Toll interacting protein (TOLLIP) increases in fibrotic lung tissue of connective tissue disease-associated interstitial lung disease and is associated with clinical outcome

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Research Article

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Abstract

Objective

There is a lack of Toll interacting protein (TOLLIP) histological data in the lung tissue of patients with connective tissue disease-associated interstitial lung disease (CTD-ILD). We aim to demonstrate the features of TOLLIP expression in the lung tissue of CTD-ILD patients and to associate TOLLIP expression with polymorphisms and clinical characteristics.

Methods

Sixteen patients diagnosed with CTD-ILD who underwent lung transbronchial cryobiopsy (TBCB) at our institution between 2015 and 2021 were recruited. TOLLIP was localized in lung TBCB specimens. Rs3750920 was genotyped. Clinical characteristics, CT and pathological scores, and follow-up data were recorded.

Results

TOLLIP expressed in both alveolar and bronchiolar epithelial cells in patients with CTD-ILD. TOLLIP expression significantly increased in fibrotic areas. The distribution of rs3750920 was C/C: 43.7%, C/T:56.3%, T/T: 0%. There was no significant difference in clinical characteristics between C/C and C/T groups. The average follow-up time was 39.61 ± 16.78 months. During the follow-up, the change in G-score of chest thin-section CT (HRCT) was moderately negatively correlated with TOLLIP expression level in alveolar epithelial cells (r=-0.596, P = 0.024) and strongly negatively correlated with TOLLIP expression level in bronchiolar epithelial cells (r=-0.739, P = 0.004).

Conclusions

TOLLIP might play an important role in the regulation of pulmonary fibrosis in CTD-ILD. Patients with higher TOLLIP expression show greater improvement of ground glass opacity on HRCT, which implies TOLLIP expression is associated with prognosis. Further research is warranted to reveal the pathophysiologic mechanisms of TOLLIP in CTD, and identify potential therapeutic strategies to mitigate these diseases.

Introduction

Interstitial lung disease is characterized by progressive breathlessness or persistent non-productive cough, and can ultimately lead to death. It is common for patients with connective tissue disease to present the clinical features of interstitial lung disease [1–4]. To date, the pathogenesis of connective tissue disease-associated interstitial lung disease (CTD-ILD) remains unclear, although multiple factors, including injury and inflammatory and immune response factors, are known to participate in this process [5].

Toll-interacting protein (TOLLIP), an inhibitor of Toll-like receptor (TLR) signalling, can inhibit the inflammatory response in humans. TOLLIP is involved in interleukin (IL)-1 signalling, and ultimately downregulates the production of proinflammatory cytokines during the inflammatory response [6]. Recent studies have recognized the importance of TOLLIP in promoting autophagy and facilitating intracellular trafficking [7–8]. Dysregulation of TOLLIP is associated with various diseases, such as Parkinson's disease, Alzheimer's disease, inflammatory bowel disease, myocardial hypertrophy, and idiopathic pulmonary fibrosis [9–13]. Several studies have focused on the association between genetic variants of *TOLLIP* and susceptibility, treatment response, and the progression of patients with CTD-ILD [14–15]. However, the location of TOLLIP in the lung tissue of patients with CTD-ILD has not yet been established. The effects of TOLLIP on CTD-ILD remain unknown.

The present study is conducted to localize TOLLIP in lung specimens from patients with CTD-ILD. The distribution and effects of rs3750920 genotype will be analysed. We aim to identify any association between TOLLIP expression and polymorphisms, clinical indices, CT and histological scores, and follow-up data. New information on TOLLIP in CTD-ILD could contribute towards improved diagnostic and predictive biomarkers for this disease.

Materials And Methods

Study subjects

Sixteen patients with CTD-ILD who underwent transbronchial lung cryobiopsy (TBLC) at our institution between 2015 and 2021 were recruited for the present study. The diagnosis of CTD-ILD was based on the 2015 European Respiratory Society/American Thoracic Society guidelines. All patients were followed up at our institution. Patient characteristics, including sex, age, treatment, chest CT scan image, smoking history, and clinical variables (such as laboratory autoantibodies and pulmonary function) were recorded.

