Effect of genotype on the disease course in idiopathic pulmonary fibrosis despite antifibrotic treatment

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Received January 1, 2021; Accepted July 6, 2021

DOI: 10.3892/br.2021.1463

Abstract. A genetic predisposition has been identified in 30% of idiopathic pulmonary fibrosis (IPF) cases. Although it is highly probable that the genotype affects the disease susceptibility and course in almost all patients, the specific genotype goes undetected. The aim of the present study was to explore the effects of variants of the genes encoding interleukin-4 (IL-4), mucin 5B (MUC5B), toll interacting protein (TOLLIP), surfactant protein A (SFPTA), transforming growth factor-β (TGF-β) and transporters associated with antigen processing (TAP1 and TAP2) on the course of IPF. A total of 50 patients with IPF were enrolled, and variants of these genes were assessed. Lung function at the time of diagnosis and after 6, 12 and 18 months, and the number of acute exacerbations and deaths in each observation period were measured. ANOVA was used to test the association between gene polymorphisms and the decrease in lung function. There was no significant effect of the gene polymorphisms on the outcomes of patients up to 6 months during the observation period. After 12 months, an effect of an IL-4 single nucleotide polymorphism (SNP) (rs 2070874) on patient outcomes was observed [relative risk (RR) for T allele: 5.6; 95% confidence interval (CI), 0.79-39.0; P=0.053]. The RR of progression in patients with the IL-4 SNP (rs 2243250) and the CT and TT genotypes was 4.3 (95% CI, 1.1-17.5; P=0.046). A total of 18 months after the diagnosis of IPF, an effect of the TOLLIP polymorphism on patient outcome was detected (rs 111521887; risk allele GC; RR: 7.2; 95% CI, 0.97-53.6; P=0.052). Thus, IL-4 and TOLLIP gene polymorphisms may represent disease course-modifying factors, but not drivers of IPF.

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Key words: idiopathic pulmonary fibrosis, gene variants, interleukin 4, toll interacting protein

Introduction

Genetic predispositions and environmental gene interactions serve crucial roles in the pathogenesis of various interstitial lung diseases, including idiopathic pulmonary fibrosis (IPF) (1-3). The most often accepted concept is related to genetically based susceptibility, which may or may not lead to impairment of the alveolar epithelium, followed by the development of lung fibrosis due to the effect of extrinsic factors, such as smoking and inhalation of pollutants in the environment (4).

Various studies have demonstrated that polymorphisms of several genes, including mucin 5B (MUC5B), genes associated with telomere integrity and genes encoding surfactants increase the risk of IPF (5-7). However, a genetic predisposition has been identified in 30% of cases, whereas in the remainder of cases, this information is lacking (8). The course of IPF is as heterogeneous as the genetic susceptibility to the development of the disease. Thus, the individual genetic background may affect both the predisposition to IPF and its course (9).

The association between the MUC5B rs 35705950 minor allele and IPF risk in the non-Hispanic white population has been published elsewhere (10). The effect of the MUC5B polymorphism on IPF outcomes has been also debated in the literature. While surfactant protein A (SFTPA) plays an important role in pulmonary host defense and repair processes in the lung parenchyma, transforming growth factor-β (TGF-β) plays a crucial role in different types of fibrotic interstitial pneumonia, including IPF (11,12). Toll interacting protein (TOLLIP) is an adaptor protein that acts as an inhibitory factor in TLR signaling. Noth *et al* (13) identified three TOLLIP SNPs associated with IPF risk. Transporters associated with antigen processing (TAP)-1 and TAP-2 gene polymorphisms are susceptibility factors in patients with hypersensitivity pneumonitis (14). TAP is crucial for antigen processing, delivering cytosolic proteins into the endoplasmic reticulum, where they bind to nascent major histocompatibility complex I (15). For both IL-4

rs 2070874 and rs 2243250, the T allele has been reported less frequently than the C allele in the European population (16). The T allele was reported to be associated with enhanced IL-4 production and was found to be associated with the response to infections, tumor susceptibility and immune-mediated disorders (17). IL-4 plays an important role in both the innate and adaptive immune responses. Both basophils and natural killer T-cells have been suggested as primary sources of IL-4 in humans (18). Impairment of alveolar epithelial cells leads to the induction of alarmin production, stimulating basophils and leading to IL-4 secretion. The major role of IL-4 concerns not only the induction of a Th2 response, but also the stimulation of dendritic cells to present antigens to other immune cells and alternative activation of macrophages. These IL-4-activated macrophages produce matrix metalloproteinase 12, a collagenolytic factor that may induce a fibrotic response to injury (19).

