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BRIEF ARTICLE

Cytokine gene polymorphisms in idiopathic pulmonary fibrosis

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Abstract

AIM: To characterize cytokine gene polymorphisms in patients with idiopathic pulmonary fibrosis (IPF) compared to healthy controls.

METHODS: Fifty-six IPF patients were involved in the study. The control population consisted of 144 healthy volunteers without history of lung disease. All of the patients were diagnosed with IPF according to the American Thoracic Society/European Respiratory Society consensus statement. Polymorphisms in the interleukin (*IL*)-*1*, *IL-1*, *IL-1R*, *IL-1RA*, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, tumour necrosis factor, interferon, transforming growth factor, *IL-1*, *IL-2*, *IL-4* and *IL-4RA* genes were characterized by polymerase chain reaction with sequence-specific primers. Statistical analysis was performed using the MedCalc statistical software. A Bonferroni correction of significance at an alpha of 0.05 was used for multiple analyses. A corrected *P* value less than 0.0023 (0.05/22) was considered significant.

RESULTS: We found significant differences in the *IL-4* promoter region polymorphisms between IPF patients and controls. Namely, polymorphisms of *IL-4* (*-590*) [computed tomography (CT) in 32 of 56 patients *vs* 27 of 144 controls; $P < 0.0001$] and *IL-4* (*-33*) (CT in 25 of 56 patients *vs* 27 of 144 controls; $P = 0.0006$) differed between both groups. With regard to haplotypes, we found differences in the frequencies for haplotype 1 of *IL-4* (*-1098*) (*-590*) (*-33*) between IPF and controls (TCC in 23 of 56, TTC in 10 of 56, and TTT in 21 of 56 patients *vs* TCC in 112 of 144, TTC in 0 of 144, and TTT in 32 of 144 controls; $P < 0.0001$). We did not find significant differences in gene polymorphism frequencies of other cytokines in the IPF group *vs* the controls.

CONCLUSION: We hypothesize that *IL-4* promoter polymorphisms could be involved in the pathogenesis of IPF, likely *via* enhancement of the T_H2 cytokine milieu with exaggerated fibroproliferative healing.

Key words: Cytokine genes; Idiopathic pulmonary fibrosis; T_H2-type immune response

Core tip: Enhanced fibroproliferation resulting in terminal fibrosis is the main feature of idiopathic pulmonary

fibrosis (IPF). Various mechanisms of alveolar damage and its healing are involved in IPF development. One of the potential contributing pathogenic factors is the genetically encoded imbalance of cytokine production. We found differences between the frequencies of interleukin (*IL*)*-4* gene promoter polymorphisms in IPF patients *vs* controls. Based on these results and on the observation that *IL-4* promoter polymorphisms can influence IL-4 production, we hypothesize that *IL-4* promoter polymorphisms could be involved in the pathogenesis of IPF, likely by enhancing the T_H2 cytokine milieu with subsequent fibroproliferative healing.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most severe interstitial lung disease (ILD) and can be characterized by uncontrolled fibroproliferation as a sequelae of the pathological pattern of the healing of multiple alveolar lesions[1,2]. The role of the regulatory, profibrotic and antifibrotic cytokines and chemokines in the pathogenesis of IPF has been the subject of much conjecture[3-6]. One of the possible mechanisms of fibroproliferative healing in IPF could be the alternative activation of alveolar macrophages (AM) under the influence of predominantly T_H2-type cytokines. These alternatively activated AMs induce an increase of collagen production by fibroblasts. This process appears to be mediated by C-C chemokine 18, which is overexpressed in the presence of interleukin (IL)-10, IL-4 and IL-13[7,8]. One of the possible mechanisms of overexpression of T_H2 cytokines is the expression level of redox-sensitive transcription factors. Kikuchi et al. observed that the expression of T_H2 cytokines, such as IL-4 and IL-13, was significantly elevated in the lungs of Nrf2-deficient mice, with an increase in the number of T_H2 cells that expressed the GATA-binding protein 3[9]. However, the pathogenesis of IPF is a complex, multi-event process in which the T_H2 cytokines might be only one of multiple pathogenic factors. Nevertheless, it seems that cytokine imbalance likely plays an important role in coordinating the healing of alveolar lesions resulting in fibrosis.