DNA extraction and genotyping

Genomic DNA was extracted using a High Pure PCR template preparation kit (ONCERT, Xiamen, China) from lung biopsy specimens. Primers were designed according to a previous study as follows [16]: rs3750920F: 5'-AGG CGT GCA GCTCAC CGC GTA GGA-3' and rs3750920R: 5'-GAG AGC CTT CTC CAT GGA CGA CCG C-3'. Real-time PCR was conducted using an Applied Biosystems QuantStudio 3D real-time PCR system (Bio-Rad, Hercules, CA, USA).

Lung tissue analysis

We next localized TOLLIP in the lung tissue of patients with CTD-ILD by performing immunohistochemistry. Serial sections of lung tissue were incubated overnight at 4°C with antibodies for TOLLIP (SAB, Baltimore, USA). At 1:400 and 1:800 dilutions of the primary antibody, TOLLIP was strongly stained in epithelial cells (**Supplementary Figure 1**). To compare differences in TOLLIP expression, we used a 1:1600 dilution of TOLLIP antibody. Immunohistochemical staining was performed according to a previously published

method [17]. Morphological examination was based on standard haematoxylin-eosin staining and immunostaining with TOLLIP.

Evaluation of TOLLIP *ex*pression

Two pathologists observed sections from low to high magnification and distinguished positively stained cells from non-specific staining. The type of positive cell was determined according to their morphology and location. After calibrating the staining intensity, the expression of TOLLIP was evaluated in different cell types. The percentage of positive cells was recorded as the expression ratio. All pathologists were blinded to the genotype and clinical data.

Pathological score evaluation

Lung tissue slides were reviewed by an experienced pulmonary pathologist. The reviewer was blinded to the genotype and clinical data of the patients. Fifteen histological features, including honeycombing (HC), fibroblastic focus (FF), smooth muscle hyperplasia (SMH), organizing pneumonia (OP), cellular interstitial pneumonia (CIP), prominent plasmocytic infiltration (Plasm), lymphoid follicles with germinal centres (LyGC), extensive pleuritis (PLE), vascular intimal thickening (VT), dense perivascular collagen (DPVC), airspace fibrin (AF), fat metaplasia (Fat), constrictive bronchiolitis (CB), bronchiectasis, and bronchiolar metaplasia (BM), were graded as 0 (none), 1 (mild), 2 (moderate), or 3 (marked).

Lung CT evaluation

G-score and F-score of chest thin-section CTs were assessed independently by two experienced pulmonary radiologists. G-score reflects the degree of ground-glass opacity, while F-score reflects the degree of fibrosis. The scoring method used was based on previous studies [18]. The final CT score was the average score of the six lung zones. All radiologists were blinded to genotype and clinical data.

Statistical analysis

All data were assessed for normality using the Kolmogorov–Smirnov test, and are presented as the mean ± SD or median (quartile). Homogeneity of variance was evaluated using Levene's test. Continuous variables were analysed using an unpaired t-test between two groups. Non-normal variables of the two groups were compared using a Mann-Whitney U test. The correlation between clinical data and follow-up data was calculated using Spearman's correlation coefficient. The strength of the correlation was described by the absolute value of r (0.00–0.10: negligible correlation, 0.10–0.39: weak correlation, 0.40–0.69: moderate correlation, 0.70–0.89: strong correlation, 0.90–1.00: very strong correlation). The reliability and reproducibility of parameters for inter- and intra-observer variability were evaluated by intraclass correlation (ICC) (≥ 0.75, excellent; < 0.75 and ≥ 0.40, moderate; and < 0.40, poor). Hardy-Weinberg Equilibrium (HWE) was assess by Chi-square (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). All statistical analysis was performed using SPSS statistical software version 23 for Windows (IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 7.0c; GraphPad Software, San Diego, CA, USA).

Results

Patients

A total of 16 patients with CTD-ILD (mean age 51.30 ± 9.36 years old; male: female ratio = 2:14) were enrolled in the present study. The demographics and characteristics of the study subjects are shown in **Table 2**.