In the present study, whether gene variants of interleukin-4 (IL-4), MUC5B, toll interacting protein (TOLLIP), surfactant protein A (SFPTA), transforming growth factor-β (TGF-β) and transporters associated with antigen processing (TAP1 and TAP2) play a role in the IPF course was investigated.

Materials and methods

Patients and study design. A total of 50 consecutive patients (median age 69.3; age range, 57-82 years); 24 males and 26 females, 38 of which were non-smokers) with IPF were enrolled in the present prospective observational study after providing signed informed content; refusal to take part in the study and refusal or unable to sign/obtain informed consent were the only exclusion criteria. The study was approved by the ethics committee of Thomayer Hospital and the Institute of Clinical and Experimental Medicine, Prague, Czech Republic. The diagnosis of IPF was based on the ERS/ATS/ALAT/JRS guidelines (20). All of the patients underwent a historical assessment focused on possible extrinsic exposures, a physical examination, blood tests including autoantibody tests, high-resolution computed tomography of the chest, including the quantification of inflammatory and fibrotic changes (high-resolution computed tomography alveolar score 0.1±0.3, high-resolution computed tomography interstitial score 2.4±0.8), lung function tests, bronchoscopy with bronchoalveolar lavage (cell percentages in retrieved fluid: Alveolar macrophages 70.4±19.7; lymphocytes 10.7±10.0; neutrophils 12.1±11.8; and eosinophils 6.5±8.6) and multidisciplinary team consultation, as is routine (21). Either surgical lung biopsy or transbronchial cryobiopsy was performed in select cases when the multidisciplinary team could not reach a diagnosis and the patient was willing and able to undergo any of these procedures.

Patients diagnosed with IPF provided blood for DNA analysis at the time of enrollment in the study. Participation in this study did not affect the management of the patients, and all patients received appropriate treatment for IPF according to their baseline lung functions, comorbidities and patient preferences. IPF treatment guidelines were followed (20).

Lung function and outcome assessment. The diffusing capacity for carbon monoxide (Dlco) was investigated using a ZAN 300 CO diffusion instrument (nSpire Health GmbH). The Dlco was measured using the single-breath method (22). Values are presented as percentages of the predicted values (23).

The patients' health status, including lung function, were re-assessed after 6, 12 and 18 months. At these time points, the patients' lung function was assessed, and data concerning the occurrence of acute exacerbations were collected.

IPF progression was defined as either a drop in the forced vital capacity (FVC) >10% and/or a drop in the Dlco >15% and/or an acute exacerbation and/or death within 6 months. The percentages of change in the FVC/Dlco were determined based on the absolute values of the previous results. Each patient with a >10% FVC decline and/or a >15% Dlco decline underwent a complete evaluation to exclude a non-IPF based etiology of deterioration (24).

Genotyping. A panel of single-nucleotide polymorphisms (SNPs) in genes associated with immune function and candidate loci for IPF susceptibility were selected for the present study (Table SI). Selection was based on previous studies published by our study team and a literature review with an emphasis on antigen processing (25). The selected SNPs were investigated in patients with IPF using MALDI-TOF MS-based MassARRAY (Agena Bioscience, Inc.) or TaqMan (Thermo Fisher Scientific, Inc.) genotyping assays. For positive controls, DNA samples were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (Coriell DNA nos. NA16689, NA17084 and NA17252). Primers were designed using Assay Design Suite version 2.2 (Agena Bioscience, Inc.), available only for registered users of the MassARRAY system.