One of the mechanisms resulting in increased production of cytokines is a polymorphism in a region of a cytokine gene that causes a change in its expression. Cytokines and their receptors are highly conserved within their coding regions. Although polymorphisms outside of the coding region do not influence the amino acid sequence, they may affect protein expression by influencing alternative mRNA splicing, mRNA stability, or transcription levels. Polymorphisms within the 5'- and 3'-regulatory regions of cytokines are particularly notable given that they can determine the transcription factor binding sites within the cytokine gene promoters and the structure of enhancers and silencers. In part, individual diversity in immune responses might be explained by such polymorphisms because they result in interindividual differences in the capacity to produce cytokines, which results in a variety of biological consequences. Hence, the investigation of IPF immunogenetics has been focused on identifying the cytokine polymorphisms that contribute to IRF or modify its severity.

Previous studies have described cytokine gene polymorphisms that could possibly be involved in IPF pathogenesis: *IL-10* (Whittington HA)[10], *IL-6* and tumour necrosis factor-receptor (*TNF-R*) II (Pantelidis P)[11], *IL-1-receptor antagonist* (*RA*) (Whyte M, Korthagen NM, Hutyrova B)[12-14], transforming growth factor-beta1 (*TGF-β1*) (Xaubet A, Son JY, Awad MR)[15-17], tumour necrosis factor-alpha (*TNF-α*) (Whyte M, Freeburn RW)[12,18] and *IL-8* (Renzoni E, Ahn MH)[19,20].

In our previous studies, we have suggested the involvement of *IL-1*, *IL-4*, *IL-12* and interferon (*IFN*) *-gamma* gene polymorphisms in the pathogenesis and clinical presentation (functional parameters, bronchoalveolar lavage fluid characteristics, high resolution computed tomography score) of sporadic IPF. Polymorphisms of the promoter region of *IL-4*, which were not previously described in any of the cited studies, were the most significant results[21-23]. Previous studies have shown that *IL-4* promoter variants, namely, *IL-4* (*-589*) *T* and *IL-4* (*-33*) *T*, could result in up to three-fold higher transcriptional activity of IL-4 *in vitro*, which was subsequently confirmed *in vivo*[24,25].

In our most recent study, we aimed to reinforce our hypothesis regarding the potential role of cytokine gene polymorphisms, namely, the high-producer *IL-4* promoter gene polymorphisms, for IPF pathogenesis in a larger number of patients.

MATERIALS AND METHODS

Ethics

The study design and the informed consent form were approved by the Central Ethical Committee of the University Thomayer Hospital and the Institute for Clinical and Experimental Medicine. All patients signed the informed consent form before submitting a blood sample for genotyping.

Study subjects

Fifty-six IPF patients were involved to our study. All patients with IPF were Caucasians [age: mean ± SD (67.29 ± 11.85), range (36-87 years), male/female (37/19)] Patients were diagnosed according to the American Thoracic Society/European Respiratory Society consensus statement[26]. We used the following criteria: insidious onset of dyspnea, bilateral basal crackles and digital clubbing, restrictive ventilatory pattern and lowered diffusion

Table 1 Demographic and basic clinical parameters of idiopathic pulmonary fibrosis patients, mean ± SD (range)

Parameters	Value
Men:women ratio	37:19
Mean age (yr)	67.29 ± 11.85
Mean FVC (%)	70.96 ± 20.60 (21-127)
TL_{CO} (%)	39.78 ± 16.63 (13-81)
BALF LY (%)	17.86 ± 18.93 (0-72)
BALF PMN (%)	15.84 ± 17.58 (1-70)
BALF EOS (%)	3.21 ± 6.95 (0-28)