Of all, 3 patients had anti-synthetase syndrome (ASS), 2 had dermatomyositis (DM), 3 had systemic sclerosis (SSc), 3 had Sjogren's syndrome (SS), 3 had IgG4 related disease (IgG4RD), 1 had rheumatoid arthritis (RA) and 1 had undifferentiated connective tissue disease (UCTD).

All recruited patients underwent autoantibody tests, including ANA, anti-SSA, anti-SSB, anti-RNP, anti-Rib, anti-scl70, anti-CCP, and anti-myositis-specific antibodies. Of all, 12 (75%) patients showed antinuclear antibodies positive and 6 (38%) patients showed anti-SSA antibodies positive.

The mean value of FVC was 77.70 ± 20.32 L, while the mean value of DLCO was 59.38 ± 21.04 mL/min/mm Hg. The mean total histological score was 10.40 ± 4.91. The mean G-score was 1.57 ± 0.45, while the mean F-score was 1.52 ± 0.50. The ICC, representing the inter-observer agreement, of the G-score was 0.791, while that of the F-score was 0.859.

Correlation between clinical features

Spearman's correlation coefficient was calculated between the total histological scores and clinical features, including FVC, DLCO, and CT scores. The total histological score was highly correlated with FVC (r=0.803, P=0.016). To explore the relationship between autoantibodies and clinical features, we divided the subjects into anti-SSA positive and anti-SSA negative groups, and compared their clinical features using the Mann-Whitney U test. The G-score of the anti-SSA-positive group was higher than that of the anti-SSA-negative group (P=0.027).

Genotype and allele distribution of rs3750920

The distribution of rs3750920 was C/C: 43.7%, C/T:56.3%, T/T: 0%. MAF of rs3750920 was 0.28. P value of HWE was 0.118. (**Table 1**). The subjects were divided into C/C group and C/T group. No significant differences in clinical characteristics, including sex, age, pulmonary function, CT score, and expression ratio of TOLLIP, were observed between the two groups (**Table 2**).

TOLLIP expression in lung specimens of patients with CTD-ILD

TOLLIP expressed in the alveolar epithelial cells as well as in the bronchiolar epithelial cells (**Fig. 1**). The expression of TOLLIP was significantly increased in fibrotic areas. Low TOLLIP expression was observed in fibroblastic foci and lymphoid follicles.

Correlation between clinical features and TOLLIP expression

Spearman's correlation coefficient was calculated between the expression ratio of TOLLIP and clinical features, including FVC, DLCO, CT scores, and histological scores. There was no correlation observed

between the expression ratio of TOLLIP and these clinical features.

Correlation between clinical features and outcome

The average follow-up time was 39.61±16.78 months. We assessed the clinical outcomes based on changes in CT scores, pulmonary function, survival, and acute exacerbation events. Fourteen patients underwent CT scan re-examination. The average interval time from baseline to re-examination was 7.72 ± 3.77 months. We calculated the correlation between change in CT scores and clinical features, including FVC, DLCO, total histological scores at baseline, and the expression ratio of TOLLIP in the lung. The change in G-score was moderately negatively correlated with the expression ratio of TOLLIP in alveolar epithelial cells (r=-0.596, P=0.024), and was strongly negatively correlated with the expression ratio of TOLLIP in bronchiolar epithelial cells (r=-0.739, P=0.004) (**Table 3**). Patients with higher TOLLIP expression in alveoli and bronchiale show greater improvement in follow-up G-score (**Fig 1**).

Since only five patients underwent re-examination of the pulmonary function test, we did not analyze this small subgroup separately. All subjects survived at the end of follow-up, while 4 (25%) patients suffered from acute exacerbation. The median time to the first acute exacerbation was 22 months. Kaplan–Meier analysis did not show any significant association between acute exacerbation and clinical features.

Discussion

To our knowledge, this is the first study on TOLLIP expression in lung samples from patients with CTD-ILD. We studied the association between TOLLIP and clinical characteristics and indexes, and found that the expression ratio of TOLLIP was negatively correlated with changes in G-score. We further analysed the rs3750920 genotype of *TOLLIP*, and found that the frequency of the minor allele T was lower than the previously reported frequency.

