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Cytokine gene polymorphisms and high-resolution-computed tomography score in idiopathic pulmonary fibrosis

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Summary
Introduction: Idiopathic pulmonary fibrosis (IPF) is a serious disease with unknown cause and the influence of cytokine gene polymorphisms is presumed in the etiology and pathogenesis of the disease. We used high-resolution computed tomography (HRCT) as a marker of disease stage and progression and compared the alveolar and interstitial score with IL-1, IL-4, IL-12, IL-1RA and IL-4RA cytokine gene polymorphisms.
Subjects and methods: The IPF patients were all Caucasians from the Czech Republic and consisted of 20 females and 10 males, with a mean age of 65 years, range 36–85. The HRCT results were evaluated by an experienced viewer using the interstitial and alveolar score scales, which were based on the IPF HRCT description system from Gay SE, Kazerooni EA, Tows GB, Lynch JP, Gross BH, Cascade PN, et al. [Idiopathic pulmonary fibrosis. Predicting response to therapy and survival. *Am J Respir Crit Care Med* 1998;**157**:1063–72]. We evaluated the polymorphisms of cytokine genes utilizing a PCR with sequence-specific primers method.
Results: The HRCT alveolar score was more pronounced in IL-4 RA (+1902) AG heterozygotes. The HRCT interstitial score was less severe in the IL-12 (−1188) AA homozygotes. According to progression of the HRCT interstitial score, the CC homozygosity at IL-1 RA (mspa 111100), the AA homozygosity at IL-4 RA (+1902) and CC homozygosity at

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IL-4(+33) positions were more frequent in patients with stable disease compared to those with progressive disease.
Conclusions: We assume from our data that the polymorphisms of IL-4, IL-4RA, IL-1RA and IL-12 genes (genes of cytokines with regulatory activity) might influence the phenotype of IPF as shown by measurable changes in HRCT investigations.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a serious disease characterized by uncontrolled fibro-production as a sequelae of alveolar injury which devastates the lung architecture.[1–5] The etiology of the disease is still unknown, but an impaired immunological response to some exogenous insult in genetically disposed people is presumed.[6–9]

Previous studies have pointed out a correlation between cytokine gene polymorphisms and IPF development. Whittington et al.[10] have studied the role of a novel IL-10 gene polymorphism, a G to A substitution at position (+43) of the start codon ensuing in lower levels of IL-10 protein secretion. Studies of the effect of IL-13 on TGF-beta-1 activation implied that transgenic mice with over-expression of IL-13 are prone to generate airway and parenchymal tissue fibrosis.[11] Pantelidis et al.[12] discovered an increased frequency of the association of the IL-6 intron 4G and the TNF-RII 1690C alleles in patients with IPF. The correlation of the increased risk of fibrosing alveolitis associated with IL-1-RA and TNF-alpha gene polymorphisms was previously described.[13] The potential role of TGF-beta 1 polymorphism was also investigated. Polymorphisms in codons 10 and 25 of the TGF-beta promoter do not predispose to the development of IPF, while proline coded at codon 10 was associated with an increase in alveolar arterial oxygen tension difference during follow up.[14] The TGF-beta 1 gene polymorphism at the position (+915) of the signal sequence, which changes codon 25 (arginine to proline) is associated with inter-individual variation in levels of TGF-beta-1 and that high production of TGF-beta 1 was present significantly more frequently in patients with pretransplant lung fibrosis and posttransplant allograft lung fibrosis.[15] Studies of other cytokine gene polymorphisms, which could influence the susceptibility to IPF development are still continuing, with often contradictory results.[16,17] In our previous study, we suspected a pathogenic role of IL-1, IL-2 and IL-4 cytokine gene polymorphisms in IPF development.[18]

However, the influence of the gene polymorphisms alone does not sufficiently elucidate the etiology and pathogenesis of the disease. We suppose that the cytokine gene polymorphisms would have been only the disease modifying and predisposing factors and might influence the pronunciation of the pathogenic changes.