BALF: Bronchoalveolar lavage fluid; FVC: Forced vital capacity; TL_{CO}: Transfer factor for CO; LY: Lymphocytes; PMN: Polymorphonuclear leukocytes; EOS: Eosinophilic leukocytes.

capacity for carbon monoxide, and typical radiological changes on high resolution tomography of the lungs (HRCT) with prevailing fibrotic changes and granulocytic bronchoalveolar lavage (BAL). A videothoracoscopic lung biopsy was performed in 16 patients who did not meet all above-mentioned criteria, and in all cases, the histopathological investigation revealed changes characteristic for usual interstitial pneumonia (UIP). We excluded other potential causes of ILD that can present with a UIP HRCT pattern, namely, hypersensitivity pneumonitis and connective tissue diseases (Table 1).

The control population of 144 unrelated individuals (24 males, 120 females) was made up of Caucasians from the Czech Republic. All control subjects were potential bone marrow donors in generally good health status, without any known diseases, at the time of the study. They did not state asthma, pulmonary fibrosis and systemic inflammatory disease in their histories. The normal controls had a mean age of 43.1 (SD = 16.17) years (range 19-80 years).

Methods

Polymorphisms in the promoter regions of *IL-1α*, *IL-1β*, *IL-1R*, *IL-1RA*, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, *TNF-α* and *IFN-γ* and the polymorphisms in the translated regions of the *TGF-β*, *IL-1β*, *IL-2*, *IL-4* and *IL-4RA* genes were characterized (Table 2).

DNA extraction

Two milliliter of peripheral venous blood were collected into ethylen-diamino-tetraacetic acid (EDTA) tubes. Isolation of genomic DNA was performed from 350 μL of blood using a DNA Blood Kit Cartridge B350 (Qiagen, Germany) on an automated BioRobot EZ1 instrument. The quality of DNA samples was checked on a spectrophotometer and DNA samples were archived at -20 ℃.

Cytokine genotyping: We evaluated the polymorphisms of thirteen different cytokine genes utilizing the CYTOKINE GENOTYPING KIT (Dynal, Biotech, Norway). The test is designed as a polymerase chain reaction (PCR) with sequence-specific primers. In detail, each well of a 48-well tray contains a specific primer pair for amplifying the desired unique sequence. The entire procedure was performed according to the manufacture's manual. The obtained pattern of positive and negative PCR was documented and interpreted according to the manufacture's worksheet.

Table 2 List of investigated cytokine gene polymorphisms

Polymorphism	Genotype
IL-1α (-889)	C/C C/T T/T
IL-1β (-511)	C/C C/T T/T
IL-1β (+3962)	C/C C/T T/T
IL-1R pst 1970	C/C C/T T/T
IL-1 RA mspa 11100	C/C C/T T/T
IL-1 RA (+1902)	A/A A/G G/G
IL-12 (-1188)	A/A A/C C/C
INF-β UTR 5644	A/A A/T T/T
TGF-β1 codon 10	C/C C/T T/T
TGF-β1 codon 25	C/C C/G G/G
TNF-α (-308)	A/A A/G G/G
TNF-α (-238)	A/A A/G G/G
IL-2 (-330)	G/G G/T T/T
IL-2 (+166)	G/G G/T T/T
IL-4 (-1098)	G/G G/T T/T
IL-4 (-590)	C/C C/T T/T
IL-4 (-33)	C/C C/T T/T
IL-6 (-174)	C/C C/G G/G
IL-6 (+565)	A/A A/G G/G
IL-10 (-1082)	A/A A/G G/G
IL-10 (-819)	C/C C/T T/T
IL-10 (-592)	A/A A/C C/C

IL: Interleukin; TNF: Tumor necrosis factor; INF: Interferon; UTR: Untranslated region.