In the present study, the expression of TOLLIP significantly increased in fibrotic areas, in the cytoplasm of both alveolar epithelial cells and bronchiolar epithelial cells. Based on previous reports, TOLLIP widely expressed in the human respiratory tract, including macrophages, primary human nasal, bronchial, and type II alveolar epithelial cells, where diffuse punctate staining was observed in the cytoplasm and cell membrane [19].

Although TOLLIP expression and localization in the lung specimens of patients with CTD-ILD has not been previously reported, the location of TOLLIP protein in IPF has been described in a previous study [20]. This study revealed that TOLLIP abundantly expressed in “aberrant basaloid cells” in the lungs of patients with IPF, whereas the total expression of TOLLIP was lower in patients with IPF than in healthy controls. Co-staining of TOLLIP and E-cadherin confirmed that the positive cells were epithelial cells.

TOLLIP expression in lung tissue of patients with CTD-ILD could be different from patients with IPF. Previous studies have demonstrated that there are some differences in the pathogenesis of pulmonary fibrosis between IPF and CTD-ILD [21–23]. For instance, endothelial cells are primarily involved in SSc-ILD, whereas the injury of alveolar epithelial cells is mainly observed in IPF [22]. Since autoimmunity is an essential

feature of CTD-ILD, immune-mediated inflammation of lung tissue is common, but the mechanisms by which the lung tissue becomes inflamed remain unclear for IPF. Therefore, the assessment of TOLLIP expression in lung tissue of patients with CTD-ILD is necessary.

We hypothesize that the increased expression of TOLLIP observed in fibrotic areas might be associated with its role in pulmonary fibrosis. Previous studies have indicated that TOLLIP participates in the inflammatory response, autophagy, and nuclear interactions [6–8, 24–25]. As an endogenous inhibitor of Toll-like receptor signalling, TOLLIP can prevent pro-inflammatory signalling by binding to IL-1 receptor-associated kinase (IRAK-1) [6]. The negative role of TOLLIP in pathogen-associated inflammation is essential for immune tolerance. Dysfunction of TOLLIP leads to overactivation of the canonical MyD88-dependent TLR2 and TLR4 signalling pathway [26–27]. The pro-fibrotic effect of TLR2 and TLR4 activation is supported by several previous studies [28–29]. The activation of TLR4 enhances the process of fibrosis in the lung by transforming danger signals into myofibroblasts, and increasing the secretion of ECM molecules [29]. Another crucial role of TOLLIP is the promotion of autophagy, which it facilitates by bridging ubiquitinated protein aggregates with LC3 and inhibiting the phosphorylation of vacuolar protein sorting 34 (VPS34), an important protein involved in autophagy [30]. Impaired autophagy and excessive ROS generation contribute to IPF progression. Anti-fibrotic agents can attenuate fibrosis in bleomycin-exposed mice by improving autophagy [31]. Although the exact molecular mechanisms remain unclear, mounting evidence suggests that TOLLIP is a protective regulatory protein in pulmonary fibrosis. Therefore, the observed TOLLIP overexpression in the lung specimens of the present cohort might be a compensatory response to pulmonary fibrosis associated with CTD.

We observed a significant correlation between the change in G-score and TOLLIP expression in both alveolar epithelial cells and bronchiolar epithelial cells, indicating that patients with higher expression of TOLLIP had better improvement of ground glass opacity signs during follow-up by CT. This correlation was not identified with the change in F-score which evaluates fibrosis and TOLLIP expression. It is possible that TOLLIP could protect lung tissue from fibrosis in the early stages of the disease. In the present study, there was no correlation between TOLLIP expression and histological patterns of ILD or CTD diagnosis, which implies that increased TOLLIP expression is closely related to pulmonary fibrosis, regardless of the cause.