High-resolution computed tomography (HRCT) of the lungs is one of the best, if not ultimately the best, method for showing typical changes in IPF. Nevertheless, the experience of the evaluating radiologist is an important condition for the interpretation of accurate HRCT changes.[19,20] This fact is very important in case we cannot obtain the biopsy specimen of lung tissue for histopathological verification of the diagnosis.[21] The extent of reticulation and honey-combing on HRCT is an important independent predictor of mortality in patients with IPF.[22] The inflammatory activity is supposed not to be the dominant feature of the disease, but in some cases the role of inflammatory changes is conceded and proved by Gallium-67 citrate scans; nevertheless, it does not seem relevant for the patient's prognosis.[23] The thin-section CT histograms of the lungs were found to correlate well with results of pulmonary function tests in IPF and therefore seem to be suitable for use as valid indexes of IPF.[24–26] Scoring systems for the extent of the interstitial changes in IPF are used for comparability of changes in time and between different patients and also to make the results understandable for other clinicians and radiologists. The scoring systems have been designed by experienced pulmonary radiologists and clinicians interested in IPF investigation.[27–32] From the pathologic point of view, the cystic lesions on HRCT sections correlated well with micromorphology of the lung tissue, but the pattern of activity (ground glass) and fibrosis (consolidation) did not correlate with the histopathologic findings.[33]

In our study, we have concentrated on the HRCT alveolar and interstitial scores at the time of diagnosis and also their dynamic changes in serial investigations and their correlations with IL-1 alpha, IL-4, IL-12, IL-1RA and IL-4 RA gene polymorphisms in patients with IPF.

The choice of the polymorphisms resulted from our previous study of cytokine gene polymorphisms in IPF, where the IL-1, and especially IL-4 polymorphisms seemed to be strongly correlated with IPF presence compared to a healthy population.[18] The group of IL-1 cytokine gene polymorphisms in IPF showed some significant results also in the previous study of Whyte et al. mentioned above.[18] The IL-4 gene polymorphisms was to our knowledge investigated in IPF patients only by our investigational group, and the results encouraged us to correlate the findings with clinical signs of the disease. The strongest correlation with IPF development showed the IL-4 polymorphism at position (−33), where the prevailing genotype in the IPF group was compared by CT to the healthy controls with CC ($P<0.0001$) and the P-value remained statistically significant even after a Bonferroni correction ($P_{corr}<0.0022$).[18] The IL-12 gene polymorphisms were involved in our study according to regulatory function of this cytokine in immune reactions, despite the fact we did not prove in our previous study the significant correlation with IPF development.[18] The pivotal idea of the choice of cytokine polymorphisms was the hypothesis of the prevalence of Th2 type of immune response in IPF leading to extensive fibrosis as an answer to multiple insults of alveolar wall.

Material and methods

Study subjects and study design

The IPF patients were all Caucasians from the Czech Republic and consisted of 20 females and 10 males, with a mean age of 65 years, range 36–85. All the patients with IPF were diagnosed according to American Thoracic Society (ATS)/ European Respiratory Society (ERS) consensus classification[34] and all signed an informed consent form. The study was approved by Central Ethical Committee of University Thomayer Hospital and Institute for Clinical and Experimental Medicine.

After the basal demographic data such as race, age and sex was collected, the HRCT of the lungs was performed at the time of the diagnosis and then repeated every 12 months (Tables 1 and 2). Polymorphisms in the promoter regions of the IL-1alpha, IL-4 and IL-12 and translated regions of IL-4RA and IL-1RA genes were characterized. The cytokine gene polymorphisms were chosen on the basis of our previous study[18] (Table 3). The results were then statistically evaluated.