Statistical analysis

The basic statistical characteristics, *i.e.*, the mean values and SD, were calculated for continuous variables (demographic and basic clinical parameters). The genotype frequencies and allele carriage frequencies in IPF were determined by direct counting and were compared with those in the control population using a Fisher's exact or χ^2 test. A Bonferroni correction of significance level at an alpha of 0.05 was used for multiple analyses. Statistical analysis was performed using the MedCalc statistical software. A P value less than 0.0023 (0.5/22) was considered significant.

RESULTS

Demographic and clinical data of the IPF group

The statistical analysis of the basic demographic data, functional parameters, and BAL fluid cell counts of the group of IPF patients is presented in Table 1.

Frequencies of cytokine gene polymorphisms in the IPF vs control group

When comparing the frequencies of gene polymorphisms between IPF individuals and controls, we found significant differences for the investigated *IL-4* promoter region polymorphisms (-590) and (-33) ($P < 0.0001$; $P = 0.0006$, respectively). In the IPF group, we more fre-

Table 3 *IL-4* (*-590*), *IL-4* (*-33*) and *IL-4 haplotype 1* polymorphisms in idiopathic pulmonary fibrosis and healthy controls *n* (%)

Locus	Genotype	Controls	IPF group	*P* value
IL-4 (*-590*)	CC	112 (77.8)	24 (42.9)	< 0.0001
	CT	27 (18.8)	31 (55.4)	
	TT	5 (3.5)	1 (1.8)	
IL-4 (*-33*)	CC	112 (77.8)	29 (52.7)	0.0006
	CT	27 (18.8)	25 (45.5)	
	TT	5 (3.5)	1 (1.8)	
IL-4 haplotype 1	GCC	0 (0.0)	1 (1.8)	< 0.0001
	TCC	112 (77.8)	23 (41.1)	
	TCT	0 (0.0)	1 (1.8)	
	TTC	0 (0.0)	10 (17.9)	
	TTT	32 (22.2)	21 (37.5)	

IPF: Idiopathic pulmonary fibrosis; IL: Interleukin.

quently observed the T allele at these positions (Table 3).

With regard to haplotypes, we observed differences in the frequency of haplotype 1 in the promoter region of *IL-4* (*-1098*) (*-590*) (*-33*) between the IPF and control groups (haplotype 1: TCC in 23 of 56 (41.1%), TTC in 10 of 56 (17.9%), and TTT in 21 of 56 (37.5%) patients *vs* TCC in 112 of 144 (77.8%), TTC in 0 of 144 (0%), and TTT in 32 of 144 (22.2%) controls; $P < 0.0001$) (Table 3).

The differences in the frequency of haplotype 2 in the promoter region of *IL-4* between IPF and controls were not significant after Bonferroni correction (Haplotype 2: GCC in 16 of 56 (28.6%), GCT in 2 of 56 (2.6%) and TCC in 35 of 56 (62.4%) patients *vs* GCC in 22 of 144 (15.3%), GCT in 0 of 144 (0%) and TCC in 117 of 144 (81.2%) controls; $P = 0.0061$). We also observed differences between IPF and controls for the frequencies of haplotype 1 polymorphisms at *IL-6* (*-174*) and (*nt565*), but these differences could not be declared as significant after Bonferroni correction [CA in 4 of 56 (7.1%) patients *vs* in 25 of 144 (17.4%) controls; $P = 0.0438$].

We did not find significant differences in gene polymorphisms frequencies for any other cytokines in the IPF group compared to the healthy population.

Hardy-Weinberg equilibrium

We tested for Hardy-Weinberg (HW) equilibrium for all examined SNPs as well. With the exception of *IL-4* (*-590*) and (*-33*), all SNPs were in equilibrium. We had to reject HW equilibrium in the control group for both *IL-4* (*-590*) and *IL-4* (*-33*) at $P = 0.05$ and in the IPF group for *IL-4* (*-590*) at $P = 0.01$ and for *IL-4* (*-33*) at $P = 0.07$. The less significant *P* value in the IPF group for *IL-4* (*-33*) was because of the smaller number of samples in that group compared to controls.

