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Analysis of IL-12 p40 subunit gene and IFN- γ G5644A polymorphisms in Idiopathic Pulmonary Fibrosis

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Abstract

Background: Genes encoding cytokine mediators are prime candidates for genetic analysis in conditions with T-helper (Th) cell disease driven imbalance. Idiopathic Pulmonary Fibrosis (IPF) is a predominantly Th2 mediated disease associated with a paucity of interferon-gamma (IFN- γ). The paucity of IFN- γ may favor the development of progressive fibrosis in IPF. Interleukin-12 (IL-12) plays a key role in inducing IFN- γ production. The aim of the current study was to assess whether the 1188 (A/C) 3'UTR single nucleotide polymorphism (SNP) in the IL-12 p40 subunit gene which was recently found to be functional and the 5644 (G/A) 3' UTR SNP of the IFN- γ gene were associated with susceptibility to IPF.

Methods: We investigated the allelic distribution in these loci in UK white Caucasoid subjects comprising 73 patients with IPF and 157 healthy controls. The SNPs were determined using the polymerase chain reaction in association with sequence-specific primers incorporating mismatches at the 3'-end.

Results: Our results showed that these polymorphisms were distributed similarly in the IPF and control groups

Conclusion: We conclude that these two potentially important candidate gene single nucleotide polymorphisms are not associated with susceptibility to IPF.

Introduction

Over the last few years there is accumulating evidence to support the paradigm that a complex network of cytokines, produced by activated CD4+ cells, governs the initiation, maintenance and resolution of an immune response [1]. CD4+ T cells can be distinguished, based on

their pattern of cytokine production, into T helper (Th)1 and (Th)2. Th1 cells secrete Interleukin (IL)-2 and IFN- γ , thus promoting cell-mediated immunity whereas Th2 cells produce IL-4, 5,10 and 13 thereby facilitating humoral immunity [2,3].

IL-12 plays a key role in promoting Th1 responses. This cytokine, produced primarily by antigen-presenting cells is a 75-kDa heterodimer composed of two disulfide-linked subunits designated p35 and p40, which are encoded by separate genes on chromosomes 3p12-3q13.2 and 5q31-33 respectively [4]. IL-12 up-regulates IFN- γ production, and IFN- γ is a powerful co-stimulator of IL-12 production [5]. Thus, a powerful positive feedback loop can develop between these cytokines that has been shown to drive type 1 immune responses in a variety of infectious diseases and many autoimmune processes [5-7]. Deficiency of this feedback might result in a shift towards a Th2 response.

A number of studies have shown that idiopathic pulmonary fibrosis (IPF) is characterized by a predominance of gene expression for Th2-type regulatory cytokines. This pattern is associated with high levels of IL-4 and IL-5 and a paucity of IFN- γ [8-10]. The paucity of IFN- γ , known for its anti-fibrotic properties, may contribute to the excessive fibroblast activation, deposition of collagen and scar formation that occurs in IPF.

Hereditary factors may contribute to the risk of developing IPF, although no specific genetic abnormality has been identified yet except in isolated families. However, the existence of familial pulmonary fibrosis, the presence of alveolar inflammation in clinically unaffected family members of patients with familial IPF and the appearance of IPF-like disease in association with inherited disorders suggest a genetic predisposition [11,12].

Genes encoding cytokines, which strongly influence the course of T cell mediated immune responses, are prime candidates for study in conditions where there is an imbalance in the T helper cytokine profile. Polymorphisms in a range of human cytokine genes have been correlated with different levels of protein production, transplant rejection, fibrosis and autoimmunity [13-16].