The HRCT investigation was performed on the SOMATOM Sensation 40 machine (Siemens AG, Berlin and Munich, Germany). The results were evaluated by an experienced viewer using the interstitial and alveolar score scales, which were based on the IPF HRCT description system of Gay et al.[29] (Tables 1 and 2). The HRCT scores were evaluated at four levels: aortic arch, hilar level, right atrium and basal parts of lungs. The values are stated in percentages and

Table 1 HRCT scoring system in IPF (based on scoring system of Gay et al.).

	Alveolar score	Interstitial score
0	0	0, no honey-combing
1	1–4%	1–4%, no honey-combing
2	4–24%	5–24%
3	25–49%	25–49%
4	50–74%	50–74%
5	75%–100%	75–100%

Table 2 HRCT score in IPF patients.

	IS I	IS II	IS III	IS IV	AS I	AS II	AS III	AS IV	Dynam. IS	Dynam. AS
13.	2	4			2	2			2	0
2.	3	3	4	4	4	3	1	2	2	1
23.	4		4		2		1		0	1
5.	3				4					
57.	3				3					
44.	4	3			3	1			2	2
60.	4	4			3	1			0	1
8.	4	4			1	2			0	2
18.	3			5	5	4		4	2	1
35.	3				2					
54.	3				3					
21.	4				3					
62.	5				3					
6.	5	5			3	3			0	0
32.	3				1					
2.	4	4	4	4	0	3	2	1	0	1
49.	4				0					
3.	3		4		2		3		2	2
31.	4	4			4	2			0	1
33.	4				1					
38.	4				2					
47.	3	3			1	1			0	0
11.	4	4	4,5	4	3	2	3	2	2	1
43.	3	4	4		1	1	1		2	0
37.	5				5					
55.	5				0					
20.	4	4	5		4	4	4		2	0

IS = interstitial score.
AS = alveolar score.
Dynamics: 0—without changes, 1—regression, 2—progression.
I—at the time of diagnosis.
II—1–11 months after diagnosis.
III—12–23 months after diagnosis.
IV—24–36 months after diagnosis.

Table 3 List of investigated cytokine gene polymorphisms.

Polymorphism	Genotypes		
IL-1alpha −889	C/C	C/T	T/T
IL-4 11098	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-4RA +1902	A/A	A/G	G/G
IL-12 −1188	A/A	A/C	C/C
IL-1 RA mspa111100	C/C	C/T	T/T

describe the extent of changes. We considered the disease to be stable for value 0 and 1 and to be progressive, for value 2 in columns showing the score changes in Table 3.

DNA extraction from the peripheral blood sample

Ten millilitres of venous peripheral blood was collected in EDTA tubes and a red cell lysis buffer was added. After 20 min, the tube was spun for 10 min at 1300*g* and the supernatant was removed. White cell lysis buffer was then added to the sediment with proteinase and SDS. The mixture was incubated on a rotator for 18 h at 37 °C. After the incubation, 6 M NaCl and chloroform was added and mixed for 15 s before being centrifuged for 25 min at 1300*g*.

The supernatant was added to 4 ml of absolute ethanol in a clean tube. The precipitated DNA was removed, resuspended in sterile water and stored at 4 °C.

Cytokine genotyping

We evaluated the polymorphisms of cytokine genes utilizing a cytokine genotyping kit (Dynal Biotech, Oslo, Norway). The test is designed as a PCR with sequence-specific primers.

The whole procedure was performed according to the manufacture's instructions. The preparation of PCR master mix consists of 140 5 l PCR Buffer, 75 5 l genomic DNA (75–120 ng/5 l), 4 5 l Taq polymerase (5 U/5 l) and 261 5 l PCR H_2O. Ten 5 l of this mixture is then dispensed to each well of the tray. The thermal cycling programme for PTC 225 DNA Engine Tetrad (MJ Research, USA) was as follows: initial denaturation 94 °C for 2 min, then 10 cycles of 94 °C 15 s and 65 °C 60 s, and then 20 cycles of 94 °C 15 s, 61 °C 50 s and 72 °C 30 s.

