i.e., chronic obstructive pulmonary disease (COPD), asthma, pulmonary tuberculosis, pneumonia or pneumoconiosis); (V) a history of systemic diseases (i.e., end-stage renal failure, liver cirrhosis, malignancy, or chronic heart and liver diseases). At the same time, 10 patients without pneumothorax associated disease were selected as the control group. The cases of the control group were selected from the same age-bracket. There are 4 patients with lung cancer, 3 patients with bronchiectasis, 2 patients with tuberculosis and 1 patient with lung abscess enrolled in the control group. All patients of them underwent thoracoscopic lobectomy or partial lobectomy. The tissues of the control group were obtained far away from the site of the primary lesions, and identified by two pathologists as normal lung tissue. The age, sex, body mass index (BMI, kg/m^2), recurrence of pneumothorax and side of pneumothorax were recorded (*Table 1*). The institutional research ethics committee of the Beijing Chaoyang Hospital approved the study, and all of the patients signed the informed consent preoperatively.

Immunohistochemistry

Expression of TβR1, Smad2, Smad3 and Smad4 in the pathological sections was assessed by an Immunohistochemical SP method staining. We purchased the primary antibodies from the Abcam Company (Cambridge, UK). Anti-TGF beta Receptor I antibody (rabbit polyclonal to TGF beta receptor I, ab31013), anti-Smad2 antibody (rabbit monoclonal to Smad2, ab40855), Anti-Smad3 antibody (rabbit monoclonal to Smad3, ab40854) and anti-Smad4 antibody (rabbit monoclonal to Smad4, ab40759). Murine anti-rabbit polyclonal antibody was selected as the secondary antibody. Paraffin blocks

Table 1 Clinical characteristics of study group

Variables	Item	Number of patients	Constituent ratio (%)
Age	<23 years	18	52.94
	≥23 years	16	47.05
Sex	Male	27	79.41
	Female	7	20.59
BMI*	Abnormal	5	14.71
	Normal	21	61.76
Recurrence	Yes	15	41.12
	No	19	55.88
Side involved	Left	27	79.41
	Right	6	17.65
	Bilateral	1	2.94

*, the body mass index (BMI) ranged from 18.5 to 24.9. BMI value does not apply to less than 18 years old minors, so we removed. The average age group was 23 years old.

were sectioned continuously at a thickness of 4 μm for each case. The sections were deparaffinized in xylene. After the xylene was subsequently removed with absolute ethanol, the slides were treated with 3% H_2O_2 which can inactivate endogenous peroxidase. The sections were incubated with the antibodies to TβR1, Smad2, Smad3 and Smad4 respectively. The reaction was followed by biotin-conjugated rabbit anti-human immunoglobulin and horseradish peroxidase-conjugated streptavidin. Diaminobenzidine was used as chromogenic substrate, and brown or crimson red precipitates were identified as positive staining. The slides were counterstained with hematoxylin and mounted with glycerol gelatin. In each experiment, a section of PSP known to express TβR1, Smad2, Smad3 and Smad4 served as a positive control, Negative control was performed in the same way without addition of the primary antibodies.

Slide evaluation

Each slide was evaluated randomly in five areas with obvious lesions. Photographs were taken at each evaluated area for records. Each slide was read and scored by two independent investigators. Immunoreactivity was evaluated using the staining intensity score and distribution score. The Immunoreactivity was scored semi-quantitatively on the basis of a well-established immunoreactivity scoring system (IRS) (9-11). The IRS is calculated by the product of the percentage of positive cells (>80%, 4 points; 51–80%, 3 points; 11–50%, 2 points; 0–10%, 1 point; 0%, 0 point) and the intensity of the staining (strong, 3 points; moderate, 2 points; mild, 1 point; and no staining, 0 point). The final scores are between 0 (no staining) and 12 (maximum staining). The IRS were divided into one of the following three groups based on the final score; negative immunoreactivity was defined as a total score of 0, low expression was defined as a total score of 1–4, and high expression was defined as a total score of >4.