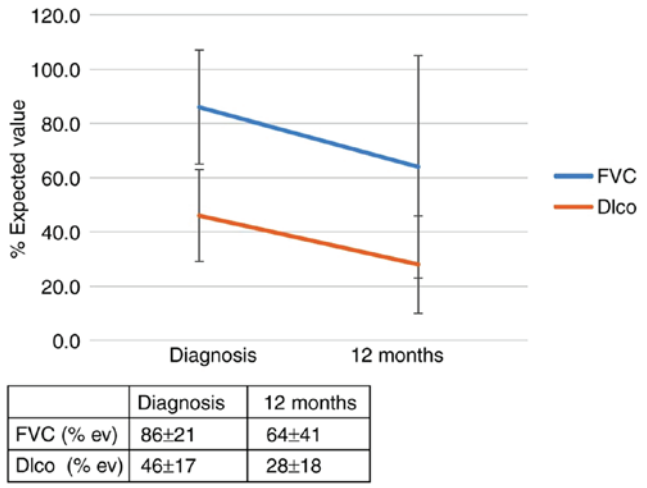
The detailed procedures for the MassARRAY-based multiplexed assay design and iPLEX-based genotyping have been described previously (26). Briefly, the DNA template (10 ng) with multiplex primers and PCR mix (iPLEX® Gold Reagent Set; Agena Bioscience) were used for single-base extension-based PCR amplification of the target region. The iPLEX-extended amplicons were spotted on the SpectroCHIP Array using the MassARRAY® Nanodispenser RS 1000 station and were genotyped using the MassARRAY platform. The distinct mass of the extended primer for the alternative alleles was traced using MassARRAY Typer version 4.0.20. For TaqMan-based genotyping, custom TaqMan® primer probes (labeled with either VIC® or 6-FAM™ dye) were designed for the SNPs *TOLLIP* rs111521887 and *TOLLIP* rs5743894 using the online Custom TaqMan® Assay Design Tool (Thermo Fisher Scientific, Inc.), and predesigned assays for C_16176216_10 and C_11464118_30 were used to genotype *IL-4* rs2243250 and *TGF-β1* rs1800471 (Thermo Fisher Scientific, Inc.), respectively, in the subjects using standard protocols. Specifically, 10 ng template DNA, LightCycler 480 Probes Master (Roche Diagnostics) and the custom TaqMan SNP genotyping assay were used. The thermocycling conditions on the LightCycler 480 system (Roche Diagnostics) were: 5 min at 95˚C; followed by 45 cycles of 95˚C for 10 sec, 60˚C for 45 sec and 72˚C for 1 sec (acquisition was performed each cycle) and then 40˚C for 30 sec. The data was obtained by endpoint analysis (27).

For assessment of the quality of the genotyping protocol, positive and negative template control samples were

Table I. Lung function at the time of diagnosis, and after 6, 12 and 18 months, as well as counts of acute exacerbations and deaths in each observation period.

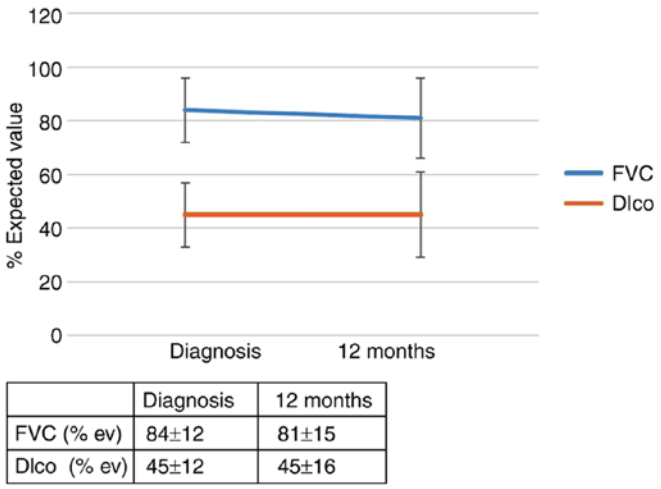
Time after diagnosis, months	Patient, n	FVC, l[a]	FVC, % pv[a]	Dlco, mmol/kPa/min[a]	Dlco, % pv[a]	Acute exacerbation, n	Death, n
0	50	2.8±0.7	81.0±14.8	3.7±1.6	44.2±11.8	0	0
6	28	2.8±0.8	83.4±17.3	3.8±1.0	46.0±16.9	0	0
12	27	2.7±0.7	79.8±14.4	3.65±4.6	43.6±13.5	2	4
18	16	2.8±0.7	77.6±14.4	3.09±1.8	46.8±11.8	0	2

[a]Mean ± standard deviation. FVC, forced vital capacity; pv, predicted value; Dlco, diffusing capacity for carbon monoxide.