After the cycling is completed, the PCR products are loaded onto a 2% agarose gel stained with ethidium bromide for electrophoresis (5 V/cm). The obtained pattern of positive and negative PCR is documented and interpreted according to the manufacturer's instructions.

Statistical analysis

Descriptive statistics were calculated (i.e. mean value and standard deviation) and derived from the quantitative variables. For discrete variables (genotype, etc.) the chi-square, resp. Fisher's exact test was used. A multiway frequency table was used for the evaluation of an association between the polymorphisms in the group of patients.

Table 4 Correlation of the HRCT alveolar score at the time of diagnosis and IL-4 RA (+1902) polymorphisms.

	AS 0+1 groups	AS 2+3 groups	AS 4+5 groups
AA	5 (31%)	10 (63%)	1 (6%)
AG	3 (27%)	3 (27%)	5 (46%)

$P < 0.05$.

Table 5 Correlation of the HRCT interstitial score at the time of diagnosis and IL-12(−1188) polymorphisms.

	IS 2+3 groups	IS 4+5 groups
AA	10 (55,5%)	8 (44,5%)
AC, CC	1 (11%)	8 (89%)

$P < 0.05$.

Table 6 Correlation of the HRCT interstitial score changes and IL-1RA (mspa 111100) polymorphisms.

	Stable disease	Progressive disease
CC	6 (75%)	2 (25%)
CT	0	4 (100%)
TT	2 (67%)	1 (33%)

$P < 0.05$.

Results

The highest HRCT alveolar score (4+5) was associated with IL-4 RA (+1902) AG heterozygotes as compared with AA homozygotes (Table 4). When we compared the results with the results of our group of 103 healthy volunteers from the previous study, the AA homozygosity at this position seemed to appear at a similar percentage in IPF patients with alveolar score 2+3 and in the healthy volunteers (63% vs. 55%).[18]

The HRCT interstitial score was less severe in the IL-12 (−1188) AA homozygotes (Table 5). When we looked at the healthy population, the prevailing genotype is also AA at this position (55%), AC and CC is present in 41% of our healthy volunteers compared to 89% seen in IPF patients with interstitial score 4+5.[18]

According to the progression of the HRCT interstitial score, the CC homozygosity at IL-1 RA (mspa 111100), the AA homozygosity at IL-4 RA (+1902) and CC homozygosity at IL-4(−33) were more frequent in patients with stable disease compared to that with progressive disease (Tables 6–8). We are aware that the subgroups of patients in these groups are limited, but when comparing the frequency of the IL-4(−33) CC polymorphism in the healthy population vs. IPF patients it can be seen that 75% of the healthy subjects in our control

Table 7 Correlation of HRCT interstitial score changes and IL-4RA (+1902) polymorphisms.

	Stable disease	Progressive disease
AA	8 (67%)	4 (33%)
AG	0	3 (100%)

$P<0.08$.

Table 8 Correlation of HRCT interstitial score changes and IL-4 (−33) polymorphisms.

	Stable disease	Progressive disease
CC	4 (100%)	0
CT	4 (36%)	7 (64%)

$P<0.08$.

group are CC homozygotes, compared to 0% of the IPF patients with progressive disease.[18] In the case of IL-1RA (mspa 111100) and IL-4 RA (+1902) polymorphisms, comparison with the previous results of the healthy population showed no reasonable explanation for any allele carriage and IPF progression.

The other polymorphisms did not show a statistically significant correlation with HRCT markers of IPF either at the diagnosis or during follow up.

No association between the polymorphisms in IPF patients was found using multiway contingency tables.

Discussion

In order to investigate the influence of our previously investigated polymorphisms on the IPF manifestation, we chose the HRCT of the lungs as the best measurable and easiest to evaluate marker of the disease.[22,24–30,32] We compared the alveolar and interstitial score and its progression in time with cytokine gene polymorphisms at the (−889) IL-1 alpha, (−1098) IL-4, (−590) IL-4, (−33) IL-4, (+1902) IL-4RA, (mspa 111100) IL-1RA and (−1188) IL-12 positions.