DISCUSSION

In our study, we have reinforced our hypothesis that *IL-4* gene promoter polymorphisms might be involved in IPF pathogenesis. The involvement of *IL-4* promoter gene polymorphisms has previously been shown to be involved in the pathogenesis of other diseases and their presentations, including: asthma severity, common variable immune deficiency, autoimmune thyroid disease, atopy, allergic rhinitis, eczema, Crohn's disease and renal transplantation outcome[27-29]. However, *IL-4* promoter gene polymorphisms have not yet been investigated, to our knowledge, in IPF Individuals who carry the T allele at the (-590) position of the *IL-4* gene, as more than half of our IPF patients did, were previously found to have a higher proportion of IL-4-producing T-helper cells (Nakashima H)[25]. IL-4 is required for the subsequent appearance of IL-4-producing cells, and thus for T_H2 lineage commitment. Regarding the biological effect of IL-4, RNA hybridization studies have demonstrated that IL-4 suppresses the expression of T_H1 and inflammatory cytokines (IL-1, IL-1 and TNF). These results suggest that IL-4 modulates monocyte production of TNF and IL-1 by down-regulating gene expression. This unique property of IL-4 may be important in regulating the immune response[30]. Regarding the direct role of IL-4 in the pathogenesis of fibrosis, *in vitro* studies have shown that this cytokine can regulate fibroblast function, including chemotaxis, proliferation, collagen synthesis, and myofibroblast differentiation[31,32]. In accordance with these observations, other reports have observed increased IL-4 expression in bleomycin-, silica- and radiation-induced lung injuries and suggested that activated macrophages represent the major source of IL-4 during the establishment of active lung fibrosis. In human studies, the progression of IPF was also shown to be associated with sustained IL-4 production[33-35]. These results strongly suggest that *IL-4* gene polymorphisms with consequent IL-4 overproduction are involved in the pathogenesis of many diseases in which the role of T_H1/T_H2 equilibrium is crucial. IPF is one such disease, and our study supports the hypothesis of an encoded genetic predisposition for T_H2 immune response to environmental stimuli, which could result in alternative activation of AMs and the triggering of pathologic healing of alveolar lesions[8]. *IL-4* promoter variants, namely, *IL-4* (*-589*) *T* and *IL-4* (*-33*) *T*, result in up to three-fold higher transcriptional activity *in vitro*, which likely results in increased IL-4 production and thus T_H2 dominant cytokine milieu[25]. We have found in our study that this high-producer *IL-4* genotype is significantly more present in IPF patients than controls. Aside from the potential direct role of *IL-4* gene polymorphisms in IPF aetiology, these polymorphisms could also have disease-modifying effects. This notion was hypothesized in our previous studies on the correlation of clinical parameters of IPF with *IL-4* gene polymorphisms, in which we put forth the suggestion of the protective role of *IL-4* (*-33*) *CC* homozygosity (the low-producer genotype) against the progression of HRCT interstitial changes[22].

The *IL-6* (*-174*) *G→C* polymorphisms were previously mentioned in relationship with IPF development in the two studies by Pantelidis *et al*[11] and Riha *et al*[36]. In our

study, the statistical significance for the difference of frequencies of this polymorphism between IPF and control individuals was not reached after Bonferroni correction. This is likely because of our investigation of a greater number of polymorphisms in one kit and because the correction for multiple comparisons substantially influenced the result. The *IL-6* (*-174*) *G→C* polymorphism appears to be functionally relevant and codes for higher IL-6 production (allele G), which was shown in neonates and later in patients with liver cirrhosis and hepatocellular carcinoma[28,37]. The role of IL-6 in the pathogenesis of fibrosing interstitial lung diseases has been hypothesized in recent studies, which supports the potential involvement of *IL-6* polymorphisms in the pathogenesis of IPF[38-40].