Statistical analysis

The statistical analyses were conducted using SPSS software (version 17.0; SPSS Inc., Chicago, III, USA). The continuous variables are presented as the mean ± standard deviation. Statistical analyses to compare two groups of data were performed using an unpaired Student's *t*-test. Ratio analysis was performed with Fisher's exact test. Correlations between low-expression and high-expression groups of TβR1, Smad2, Smad3 and Smad4 with age, sex, BMI, recurrence and side of pneumothorax were analyzed using the chi-square test or Fisher's exact test. P values less than 0.05 were considered statistically significant.

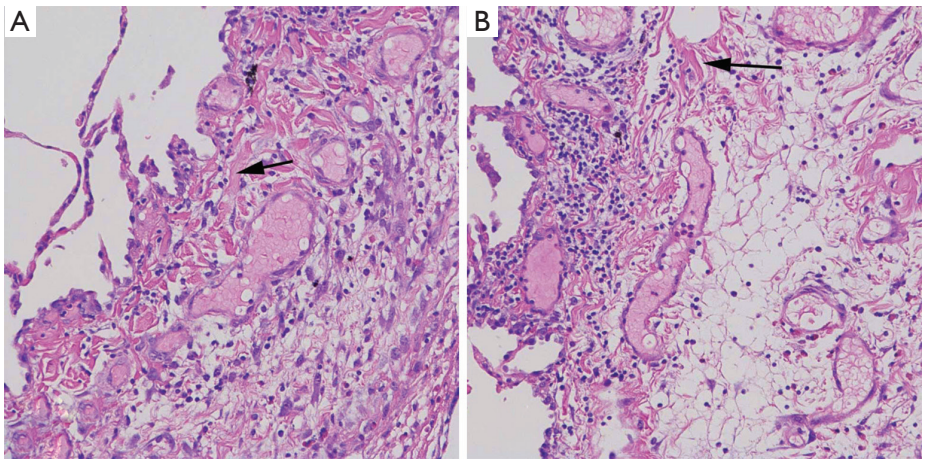

Figure 1 Pulmonary bulla combined with fibrotic representation in H&E staining. (A) and (B) are H&E staining of pulmonary bulla pathological section of different PSP patients (original magnification ×400). The light pink tissue where the arrow points to refers to fibrous tissue.

Results

(I) Pulmonary bulla combined with fibrotic representation in H&E staining

By H&E staining of pulmonary bullae tissue sections, we found a large number of fibrous tissue around the bulla tissue. It suggested that there was frequent representation of pulmonary fibrosis in pulmonary bullae tissue (*Figure 1*).

(II) The expression of TβR1, Smad2, Smad3 and Smad4 at the site of bullae tissue and normal tissues from the same PSP patients

To investigate the expression levels of TβR1, Smad2, Smad3 and Smad4 both in the bullae tissue and in normal tissues of the same PSP patients, the immunohistochemistry staining was performed in specimens of 16 cases. Observing through microscope, the expression of TβR1, Smad2 and Smad4 in the PSP bullae tissue were obviously concentrated staining. We found that TβR1, Smad2 and Smad4 mainly expressed in the alveolar type II cell. TβR1 mostly expressed in the membrane, while a tiny portion of its expression can be observed in the cytoplasm. Smad2 and Smad4 mostly expressed in the cytoplasm as well as in the nucleus. In the contrary, the normal lung tissue around the bullae in the same section showed less obvious staining (*Figure 2*).

The results of Fisher exactness analysis showed that the expression levels of TβR1, Smad2 and Smad4 in pulmonary bullae were significantly higher than that in normal lung tissue (P=0.001, 0.023, 0.015 respectively) (*Table 2*). These results suggested that TβR1, Smad2 and Smad4 highly expressed in the bullae area of patients with PSP. The expression of Smad3 in pulmonary bullae was not showed significantly different from that in normal lung tissues (P=0.172) (*Table 2*). But observation through microscope showed that the coloration intensity and density of Smad3 in the bullae tissue was obviously higher than that in the normal tissues around the bullae. Smad3 mostly expressed in the cytoplasm and the nucleus (*Figure 2*).