TOLLIP is associated with some clinical characteristics, the treatment response, and prognosis of ILD. A genome-wide association study (GWAS) correlated additional common variants with susceptibility and mortality of IPF in three stages. Three *TOLLIP* SNPs (rs111521887, rs5743894, and rs5743890) were identified in this GWAS, of which the minor alleles rs111521887_G and rs5743894_G presented a risk for IPF [32]. The minor allele rs5743890_G was protective for susceptibility to IPF, but was also a risk factor for mortality, whereas the major allele rs5743890_A affected the progression of IPF. Interestingly, this study demonstrated an association between *TOLLIP* SNPs and TOLLIP expression. Compared with homozygous carriers of common alleles, TOLLIP expression decreased in the lung tissue of minor allele carriers, by 20% in patients with rs5743890_G, by 40% in those with rs111521887_G, and by 50% in those with rs5743894_G. The decreased TOLLIP expression observed in minor allele carriers indicates that TOLLIP deficiency might contribute to the pathogenesis of IPF.

In addition to GWAS, some clinical retrospective studies have also demonstrated the function of TOLLIP in IPF. A retrospective study demonstrated that the minor allele (C) in *TOLLIP* rs5743890 was associated with worse survival and disease progression in patients with IPF [33]. In a post-hoc exploratory analysis of patients enrolled in the clinical trial “Evaluating the Effectiveness of Prednisone, Azathioprine, and N-Acetylcysteine in Patients with Idiopathic Pulmonary Fibrosis”, a significant correlation was observed between the response to N-acetylcysteine (NAC) therapy and rs3750920 of *TOLLIP* [34]. These results were replicated in an independent cohort of patients drawn from the University of Chicago (UChicago) and “The INSPIRE Trial: A Study of Interferon Gamma-1b for Idiopathic Pulmonary Fibrosis”, which included 405 individuals from the two cohorts, and a significant interaction between NAC therapy and rs3750920 was confirmed. In another retrospective study of patients with IPF and IPAF, rs3750920 T/T showed an increased frequency in the positive ANA group, in which NAC exposure seemed to be beneficial for transplant-free survival [35]. In a Japanese population study, the frequency of the minor T allele of rs3750920 was low in patients with fibrotic ILD, especially in non-IPF patients, which might be an indicator of better survival in these patients [14]. In an inception cohort study on early rheumatoid arthritis (RA), *TOLLIP* rs111521887 was associated with pulmonary fibrosis in RA [36].

In the present study, we analysed the association between the rs3750920 genotype distribution and clinical characteristics. The frequencies of C/C and C/T were 43% and 57%, respectively, whereas T/T was not identified. MAF was 0.28, which was obviously lower than that from 1000 Genomes data. A Japanese study on rs3750920 genotype of patients with fibrotic ILD showed C/C, C/T, and T/T frequencies of rs3750920 were 63%, 30%, and 7%, respectively. We hypothesize that the minor T allele of rs3750920 may present a lower frequency in Asian patients with fibrotic ILD. No association between rs3750920 and CTD-ILD progression was observed in the present study.

The critical function of TOLLIP has been evaluated in several diseases, including neurodegenerative diseases, pulmonary diseases, cardiovascular diseases, gastrointestinal diseases, and the pathogen immune system response [9–13, 37–38]. The role of TOLLIP in CTD remains unclear. However, the genotypes of rs3750920 might be associated with the presence of ANA in patients with fibrotic ILD [35]. In *in vivo* animal model research, SSc individuals presented high TLR4 expression in skin fibrosis, and might show an optimal therapeutic response to selective inhibitors of TLR4 [39]. Based on previous studies and the functional importance of TOLLIP, we postulate that it may also participate in the pathogenesis of rheumatoid diseases, which warrants ongoing investigation.

The present study has several limitations. First, the small sample size limits the association analysis and the generalizability of the results. Second, the disease severity of the recruited participants was mild, so they were able to withstand TBLC. This could explain why all patients survived during the follow-up period. Finally, although we observed increased TOLLIP expression in patients with CTD-ILD and associated this with clinical characteristics, further *in vivo* and *in vitro* studies are required to explore the mechanisms by which TOLLIP participates in CTD-ILD.