	Diagnosis	12 months
FVC (% ev)	86±21	64±41
Dlco (% ev)	46±17	28±18

Figure 1. Significant effect of T allele (interleukin-4, rs 2070874) on lung function decline (FVC and Dlco) after 12 months. FVC and Dlco are expressed as a percentage of the predicted value, mean ± standard deviation. FVC, forced vital capacity; Dlco, diffusing capacity for carbon monoxide.



	Diagnosis	12 months
FVC (% ev)	84±12	81±15
Dlco (% ev)	45±12	45±16

Figure 2. Effect of the CC genotype (interleukin-4, rs 2070874) on lung function decline (FVC and Dlco) after 12 months. FVC and Dlco are expressed as a percentage of the predicted value, mean ± standard deviation. FVC, forced vital capacity; Dlco, diffusing capacity for carbon monoxide.

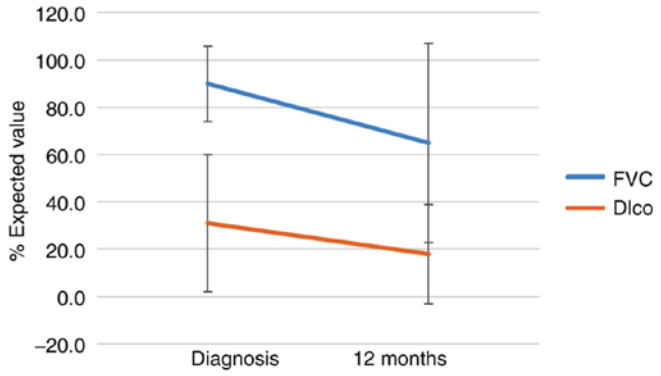
included in each assay plate. For each SNP, the genotype data were manually verified using the call cluster plot (26). Measurements were performed in duplicates. The datasets generated and analyzed during the current study are available in the European Variation Archive (EVA) repository (ebi.ac.uk/ena/data/view/PRJEB44734).

Statistical analysis. A repeated measures ANOVA (with data exhibiting sphericity) and grouping factors was used to test the association between the gene polymorphisms and lung function decline at the 6-, 12- and 18-month follow-up assessments (FVC and Dlco). Bonferroni corrections were applied for multiple comparisons. Two-way frequency tables were analyzed using Fisher's exact test to assess an effect of nucleotide polymorphisms on patient outcomes. Relative risk (RR, calculated by dividing the percentage of subjects with progression for each allele) with 95% confidence intervals (CI) were calculated for 'risk' alleles in comparison with 'protective' alleles (MedCalc Software, Ltd., version 14.8.1). All tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference. Comparisons with $P<0.06$ are considered 'clinically significant'. Associations between gene polymorphisms and lung functions are graphically illustrated, documenting FVC and Dlco at the time of diagnosis [mean ± standard deviation(SD)] and FVC and Dlco at the time for which effect of gene polymorphisms were found (mean ± SD).

Results

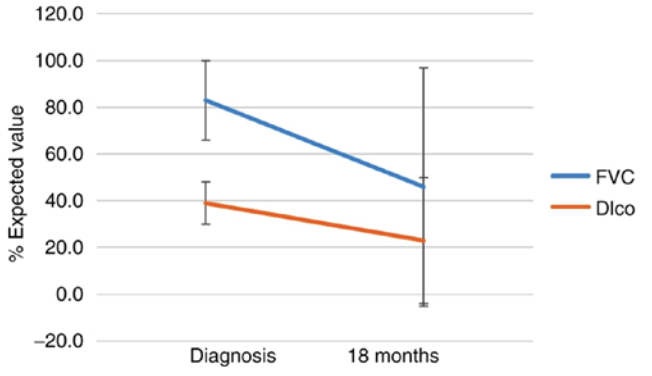
The lung function at the time of diagnosis, and after 6, 12 and 18 months, as well as the counts of acute exacerbations and deaths in each observation period are summarized in Table I. An acute exacerbation was defined following criteria published by Collard *et al* (28).

