Analysis of Tumor Necrosis Factor-α, Lymphotoxin-α, Tumor Necrosis Factor Receptor II, and Interleukin-6 Polymorphisms in Patients with Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is characterized by chronic inflammation that is associated with structural damage of the lung and fibrosis. Although the etiology of IPF is unknown, it is likely to involve an interaction between environmental and multiple genetic components. Animal models of pulmonary fibrosis have shown that proinflammatory mediators are critical at both the inflammatory and fibrotic stages of the disease. Genetic variants exist in genes encoding proinflammatory mediators, as well as in genes encoding their receptors, which makes these genes candidates for the pathogenesis of IPF. In the present study, we examined 12 biallelic polymorphisms in the genes for tumor necrosis factor (TNF)-α (+488[G/A], −238[G/A], −308[G/A]), lymphotoxin (LT)-α (+720[C/A], +365[C/G], and +249[A/G], determining haplotypes LT-α1 to LT-α4), tumor necrosis factor-receptor 2 (TNF-RII) (gb:M32315: 676[T/G], 1663[A/G], 1668[T/G], 1690[C/T]), and interleukin- (IL)-6 (promoter −174[G/C], intron 4[A/G]). We also examined the haplotypes determined by the three biallelic polymorphisms in each of the TNF-α and LT-α genes. As compared with a normal control population, the IPF group showed no significant deviations in genotype, allele, or haplotype frequencies. Surprisingly, in the IPF population, but not in the control population, an increased frequency of cocarriage of the IL-6 intron 4G and the TNF-RII 1690C alleles was observed, despite the location of the two genes on different chromosomes. Moreover, using impairment of carbon monoxide transfer (D_{LCO}) adjusted for duration of dyspnea as a marker of rapidity of disease progression, we found that the IL-6 intron 4GG genotype was the only genotype independently associated with lower D_{LCO} levels. These findings, if independently confirmed, will be the first to suggest that disease progression in IPF may be linked to a particular genetic marker or to functional polymorphisms in other genes near that marker.

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disorder of the lung of unknown etiology that is characterized by a chronic inflammatory process (alveolitis) and interstitial fibrosis with variable degrees of severity. Although the initiating agent for IPF is unknown, the pathogenesis of pulmonary fibrosis appear to be driven by persistent inflammation characterized by the differential induction of several lower respiratory tract (LRT) extracellular mediators, of which proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 are central (1–4). It appears increasingly likely that there is a genetic predisposition to IPF. This is suggested by the existence of familial forms of IPF, the presence of exaggerated inflammatory activity in the lungs of otherwise unaffected family members of patients with IPF (5, 6), and the failure of exposure to fibrogenic agents such as bleomycin and asbestos to lead to lung fibrosis in all individuals. Although the nature of the genetic component in IPF is unknown, good pathogenic candidates include polymorphisms in genes for proinflammatory cytokines and their receptors. In the present study, we assessed single nucleotide polymorphisms (SNPs) in four candidate genes: the genes for the TNF cluster (TNF-α/lymphotoxin (LT)-α), the high-affinity receptor for TNF-α and LT-α; tumor necrosis factor-receptor 2 (TNF-RII), and the proinflammatory cytokine IL-6.

The TNF-α and LT-α genes are located adjacent to each other in the major histocompatibility complex class III region, on chromosome 6p21.3. TNF-α and LT-α act via two receptors: the 55-kD TNF-RI and the 75-kD TNF-RII, whose cell-surface expression is necessary for the development of lung fibrosis (7, 8). In patients with IPF, there is increased TNF-α expression by alveolar and interstitial macrophages and type II epithelial cells (9–11). Furthermore, TNF-α inhibition and TNF-α overexpression studies in animal models of fibrosing alveolitis (FA) have established TNF-α as a critical mediator in the development of lung fibrosis (12). Significantly, approximately 60% of variation in TNF-α production is considered to be genetically determined (13). Gene knockout studies in mice have also shown that TNF-α/LT-α double-deficient mice are resistant to bleomycin-induced lung fibrosis (14), and in the hapten-immune model of pulmonary fibrosis in hamsters, LT-α has been shown to participate in the immunopathogenesis of pulmonary fibrotic disease (15). Moreover, at the genetic level, different LT-α gene haplotypes have been associated with variations in the *in vitro* levels of TNF-α production by inflammatory cells, which may be primary or due to extended linkage with the haplotypes in the TNF-α gene (16).

IL-6 promotes fibrogenesis either alone or in concert with TNF-α (1, 17), and there is a complex interaction and cross-regulation between the two genes (18–20). IL-6 levels in bronchoalveolar lavage fluid from patients with IPF are significantly higher than in normal subjects (21). Moreover, in the mouse model of bleomycin-induced lung fibrosis, the segregation between the fibrosis-sensitive C57BL/6 mouse strain and the fibrosis-resistant BALB/c mouse strain has been linked to differences between the two strains in the inducibility of IL-6 messenger RNA (mRNA) upon bleomycin exposure (22).

Against this background, we evaluated associations between 12 SNPs within the TNF-α (3), LT-α (3), TNF-RII (4), and IL-6 (2) genes and the development and progression of

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IPF. The polymorphisms assessed in this study have been previously extensively examined in a number of disorders characterized by exaggerated immune modulation.