(III) Expression of TβR1, Smad2, Smad3 and Smad4 in lung tissue of PSP patients and control group

There were 34 PSP patients included as the study group. And 10 patients without pneumothorax associated disease were selected as the control group. The constituent ratio of TβR1, Smad2, samd3 and Smad4 semi-quantitative score reaching "++" of the study group were significantly higher than that of the control group (*Table 3*).

The IRS scores of TβR1, Samd2 and Smad4 of the study group were significantly higher than that of the control group, and the differences were statistically significant (P=0.012, 0.031, 0.000 respectively) (*Table 4*). Observing through microscope, the expression of TβR1, Smad2 and Smad4 in the PSP bullae tissue were obviously concentrated staining. We found that TβR1, Smad2 and Smad4 mainly

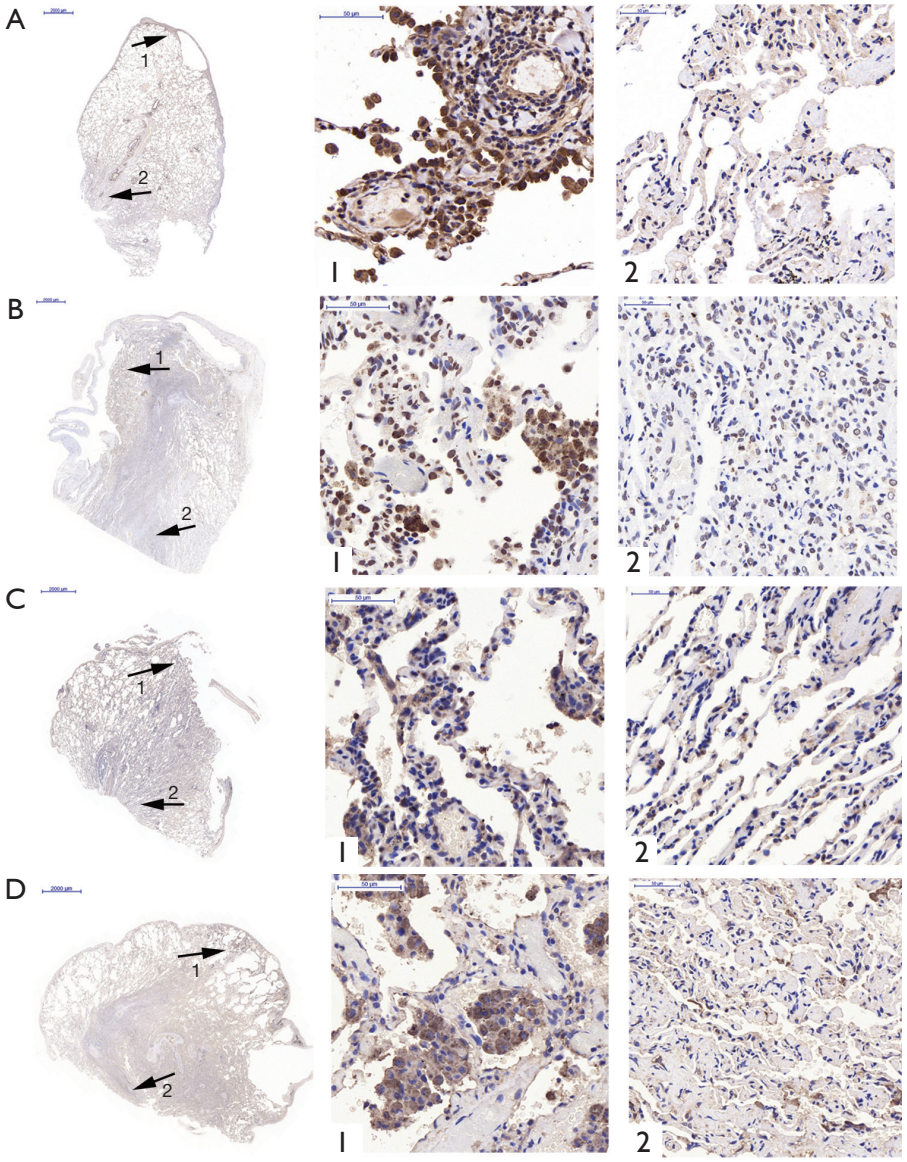

Figure 2 Expressions of TβR1, Smad2, Smad3 and Smad4 in PSP patients. Expressions of TβR1, Smad2, Smad3 and Smad4 in PSP patients are detected by IHC staining SP method. (A-D) Pictures are pulmonary bullae from different PSP patients. Pictures 1 and 2 represent 1 and 2 position (arrow) in the left picture (A-D). (A) It showed that TβR1 mainly expressed in the alveolar type II cell. It mostly expressed in the membrane, while a tiny portion of its expression can be observed in the cytoplasm. The pulmonary bullae tissue which can be seen obviously concentrated staining was strong positive (original magnification ×400, picture 1). In the contrary, the normal lung tissue around the bullae in the same section shows less obvious staining than the bulla tissues. (original magnification ×400, picture 2); (B) it showed that Smad2 mainly expressed in the alveolar type II cell. It mostly expressed in the cytoplasm and the nucleus. The pulmonary bullae tissue which can be seen obviously concentrated staining was strong positive (original magnification ×400, picture 1). In the contrary, the normal lung tissue around the bullae in the same section shows less obvious staining than the bulla tissues. (original