No effect of the selected gene polymorphisms during the observation period was found on the patient outcomes in the first 6 months. However, 12 months after diagnosis, an effect of the IL-4 SNP (rs 2070874) on patient outcomes was observed; RR of progression for the T allele: 5.56 (95% CI, 0.79-39.0; $P=0.053$). The RR of progression in patients with the IL-4 SNP (rs 2243250) and the CT and TT genotypes (allele CC was found to be protective) was 4.3 (95% CI, 1.1-17.5; $P=0.046$). The effect of the genotype IL-4 (rs 2070874) on the outcome of patients with IPF 12 months after diagnosis is summarized in Figs. 1 and 2 and in Table SIIA. The effect of the genotype IL-4 (rs 2243250) on the outcomes of patients with IPF 12 months after diagnosis is shown in Figs. 3 and 4



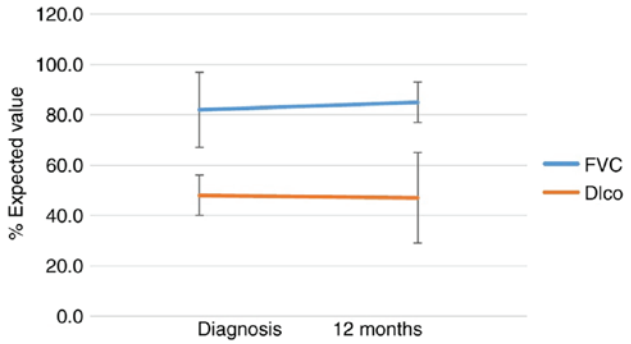
	Diagnosis	12 months
FVC (% ev)	90±16	65±42
Dlco (% ev)	31±29	18±21

Figure 3. Significant effect of the T allele (interleukin-4, rs 2243250) on lung function decline (FVC and Dlco) after 12 months. FVC and Dlco are expressed as a percentage of the predicted value, mean ± standard deviation. FVC, forced vital capacity; Dlco, diffusing capacity for carbon monoxide.



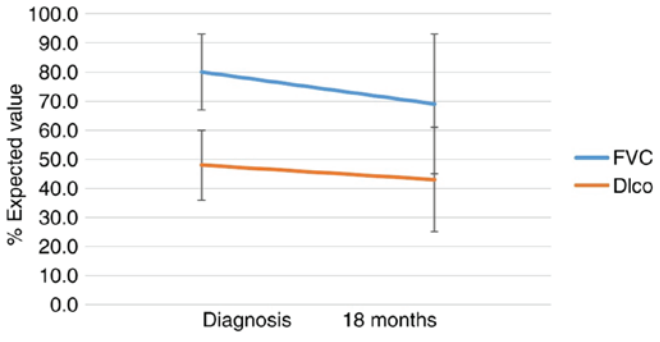
	Diagnosis	18 months
FVC (% ev)	83±17	46±51
Dlco (% ev)	39±9	23±27

Figure 5. Significant effect of G allele (toll interacting protein, rs 111521887) on lung function decline (FVC and Dlco) after 18 months. FVC and Dlco are expressed as a percentage of the predicted value, mean ± standard deviation. FVC, forced vital capacity; Dlco, diffusing capacity for carbon monoxide.



	Diagnosis	12 months
FVC (% ev)	82±15	85±8
Dlco (% ev)	48±8	47±18

Figure 4. Effect of the CC genotype (interleukin-4, rs 2243250) on lung function decline (FVC and Dlco) after 12 months. FVC and Dlco are expressed as a percentage of the predicted value, mean ± standard deviation. FVC, forced vital capacity; diffusing capacity for carbon monoxide.