Recently, a complete genomic sequence analysis of the IL-12 gene encoding its p40 subunit identified several intronic polymorphisms and a Taq I (A/C) single nucleotide polymorphism in the 3' untranslated region of the IL-12 p40 gene at position 1188 [17] which was also found by another group [18]. This polymorphism was recently found to be functional [19] and associated with susceptibility to insulin dependent diabetes mellitus and multiple sclerosis [20,21]. Wu et al, [22] have described a G/A polymorphism at position 5644 in the 3' untranslated region of the IFN- γ gene (Accession No, M37265). Bream et al suggest that this SNP is located in the 3' flanking region [23] but De Capei et al [24] positions the polymorphism in the 3'UTR, consistent with Wu et al.

The 3'UTR region plays an important role in the expression of many eukaryotic genes by governing mRNA stability, localizing mRNA, and regulating translation efficiency and any polymorphism in this region of the gene might affect gene expression.

Against this background, we examined the distribution of these two 3'UTR polymorphisms, selected because one of them has been shown to be functional and the other located in a region that likely affects mRNA stability in 73 IPF patients and 157 healthy controls.

Methods

Patients

IPF patients were selected to be white UK Caucasians. The mean age of the IPF patients (n = 73) was 62.5 \pm 1.1 years (56 males and 17 females).

The diagnosis of IPF was made using the ATS/ERS definition criteria: exclusion of all known causes or associations with lung fibrosis; bilateral crackles on auscultation; the presence of typical features on chest high resolution computerized tomography, a restrictive pulmonary deficit and/or reduced gas transfer measurements, and the absence of bronchoalveolar lavage features that might suggest an alternative diagnosis. In 23 of 73 patients, the diagnosis of fibrosing alveolitis was confirmed by surgical biopsy.

Informed patient consent was obtained from all subjects and authorization was given by the Ethics Committee of the Royal Brompton Hospital.

Control subjects

All control subjects (n = 157) were white UK Caucasian cadaveric renal allograft donors collected at the Oxford Transplant Centre (Churchill Hospital, Oxford). The representative nature of this control population for UK Caucasians has previously been demonstrated in HLA genotyping studies [25].

Sequence Specific Primers-Polymerase Chain Reaction (SSP-PCR)

Polymorphisms were determined using SSP-PCR methodology that utilizes sequence specific primers with 3'-end mismatches and identifies the presence of specific allelic variants, by PCR amplification.

For the identification of the IL-12 biallelic polymorphism corresponding to position 1188 (A/C) we used the sequence-specific reverse primers: 5'TTG TTT CAA TGA GCA TTT AGC ATC T and 5' GTT TCA ATG AGC ATT TAG CAT CG in combination with the consensus forward primer 5'ATC TTG GAG CGA ATG GGC AT at a final

Table 1: Genotype and allele frequencies of the *IL-12* p40 gene, position 1188 3'UTR polymorphism in patients with IPF and controls

Genotype	IPF [n = 73] (%)	Controls [n = 157] (%)
AA	43 (59)	96 (61)
AC	28 (38)	50 (32)
CC	2 (3)	11 (7)
Allele		
A	114 (78)	242 (77)
C	32 (22)	72 (23)

Percentages are in parentheses p values: no significance, IPF versus control subjects

Table 2: Genotype and allele frequencies of the *IFN-γ* position 5644 3'UTR polymorphism in patients with IPF and controls

Genotype	IPF [n = 73] (%)	Controls [n = 157] (%)
AA	29 (40)	56 (36)
AG	36 (49)	77 (49)
GG	8 (11)	24 (15)
Allele		
A	94 (64)	189 (60)
G	52 (36)	125 (40)

Percentages are in parentheses p values: no significance, IPF versus control subjects

concentration of 3.8 µg/µl with an expected product size of 780 bp.