Conclusion

TOLLIP highly expressed in the fibrotic areas of lung tissue from patients with CTD-ILD. Higher TOLLIP expression was associated with better improvement of ground glass opacity on HRCT. The frequency of rs3750920_T was less than that reported by other studies, suggesting that the number of carriers of the minor T allele of rs3750920 might be fewer in Asian populations. Considering the immunoregulatory role of *TOLLIP*, further research is warranted to unveil the pathophysiological mechanisms of *TOLLIP* in CTD, and to identify novel therapeutic strategies to mitigate these diseases.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from all subjects. The study is complied with the Declaration of Helsinki and is approved by the ethics committee of West China Hospital (No. 779 in 2019).

Consent for publication

All authors have agreed to be so listed and have seen and approved the manuscript for publication.

Availability of data and materials

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

Competing interests

The authors have declared no conflicts of interest.

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Author's contributions

Jiang conceived and guided this study. Cui searched the literature; Li and Zou did the lung tissue analysis; Jiang and Li did the TOLLIP expression evaluation; Cai and Lyu did the CT score evaluation; Cui organized data.

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Tables

Table 1 The genotype and allele frequency of rs3750290 in the study cohort

SNP	Chr.	Position	Gene	Genotype（n）			Alleles(n,AF%)		MAF	HWE p value	MAFc
				CC	CT	TT	C	T			
rs3750920	11	1288726	TOLLIP	7	9	0	23 (71.9)	9 (28.1)	0.28	0.118	0.36

MAF: minor allele frequency; AF: allele frequency; HWE：Hardy-Weinberg Equilibrium; MAFc: MAF from 1000 Genomes data.

Table 2 Demographics and characteristics of the study participants

Variables	C/C (n=7)	C/T (n=9)	P value
Age (year)	48.33±10.37	44.75±11.62	0.623
Sex (M/F)	1/6	1/8	
Smoker (y/n)	1/6	1/8	
CTD diagnosis*			
ASS	1	2	
DM	1	1	
SSc	2	1	
SS	2	1	
IgG4RD	1	2	
RA	0	1	
UCTD	0	1	
Pulmonary function			
FVC (L)	80.20±24.99	73.53±12.33	0.688
DLCO(mL/min/mmHg)	69.66±16.04	42.24±18.19	0.067
CT scores			
G-score	1.64±0.50	1.51±0.43	0.423
F-score	1.67±0.48	1.39±0.50	0.122
Expression ratio of TOLLIP			
Alveoli	22.85±39.03	28.33±29.79	0.292
Bronchiale	18.57±36.70	32.50±34.94	0.307
Treatment			
Prednisolone	5 (71%)	5 (55%)	
NAC	4 (57%)	5 (55%)	
Nintedanib	0	0	
Pirfenidone	1 (14%)	2 (28%)	

SS: Sjögren's syndrome; ASS: Anti-synthetase syndrome; UCTD: Undifferentiated connective tissue diseases; SSc: Systemic sclerosis; IgG4RD: IgG4 related disease; DM:dermatomyositis. RA: rheumatoid arthritis. FVC: Forced vital capacity; DLCO: Diffusing capacity for carbon monoxide. CT: Computed tomography; G-scores: Ground glass opacity score; F-scores: Fibrosis score; NAC: N-acetylcysteine.

Table 3 Association between expression ratio of TOLLIP and CT score changes

Variables	ΔG-Score		ΔF-Score	
	r	P value	r	P value
Expression ratio of TOLLIP				
Alveoli	-0.596	0.024*	-0.324	0.258
Bronchiale	-0.739	0.004*	-0.407	0.167

ΔG-scores: The change of ground glass opacity score; ΔF-scores: The change of fibrosis score; *P<0.05