	Diagnosis	18 months
FVC (% ev)	80±13	69±24
Dlco (% ev)	48±12	43±18

Figure 6. Effect of the CC genotype (toll interacting protein, rs 111521887) on lung function decline (FVC and Dlco) after 18 months. FVC and Dlco are expressed as a percentage of the predicted value, mean ± standard deviation. FVC, forced vital capacity; Dlco, diffusing capacity for carbon monoxide.

and Table SIIB. Call cluster plots showing distribution of rs 2070874, rs 2243250 and rs 111521887 genotypes from mass spectrometry are included in Fig. S1A-C, respectively.

A total of 18 months after the diagnosis of IPF, a clinically significant effect of the TOLLIP polymorphism was detected on patient outcome (rs 111521887, risk allele GC; RR, 7.2; 95% CI, 0.97-53.6; P=0.052). The effect of the genotype of TOLLIP (rs 111521887) on the outcome of patients with IPF 18 months after diagnosis is shown in Figs. 5 and 6 and in Table SII. No relationship was found between the patient outcomes and polymorphisms of the MUC5B, TGF-β, TAP1, TAP2 and SFTPA genes.

Discussion

In the present study, the effects of IL-4, MUC5B, TGF-β, TOLLIP, TAP1, TAP2 and SFTPA gene polymorphisms on the IPF course over 18 months were evaluated. The results showed an effect of the IL-4 (rs 2070874 and rs 2243250) polymorphism on disease progression 12 months after diagnosis of IPF and an effect of the TOLLIP gene polymorphism (rs 111521887) on IPF outcomes 18 months after diagnosis. No relationship was found between the patient outcomes and polymorphisms of the MUC5B, TGF-β, TAP1, TAP2 and SFTPA genes in the study group.

The patient data were evaluated at the time of diagnosis and again 6, 12 and 18 months after diagnosis. No studies have evaluated the optimal visit interval to the best of our knowledge, yet most clinical trials evaluate patient outcomes after 12 months. As cases of rapid IPF progression have been described and longitudinal data were available for most of the enrolled subjects, data from months 6 and 18 were also included into the study protocol. IPF is the most aggressive form of progressive fibrosing interstitial lung disease, and a 6-month interval and 10% decline in FVC were found to be meaningful in a previous study (29). A Dlco decline of

>15% was incorporated as a marker of disease progression. The reason for including Dlco is explained in the study by Nathan *et al* (30); an FVC decline during the first year of follow-up may not be predictive of further progression of the disease. Despite the Dlco limitations, adding this parameter to the FVC decline is a reasonable means of defining the progression of IPF (29).

SFTPA mutations are detected primarily in patients with familial interstitial pneumonia or those with a concomitant diagnosis of lung fibrosis and lung cancer (31). However, no patient in the present study had a history of familial interstitial pneumonia or lung cancer, possibly explaining why no associations were found between SFTPA gene polymorphisms and prognosis.

There are several literature reports concerning various TGF-β gene polymorphisms and either the risk of developing IPF or the disease outcome. For example, the rs 1800470 variant has been reported to be a significant risk factor for IPF (32). The TGF-β polymorphism (rs 1800470) can modify the expression of the protein, and the G allele was shown to increase TGF-β secretion (33). However, not only the concentration of TGF-β but also the concentration of TOLLIP orchestrates the process of fibrosis. TOLLIP antagonizes TGF-β signaling by degrading TGF-β1 receptors through SMAD7-dependent mechanisms, and thus dampens the profibrotic cascade (34).

Noth *et al* (13) identified three TOLLIP SNPs associated with IPF risk; two of these (rs 111521887 and rs 5743890) were associated with an increased risk of IPF, and the third (rs 5743894) was associated with a lower risk of the disease. However, if subjects carrying this mutation develop IPF, they have a higher risk of mortality (34). Both the rs 111521887 and rs 5743894 variants are associated with a 20-50% reduction in TOLLIP mRNA expression. Despite the low number of studied subjects, the present study showed that the effects of different gene polymorphisms on the disease course may be time dependent, the effect of the TOLLIP rs 111521887 polymorphism on IPF outcomes were not observed until 18 months after the IPF diagnosis. Thus, it may not be a driver of the disease, but instead modify/enhance the fibrotic process. Since TOLLIP and TGF-β interact with each other, a more complex evaluation of the effects of multiple gene polymorphisms is required.