The *IFN-γ* polymorphism at position 5644 (A/G) was identified by the sequence-specific forward primers: 5'CCT TCC TAT TTC CTC CTT CG and 5'ACC TTC CTA TTT CCT CCT TCA in combination with the consensus reverse primer 5'GTC TAC AAC AGC ACC AGG C at a final concentration of 7.7 µg/µl with an expected product size of 298 bp. *IL-12* specific primers were used in conjunction with control primers amplifying a 256-bp fragment of the human adenomatous polyposis coli gene (primers 210/211) and the *IFN-γ* primers in conjunction with control primer mix amplifying a 796-bp fragment of the DRB gene (primers 63/64) [26,27]. All PCR reactions were carried out under identical conditions and as previously described [26,27].

Data Analysis

The genotype frequencies, allele carriage frequency and allelic frequency were determined by direct counting and they were compared with those in the control population initially using a 2 × 3 genotype contingency table and chi² followed by a 2 × 2 contingency table and chi² analysis for the individual positions. A p value less than 0.05 was considered significant. Statistical power calculations were carried out using the PS Program [28].

Estimate of power for a genetic association study is dependent on allelic frequency and the proportion of the phenotypic variant attributed to the allele (relative risk). On the basis of our patient sample size (n = 73), the ratio of controls to cases, the frequency of rarer alleles in the control population *IL-12* (0.23) and *IFN-γ* (0.4), we estimated that for our study to achieve 80% power at 5% significance the relative risk attributed to the rarer allele has to be 2.352 (95% Confidence Interval [CI] 1.79–3.01) for *IL-12* and 2.2 (95% CI 1.6–2.94) for *IFN-γ*. For a moderate statistical power of 60% an attributable relative risk of 1.97 (95% CI 1.51–2.53) for *IL-12* and 1.88 (95% CI, 1.4–2.5) for *IFN-γ* was required.

Results

Tables 1 and 2 summarize the genotype, and allele frequencies for *IL-12* p40 and *IFN-γ* gene polymorphisms in patients with IPF and controls.

Both polymorphisms, in all study populations, were in Hardy-Weinberg equilibrium.

Comparisons between the genotype and allelic frequencies in the IPF and control populations did not reveal significant frequency differences between the two groups either for the *IL-12* p40 or *IFN-γ* polymorphisms.

Discussion

The identification of differential cytokine patterns in patients with IPF or animal models of pulmonary fibrosis have provided evidence that the imbalance in the expression of Th1 and Th2 cytokines may be a central mechanism in the development and progression of pulmonary fibrosis in IPF. Interleukin-12 and IFN- γ play key roles in this process. Recently, we examined whether allelic variants in the gene coding for TNF- α , another key mediator in the development of IPF, contribute to the development of IPF [26]. A logical progression from that work was to evaluate whether SNP's located on the genes coding for IL-12 and IFN- γ are associated with the development of IPF.

In the current study we investigated the distribution of a polymorphism in the 3'UTR regions (known to affect mRNA stability) of the *IFN- γ* gene at position 5644 in the 3'UTR region (Accession No, M37265) and a functional polymorphism in the *IL-12* p40 subunit gene at position 1188 in the 3' UTR region [17-19] in patients with IPF and healthy controls. No differences were found between these populations with regard to their allelic distributions. However, the size of our patient sample ($n = 73$), although small for extensive genetic analysis studies is as large as other studies for a disease as uncommon as IPF. Our estimates of statistical power indicated that if the disease phenotype is attributable to the presence of the rarer allele in the two loci examined, these alleles would have to confer a relative risk greater than 2.2 for 80% power at 5% significance to be identified with these patient numbers. Thus, if these loci have any influence on disease risk, it must be relatively small.

The rationale of examining *IFN- γ* and *IL-12* gene polymorphisms in IPF is supported by a number of studies. First, Wallace et al have indicated that while there is evidence for both a type 1 (characterized by IFN- γ) and type 2 response (characterized by IL-4 and IL-5) in the lung interstitial inflammatory cells from patients with IPF, the type 2 pattern of cytokines appears to predominate [9]. Similarly, Majumdar et al demonstrated, in a quantitative study of open lung biopsies, that in IPF the ratio of IL-5 to IFN- γ was significantly higher than in the patients with fibrosing alveolitis associated with scleroderma (FASSc) and control subjects, with IFN- γ under-expression in IPF contributing equally to this increase [8].