magnification ×400, picture 2); (C) the Smad3 expressed in pulmonary bullae tissue and normal lung tissue of PSP patients, but the coloration intensity and density of Smad3 in bullae tissue was higher than that in normal lung tissue. The Smad3 mostly expressed in the cytoplasm and the nucleus; (D) it showed that Smad4 mainly expressed in the alveolar type II cell. It mostly expressed in the cytoplasm and the nucleus. The pulmonary bullae tissue which can be seen obviously concentrated staining was strong positive (original magnification ×400, picture 1). In the contrary, the normal lung tissue around the bullae in the same section shows less obvious staining than the bulla tissues (original magnification ×400, picture 2). The scale bar showed in (A-D) is 2,000 μm. The scale bar showed in picture 1 and 2 is 50 μm.

Table 2 The expression levels of TβR-1, Smad2, Smad3 and Smad4 both in the bullae tissue and in normal tissues

Cytokine	Research object	Positive expression (+)	Negative expression (–)	P value
TβR-1	Pulmonary bullous	16	0	0.001
	Normal lung tissue	3	13	
Smad2	Pulmonary bullous	14	2	0.023
	Normal lung tissue	7	9	
Smad3	Pulmonary bullous	15	1	0.172
	Normal lung tissue	11	5	
Smad4	Pulmonary bullous	15	1	0.015
	Normal lung tissue	8	8	

The pulmonary bullous group and the normal lung tissue group come from the same PSP patients. PSP, primary spontaneous pneumothorax. P<0.05 was considered statistically significant.

Table 3 Constituent ratio of expression levels of TβR-1, Smad2, Smad3 and Smad4 in PSP bullous tissue and control group

Cytokine	Group	High expression (++)*	Constituent ratio (%)
TβR-1	Research group	24/34	70.59
	Control group	4/10	40.00
Smad2	Research group	24/34	70.59
	Control group	5/10	50.00
Smad3	Research group	22/34	64.71
	Control group	4/10	40.00
Smad4	Research group	29/34	85.29
	Control group	4/10	40.00

*, represents high expression defined as the IRS score of >4. The research group was PSP pulmonary bullous group; the control group was normal lung tissue group. PSP, primary spontaneous pneumothorax.