Both TAP 1 and TAP 2 polymorphisms may result in TAP1/2 protein deficiency (reported for rs 1057141, rs 1135216 and rs 241447), which leads to bare lymphocyte syndrome (35). They were also found to be associated with chronic infections and impaired antigen clearance, which could potentially lead to chronic exposure to potentially harmful substances, and enhance the risk of persistent lung damage (36). Exposure to inhalation of antigens, as well as their role in IPF pathogenesis, has been widely discussed as an important trigger of lung injury, followed by impaired healing (37). However, neither persistent viral infection nor chronic exposure to inhalation of antigens represent major contributors to IPF pathogenesis. This may explain why no association between TAP gene polymorphisms and IPF outcomes were found in the present study.

Although certain studies have suggested that the MUC5B minor allele does not affect IPF survival (38,39), others have challenged the initial study of Peljto *et al* (38), declaring an improved prognosis in patients with IPF with the minor allele and, after adjusting for index event bias, proposing a significant association of MUC5B polymorphism and decreased survival (40,41). The results of the present study support the prognostic benefit of the rs35705950 polymorphism. Previous study has suggested an effect of the MUC5B variant on the bacterial burden in patients with IPF, possibly explaining its positive effect on the evolution of the disease (41).

It is difficult to establish the specific effect of IL-4 gene polymorphisms in the very complex setting of an immune response. Certain polymorphisms affect the concentration of the encoded protein; however, as different levels may be evident in different compartments and other gene-protein interactions or protein-protein interactions may occur, the effects are not easily assessed. This situation is further complicated by the detection of the splice variant IL-4δ2 mRNA, which is hypothesized to regulate IL-4 production and acts as a factor to abrogate the Th2 response. However, the regulation of its production is currently unknown, and the effect of IL-4 gene polymorphisms on alternative splicing and production of the IL-4δ2 mRNA isoform remains elusive (42,43). This may lead to the assumption that one patient with IPF may carry more than one gene mutation, and that multiple genetic factors and mechanisms might play roles in IPF progression, as reported in the study by Dressen *et al* (44) in which variations of the course of IPF according to the patient's genetic background was observed. They showed that patients with telomere complex-associated mutations had a more rapidly progressive IPF course than those with MUC5B mutations, and suggested that different pathogenetic events may lead to the same fibrotic outcome in affected subjects. Interestingly, neither MUC5B nor most of the identified IPF-associated telomere complex gene mutations were identified as prognostic factors in a study using a functional genomic model approach (45).

The major limitation of the present study was the number of enrolled patients and the relatively short observation period. Despite finding data associating IL-4 and TOLLIP gene polymorphisms with the course of IPF, additional studies with larger patient cohorts are required to further explore the significance of the findings. The presented results should be interpreted with caution; however, they may suggest that a broader approach, involving not only the risk of developing the disease but also the disease course, should be implemented in future studies.

In conclusion, the identified IL-4 and TOLLIP gene polymorphisms may represent disease course-modifying factors, but not drivers of IPF.

Acknowledgements

Not applicable.

Funding

The present study was supported by funding from the Ministry of Health, Internal Grant Agency, Czech Republic (grant no. LF_2020_004/2021_014), Palacky University, Olomouc, Czech Republic (grant no. 61989592), Thomayer Hospital, Prague, Czech Republic (grant no. TN00064190) and in part from ENOCH (grant no. (CZ.02.1.01/0.0/0.0/16_019/0000868).

Availability of data and materials

The datasets generated and/or analyzed during the present study are available in the European Variation Archive repository (https://www.ebi.ac.uk/ena/data/view/PRJEB44734).

Author's contributions

MS designed the study, collected patient data and blood samples, and wrote the manuscript. AK and KS performed the laboratory studies. JS performed the statistical analysis. MV and MP assisted with study design. All authors have read and approved the final manuscript. MS, MP and KS confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Thomayer Hospital and Institute of Clinical and Experimental Medicine, Prague, Czech Republic. Written informed consent was obtained from all enrolled subjects.

Patient consent for publication

Patient consent for publication was obtained as part of the written informed consent.

Competing interests

The authors declare that they have no competing interests.

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