Furthermore, Prior and Haslam have reported that in contrast with patients with sarcoidosis, a predominantly Th1 disease, very few patients with IPF and FASSc have elevated plasma levels of IFN- γ . However, as in sarcoidosis, those with the highest levels responded to corticosteroids [10]. Moller et al, also, have showed that in contrast to patients with sarcoidosis, only one of six patients with IPF had detectable levels of IFN- γ in the bronchoalveolar lav-

age fluid (BAL). In addition, IL-12 p40 protein was detected in BAL from four of six patients with IPF. However, although not significantly different, the median level was less than half that observed in sarcoidosis. In contrast, significantly higher levels of IL-10 were found in BAL fluid from patients with IPF than from patients with sarcoidosis and normal controls, indicating that the cytokine profile between the two diseases is quite different [29].

The paucity of IFN- γ , and the predominance of Th2 type cytokines (IL-4 is an important mediator of fibroblastic activation) may favour the development of progressive fibrosis in IPF. This is supported by the knowledge that IFN- γ inhibits fibroblast collagen synthesis in vitro [30,31] and attenuates bleomycin-induced lung fibrosis in the mouse model of lung fibrosis [32]. Furthermore, levels of IFN- γ are inversely related to the levels of type III procollagen in the BAL of IPF patients [33].

IL-12 has been shown to play a central role in the development of type 1 immune responses. Thus, deficient IL-12 activity may result in a shift towards a Th2 response. The *IL-12* p40 subunit gene at position 1188 3'UTR region has already been examined in a number of autoimmune diseases. Hall et al showed that this polymorphism was not associated with rheumatoid arthritis, Felty's syndrome or large granular lymphocyte syndrome with arthritis or multiple sclerosis [18]. However, in other studies the polymorphism was found to be associated with susceptibility to multiple sclerosis and type 1 diabetes mellitus. [20,21].

Polymorphisms in the human cytokine genes have been associated with different levels of protein production. Recent studies have shown that the 1188 3'UTR *IL-12* p40 polymorphic site is biologically relevant. Seegers *et al* have demonstrated that the presence of the rarer allele was correlated with increased IL-12 p70 secretion by stimulated monocytes [19].

Surprisingly, a recent study has found that the proximal promoter and exonic regions of the *IFN- γ* gene are invariant in a Caucasian cohort [23]. In the same study, of the three intronic and one 3'UTR single nucleotide variations identified, only the alleles in the 3'UTR locus altered transcription element DNA-binding ability. This suggests that this region of the gene could be of vital importance in the regulation of *IFN- γ* gene expression.

Although in the current study we did not observe an association between the polymorphic loci examined and IPF, we cannot exclude that other polymorphic variations within these genes [17,23] or their receptors [34] may denote susceptibility to IPF. In this regard, Tanaka et al have reported that a polymorphism within the *IFN- γ* receptor gene may result in a shift to Th2 response and

this shift may increase susceptibility to systemic lupus erythematosus [34]. Thus, further studies are needed to evaluate whether other polymorphisms in genes regulating IL-12 and IFN- γ production are involved in IPF susceptibility and the present study, although negative, will hopefully direct subsequent work in this direction.

Conclusion

In conclusion, in the current study we evaluated the distribution of single nucleotide polymorphisms in the 3'UTR region of two important candidate gene – *IL-12* and *IFN- γ* but found no direct association with susceptibility to IPF. However, further studies are needed to establish whether the under representation of these mediators in IPF is linked to other genetic variations in genes coding for proteins associated with their regulation.

Abbreviations

IL-12 = Interleukin 12, IFN- γ = Interferon – gamma IPF = Idiopathic Pulmonary Fibrosis 3'UTR = 3' Untranslated Region

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