Table 4 Expression of TβR-1, Smad2, Smad3 and Smad4 in lung tissue of PSP bullous tissue and control group (IRS)

Cytokine	Group	IRS ($\bar{x}\pm s$)	*t*	P value
TβR-1	Research group	5.5118±2.6474	2.622	0.012
	Control group	3.1200±2.0767		
Smad2	Research group	6.1529±2.9394	2.284	0.031
	Control group	4.4400±1.7558		
Smad3	Research group	4.2529±1.7193	1.503	0.140
	Control group	3.2600±2.2132		
Smad4	Research group	5.5235±1.8297	4.767	0.000
	Control group	3.4600±0.9430		

The research group was PSP pulmonary bullous group; the control group was normal lung tissue group. PSP, primary spontaneous pneumothorax; IRS, immunoreactive score. P<0.05 was considered statistically significant.

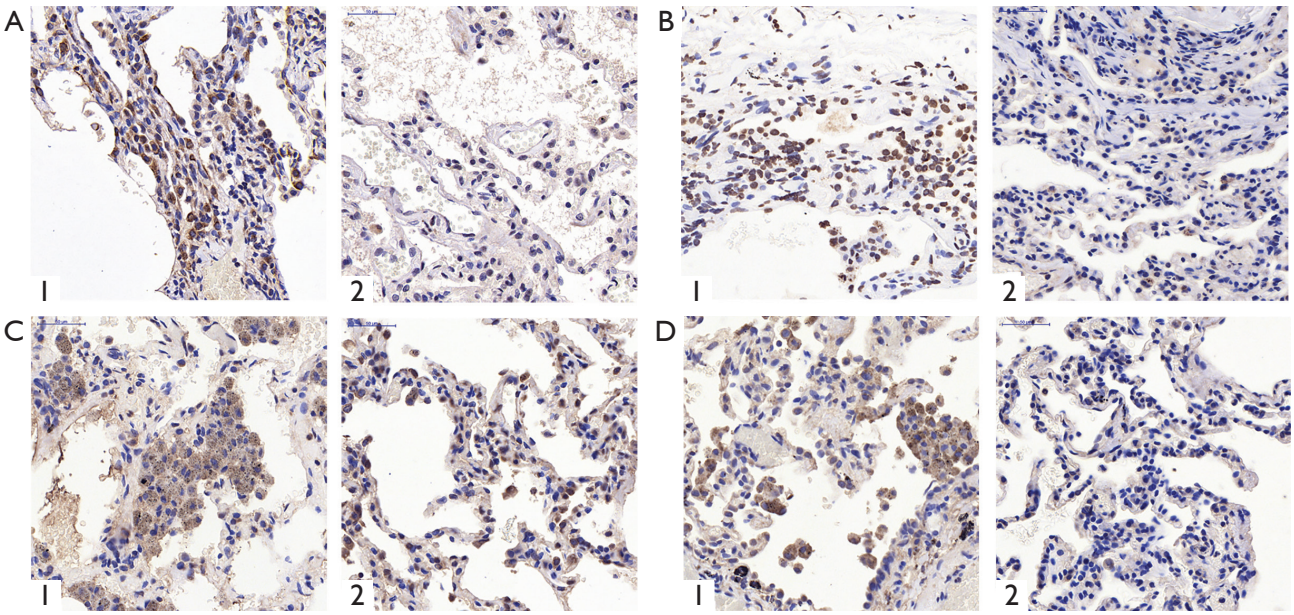

Figure 3 Expressions of TβR1, Smad2, Smad3 and Smad4 in PSP bullae tissue and normal lung tissue of the control group. Expressions of TβR1 (A), Smad2 (B), Smad3 (C) and Smad4 (D) in PSP bullae tissue and the normal lung tissue of the control group are detected by IHC staining SP method. Picture 1 represents the expression of these cytokines in the pulmonary bullae tissue. Picture 2 represents the expression of these cytokines in the normal lung tissue of the control group. (A) It showed that TβR1 mainly expressed in the alveolar type II cell. It mostly expressed in the membrane, while a tiny portion of its expression can be observed in the cytoplasm. The pulmonary bullae tissue which can be seen obviously concentrated staining was strong positive (original magnification ×400, picture 1). In the contrary, the normal lung tissue of the control group shows less obvious staining than the bullae tissue (original magnification×400, picture 2); (B) it showed that Smad2 mainly expressed in the alveolar type II cell. It mostly expressed in the cytoplasm and the nucleus. The pulmonary bullae tissue which can be seen obviously concentrated staining was strong positive (original magnification ×400, picture 1). In the contrary, the normal lung tissue of the control group shows less obvious staining than the bullae tissue (original magnification×400, picture 2); (C) the Smad3 expressed in pulmonary bullae tissue of PSP patients and normal lung tissue of the control group, but observation through microscope showed that the coloration intensity and density in the bullae tissue was higher than that in the normal lung tissue of the control group. The Smad3 mostly expressed in the cytoplasm and the nucleus; (D) it showed that Smad4 mainly expressed in the alveolar type II cell. It mostly expressed in the cytoplasm and the nucleus. The pulmonary bullae tissue which can be seen obviously concentrated staining was strong positive (original magnification ×400, picture 1). In the contrary, the normal lung tissue of the control group shows less obvious staining than the bullae tissue (original magnification ×400, picture 2). The scale bar showed in picture (A-D) is 50 μm.