Figures

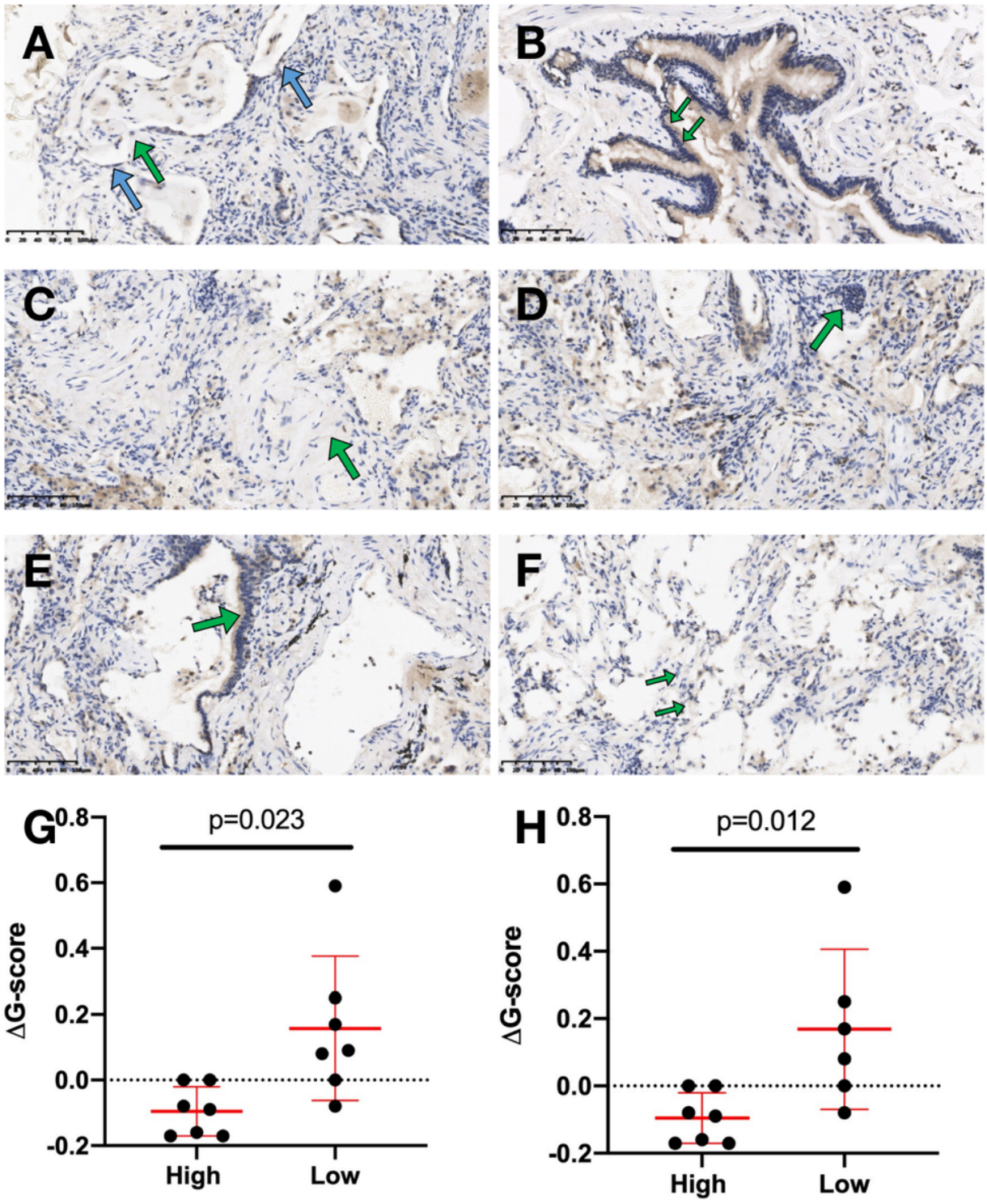

Figure 1

TOLLIP expression in bronchial mucosa and alveolar cells in patients with CTD-ILD. Section of lung tissue from a patient with CTD-ILD show high TOLLIP expression in type 1 (blue arrows) and type 2 (green arrow) alveolar epithelial cells (A). Strong staining in bronchial mucosa (green arrow) associated with fibrotic regions in patients with CTD-ILD (B). Section of lung tissue from patients with CTD-ILD show low TOLLIP expression in fibroblastic foci (C, green arrow) and lymphoid follicles (D, green arrow). High TOLLIP expression in distal small airways (E, green arrow) and fibrotic regions (F, green arrow) can be also observed.

Patients with higher TOLLIP expression in alveoli (G) and bronchiale (H) show greater improvement in follow-up G-score.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfigure1.docx