Cytokine gene polymorphisms and cytokine milieu are only a part of the net of potential mechanisms and factors leading from alveolar epithelial injuries to pathological fibroproliferative healing and eventual fibrosis. Chemokines, reactive oxygen species, antioxidants, transcription factors, growth factors and enzymes (particularly metalloproteinases and their inhibitors) are also known to be involved.

We are aware that our group of patients is not large compared to other genetic studies, but IPF is a rare disease and our study was performed at one center. We also recognize that the control and IPF patient group differ in sex and age and that this variation could influence our results. This is because IPF is more prevalent in men, not because of the sampling. Nevertheless, there are not any data in the literature on the influence of age and sex on genetic polymorphisms of cytokines, and we posit that the occurrence of cytokine polymorphisms might not be influenced by these variables.

When considering the influence of HW equilibrium, we found disequilibrium for IL-4 (-590) and for IL-4 (-33) in both the control and the IPF groups. The deviation from HW equilibrium is likely because we cannot expect Mendelian inheritance in the Czech population as in other European populations. We suggest that the influence of migration, random mutations, selection pressure and non-panmixia is similar for both populations, *i.e.*, our IPF and control group. Therefore, we consider our results as significant despite the suboptimal size of our cohorts from the genetic perspective[41]. Optimally, multi-centre studies of the effect of cytokine genetic polymorphisms in IPF will be required to support our findings.

COMMENTS

Background

Idiopathic pulmonary fibrosis (IPF) is a serious primary fibrosing interstitial lung disease with an aetiology that is not known to date. Multiple pathogenic pathways and factors contribute to the pathological pattern of healing of alveolar damage in the lungs of individuals with IPF. One of the predisposing factors that are responsible for IPF development is likely the genetically encoded type of immune response, *i.e.*, cytokine gene polymorphisms. Polymorphisms of the cytokine genes, namely, that of T_h2-type cytokines, can cause an imbalance in the cytokine milieu with T_h2 prevalence, which can then contribute to triggering fibroproliferative healing in the lungs.

Research frontiers

Knowledge of the processes contributing to the development of lung fibrosis could help find a way to influence the pathways involved in IPF pathogenesis. Thus, research of the mechanisms and factors of fibroproliferative healing in the lungs helps to find potential new drugs for treatment of this serious disease.

Innovations and breakthroughs

Previous studies of the immunogenetics of IPF revealed some cytokine gene polymorphisms that could potentially be involved in fibroproliferative healing. Namely, genetic polymorphisms of transforming growth factor, tumor necrosis factor, interleukin (*IL*)-*1RN*, *IL-6* and *IL-8* might be involved in IPF pathogenesis. The authors believe that T_h2 prevalent cytokine milieu could be caused by increased production of IL-4 through its polymorphisms. The authors' previous study on 30 patients with IPF suggested involvement of *IL-4* genetic polymorphisms in IPF development. The authors' studies have shown that *IL-4* polymorphisms, namely, at loci causing higher expression of the cytokines, could be related to more severe fibrotic changes in IPF and also influence chemokine milieu in the lungs. In this study, they have tested the frequencies of cytokine gene polymorphisms in an extended group of IPF patients to further support their previous findings.

Applications

The results support a reason to investigate genetic polymorphisms in patients with IPF to find patients whose enhanced fibroproliferation in the lung could be driven by increased expression of T_h2 cytokines, namely, *IL-4*. This subgroup of IPF patients might profit from treatment to block the IL-4 pathogenetic pathway.

Terminology

The polymorphisms of the genes indicate that some loci are variable, and different individuals and populations can bear different alleles. These different alleles can cause changes in expression of the gene, *i.e.*, influence the production of the cytokine and cause changes in cytokine milieu.

Peer review

This study reported on significant differences in polymorphisms in the *IL-4* and *IL-6* promoter regions between IPF patients and controls. This study is an extension of prior work in a larger cohort of IPF patients and presents novel and important results. The article covers a topic of clinical relevance and novelty. Their findings could have future implications in understanding the pathogenesis of IPF and its treatments. The article is well structured and presents no ethical problems.

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