expressed in the alveolar type II cell. TβR1 mostly expressed in the membrane; while a tiny portion of its expression can be seen in the cytoplasm. Smad2 and Smad4 mostly expressed in the cytoplasm as well as in the nucleus. In the contrary, the normal lung tissue of the control group showed less obvious staining than the bulla tissues (*Figure 3*). These results indicated that the expression of TβR1, smd2 and Smad4 of the study group were higher than that in the control group. The IRS score of Smad3 of the study group was higher than that of the control group, but the difference was not statistically significant (P=0.140) (*Table 4*). But observation through microscope showed that the coloration intensity and density of Smad3 in the bullae tissue was obviously higher than that in the normal tissues of the control group (*Figure 3*).

Those results suggested that TβR1, Smad2 and Smad4 highly expressed in the bullae tissues of PSP patients.

(IV) The association of expression levels of TβR1, Smad2 and Smad4 and the clinical characteristics of PSP patients

The expression of TβR1, Smad2, Smad3 and Smad4 were not statistically correlated with the clinical characteristics of PSP patients mentioned below, such as age, sex, BMI,

Table 5 The relationship between the expression level of each protein in PSP bullae and the clinical characteristics of patients

Variables	Item	Constituent ratio (%)	TβR-1	Smad2	Smad3	Smad4
Age	<23 years	52.94	0.743	0.745	0.427	0.378
	≥23 years	47.05				
Sex	Male	79.41	0.596	0.836	0.956	0.596
	Female	20.59				
BMI	Abnormal	14.71	0.453	0.658	0.359	0.827
	Normal	61.76				
Recurrence	Yes	41.12	0.057	0.722	0.938	0.904
	No	55.88				
Side involved	Left	79.41	0.059	0.233	0.236	0.726
	Right	17.65				

PSP, primary spontaneous pneumothorax.

recurrence and side involved (P>0.05) (*Table 5*). It suggested that the expression of cytokines may be not related to these clinical characteristics.

Discussion

According to our results, TβR1, Smad2 and Smad4 highly expressed in pulmonary bullae tissue tested by immunohistochemistry staining, compared with that in normal lung tissues. Thus we speculated that the high expression of TβR1, Smad2, and Smad4 might play a role in the development of pulmonary bullae.