In our previous study,[18] we investigated a wide spectrum of cytokine genes with special attention paid to IL-4 and found statistically significant results for IL-4 polymorphisms at positions (−590) (CT) and (−33) (CT) in IPF patients. These results support the idea of the pathogenic role of the IL-4 promotor polymorphisms in IPF and are in agreement with the work of Jakubzick et al., describing the influence of IL-4 on lung cells, especially in fibroblasts.[35] However, the functional consequences of the described IL-4 polymorphisms, i.e. whether these polymorphisms influence the amount of produced IL-4, or induce changes in its affinity to the IL-4 receptors on lung fibroblasts, are not known.

We found that the higher alveolar score, i.e. a greater extent of active changes at the time of diagnosis, in IL-4RA (+1902) AG heterozygotes. The lower interstitial score, i.e. the less extent of the fibrotic chages, was found in the IL-12 (−1188) AA homozygotes compared to AC heterozygotes. According to the progression of the disease in serial HRCT investigations, the CC IL-1RA (mspa 111100) homozygotes had a lower grade of progression of the interstitial score (i.e. fibrosis) than the CT heterozygotes and TT homozygotes. Similarly, the IL-4RA (+1902) AA homozygotes had lower progression of the interstitial score than the AG heterozygotes and CC IL-4 (−33) homozygotes had lower interstitial score progression than the CT heterozygotes at this position.

We found most promising the results which showed a reasonable correlation with results previously seen in our healthy volunteer group.[18] I.e. the ones where a carriage of some allele was different or similar in frequency in healthy vs. IPF patients and prone their carrier to have more or less severe HRCT score. Such correlations were seen for IL-4RA(+1902) AA homozygosity seen in similar fashion in IPF patients with a higher alveolar score and in healthy patients.

IL-12 is a key regulator of the polarisation of immune responses to T helper 1 or 2 pathways and plays a role in autoimmune and infectious diseases.[36] Nevertheless, Latsi in his study did not find any association with susceptibility to IPF and neither did we in our previous work.[18,37] According to the IL-12(−1188) polymorphisms in our study, the most frequent one in the group of healthy volunteers is AA and these homozygotes in IPF group had a lesser extent of interstitial changes, e.g. a more favourable outcome compared to the homozygous or heterozygous carriers of allele G, who had the worst interstitial score 4+5.

The genotype preserving IPF patients in our group from rapid progression of interstitial changes was IL-4(−33) CC homozygosity, which appeared also in majority of healthy subjects in our control group (75%) and in none of the patients with rapid progression.[18]

IL-1 alpha −889 did not seem to be correlated either with the interstitial and alveolar score at the time of diagnosis or with changes in the score during follow-up. But the other cytokine polymorphism from IL-1 group, the investigated IL-RA mspa 111100 polymorphism, showed greater probability of having a stable disease without progression of the interstitial score for CC homozygotes. This homozygosity is the least frequent genotype in heatlhy population which makes the function of this polymorphism in IPF unclear.

We are aware that the number of patients in our study group is not large enough to generate the results with greater statistical significance, but we suppose from our results that the polymorphisms of IL-4, IL-4RA, IL-1RA and IL-12 genes (genes of cytokines with regulatory activity) could influence the phenotype of the idiopathic pulmonary fibrosis shown as measurable changes in HRCT investigations. Our next goal is to enlarge the IPF group to obtain greater statistical sensitivity and to test the functional relevance of the polymorphisms in IPF with help of gene expression of cytokines m-RNA i bronchoalveolar lavage fluid.

We are aware that cytokine gene polymoprhisms are obviously not the only factor influencing the IPF development and progression and could be a result of the synergic effect of manifestation of variation of cytokine genes and imbalance in tissue remodelling mediators.[38]

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