Transforming growth factor beta superfamily is reported to participate in the mediation of cell regulating, growth, differentiation, apoptosis and interstitial synthesis (12). One of its family members, TGF-β1, which is focused on by many researches, is demonstrated to be involved in the induction, initiation and development of pulmonary fibrosis. Das *et al.* found that TGF-β1 had a fibrotic effect on either mice or human lung, by *in vitro* and *in vivo* experiments (13). In addition, anti-fibrosis drug IFN-gamma-1b can significantly reduce the level of TGF-β1 in lung tissue (14). These results hint that TGF-β1 plays an important role in pulmonary fibrosis. The fibrosis effect of TGF-β1 is depends on the TGF-β/Smads signaling conduction pathway (15).

The development of PSP may be related to pulmonary fibrosis. Some studies suggest that the histopathologic feature of PSP lesion is alveolar collapse accompanied by fibroelastosis. And these pathological findings are not secondary to pneumothorax but have existed before the episode of pneumothorax. We have verified this finding by H&E staining experiments of pulmonary bullae tissue (*Figure 1*). One hypothesis is that as thickened pleura with fibroelastosis becomes less compliant, and pleural thickening is uneven, areas of thinner apical visceral pleura, which may occasionally be at the site of subpleural bullae, receive a higher negative pressure and thus become likely to rupture (5). During developing of pulmonary fibrosis, the epithelial-mesenchymal transition (EMT) of fibroblasts is an important mechanism through which fibrous cells are evolved. In alveolar epithelial cells, activated TGF-β mediates EMT by activating the Smad pathway and the non-Smad pathway. At the same time, some studies have shown that inhibitors of Smads pathway can prevent the pulmonary from fibrosis by block the TGF-β/Smads pathway (15,16).

It can be seen that the main components of the TGF-β/Smads signal pathway play an important role in the progression of pulmonary fibrosis. Our results showed that TβR1, Smad2 and Smad4 in the pathways highly expressed in pulmonary bullae tissue, suggesting that the development of PSP bullae may be related to pulmonary fibrosis.

Furthermore, when the immunohistochemistry staining results of bullae tissue of 34 PSP patients were compared to the normal lung tissues of control group, it shows that the expression of TβR1, Smad2 and Smad4 were significantly higher than that of control group (P<0.05). The expression

level of Smad3 was not statistically difference between the study group and the control group, but the absolute value of the expression in the PSP bullae tissue was higher than that in the normal control group. These results suggested that TβR1, Smad2, Smad4 and Smad3 may be related to the development of PSP bullae.

It is thought that TGF-β and its downstream cytokines which highly express in PSP bullae might induce pulmonary fibrosis. TβR is a receptor protein that can bond tightly with TGF-β on the surface of cell membrane. TβR1 and TβR2 are the main receptors involved in TGF-β signal transduction. After activated, TGF-β must be combined with TβR to form the transmembrane complex which can induce fibrosis. As its downstream proteins, Smads family then conducts signaling from the membrane surface into the nucleus. The activated TGF-β firstly binds to TβR1 and TβR2, forming a complex which in turn activates the downstream Smad pathway. After the activation of Smad families, activated Smad2, Smad3 and Smad4 can enter the nucleus and regulate transcription of downstream genes. Smad4 is a core transduction of the TGF-β signaling pathway, and only with the presence of Smad4 can the TGF-β signal be transferred into the nucleus to regulate transcription (17-19). It indicates that TβR1, Smad2, Smad3 and Smad4 play an important role in TGF-β signaling pathway.

In this study, we found that TβR1, Smad2 and Smad4 highly expressed in PSP bullae. Because TβR1, Smad2 and Smad4 are important factors in TGF-β signaling pathway which mediates pulmonary fibrosis, it hints that the development of PSP may be related to pulmonary fibrosis.

In summary, the results of our study suggested that TβR1, Smad2 and Smad4 might play a role in the development of PSP bullae. Yet, the pathogenesis of pulmonary bullae formation in patients with spontaneous pneumothorax is not yet clearly understood, and the associated hypotheses need further study to be confirmed.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was approved by the Institutional Research Ethics Committee of Beijing Chaoyang Hospital, Capital Medical University (ID: 2016-S-121). All the patients signed the informed consent preoperatively. The study outcomes will not affect the future management of the patients.

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