METHODS

Sequence-Specific Primers and Polymerase Chain Reaction

Polymorphisms were determined with a methodology making use of sequence-specific primers (SSPs) and the polymerase chain reaction (PCR) that utilizes SSPs with 3′ -end mismatches and identifies the presence of specific allelic variants through PCR amplification. For indentifying the polymorphisms in the TNF-RII gene, we used the primer sequences and primer mixtures that we had previously described (23). For the polymorphisms in the TNF-α and LT-α genes, we used the primer sequences (with minor modifications) and primer mixtures previously described by Fanning and colleagues (24). Combinations of forward and reverse allele-specific primer were used to identify the *cis*/*trans* orientation of the alleles and thus the haplotypes in both the TNF-α and LT-α genes. For the biallelic polymorphism in intron 4 (A/G) of the IL-6 gene, we used the SSPs and conditions previously described by Koss and coworkers (25). For identification of the biallelic promotor polymorphism −174(G/C) in the IL-6 gene we used the sequence-specific reverse primers 2133:5′–AATGTGACGTCCTTTAGCATC and 2134:5′–AATGTGACGTCCTTTAGCATG in combination with the consensus forward primer 5′-TCGTGCATGACTTCAGCTTTA at a final concentration of 7.68 ng/μl, with an expected PCR product size of 237 bp (the IL-6[−174] SSP–PCR sequences were kindly provided by Dr. S. E. Marshall) of the Oxford Tissue Typing Center.

The polymorphic variants examined in this study are shown in Table 1.

PCR Conditions

All PCR reactions were run under identical conditions and as previously described (26), in a final volume of 13 μl overlaid with 10 μl of mineral oil. Each reaction mixture consisted of 5 μl of the appropriate primer mix and 8 μl of PCR reaction mixture (the final concentration of the PCR reaction mixture was ×1 PCR buffer (Bioline, London, UK), 160 μM of each deoxynucleotide triphosphate (Bioline), 2 mM $MgCl_2$, 0.3 U *Taq* polymerase (Bioline), and 0.01 to 0.1 μg DNA per well in 96-well plates. PCR amplifications were done in an MJ Research (Waltham, MA) PTC-200 machine. The cycling parameters for the 13-μl reactions were 96° C for 1 min, followed by five cycles of 96° C for 25 s, 70° C for 45 s, and 72° C for 25 s; 21 cycles of 96° C for 25 s, 65° C for 50 s, 72° C for 30 s; and four cycles of 96° C for 30 s, 55° C for 60 s, and 72° C for 90 s. To the completed PCR reaction, we added 10 μl of Orange G loading buffer and loaded the entire product onto a 2% agarose–×0.5 Tris–borate–ethylenediamine tetraacetic acid gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was done for 20 min at 200 V/cm^2, and the gel was photographed under ultraviolet light (320 nm). The presence of an allele-specific band of the expected size, in conjunction with a control band, was considered to be positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band was considered to be evidence for the absence of an allele.

TABLE 1. SINGLE NUCLEOTIDE POLYMORPHISMS EXAMINED IN STUDY

TNFα (24)*:	+488 (G/A)	−238 (G/A)	−308 G/A)
Haplotypes:	TNF-1 G	G	G
	TNF-2 G	G	A
	TNF-3 A	G	G
	TNF-4 G	A	G
LT-α (24)*:	+720 (C/A),	+365 (C/G),	+249 (A/G)
Haplotypes:	LT-α–1 C	C	A
	LT-α–2 A	G	G
	LT-α–3 C	G	A
	LT-α–4 A	C	A
TNF-RII (23):	†Nucleotide 676 (T/G) (exon 6) (TNF-RII[676T/G])		
	†Nucleotide 1663 (A/G) (exon 10) (TNF-RII[1663A/G])		
	†Nucleotide 1668 (T/G) (exon 10) (TNF-RII[1668T/G])		
	†Nucleotide 1690 (C/T) (exon 10) (TNF-RII[1690T/G])		
IL-6:	Intron 4 (A/G) (IL-6[intron4A/G]) (25)		
	Promoter-174 (G/C) (IL6[-174G/C]) (31)		

Definition of abbreviations: IL-6 = interleukin-6; LT-α = lymphotoxin-α; TNF-α = tumor necrosis factor-α; TNF-RII = tumor necrosis factor-receptor 2.

* Of the eight potential haplotypes determined by the three biallelic polymorphisms in the TNF-α and LT-α genes, only four have been reported in the white British population (24). The table describes these haplotypes using the nomenclature given by Fanning and colleagues (24).

† Nucleotide numbering refers to the TNF-RII mRNA sequence accession number gb:M32315.

Patients

All IPF patients were white and from the United Kingdom with the Southeast of England the major patient population referral base. The age of the IPF patients (n = 74) was 61.6 ± 1 (mean ± SEM) yr. The study population consisted of 57 males and 17 females. Mean forced vital capacity (FVC) and gas transfer for carbon monoxide ($D_{L_{CO}}$) were 83.4 ± 2.8% and 50 ± 2.4%, respectively.

The diagnosis of IPF was made according to the following criteria: bilateral crackles on auscultation; exclusion of all known causes or associations with lung fibrosis; presence of typical features on chest high-resolution computed tomography; and a restrictive pulmonary deficit and/or reduced gas transfer measurements. In 23 of 74 patients the diagnosis of FA was confirmed by surgical biopsy.

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

Control Subjects

All control subjects were white cadaveric renal allograft donors from the United Kingdom, collected from the Southeast of England by the Oxford Transplant Centre, Churchill Hospital, Oxford. The representative nature of this control population for white members of the British population in the whole of England has previously been demonstrated in human leukocyte antigen genotyping studies (26). The biallelic polymorphisms in the IL-6 gene were examined in 100 unrelated white control subjects from the United Kingdom. The same 100 control subjects were also part of the control population from which the control polymorphism data for the TNF-α, LT-α, and TNF-RII genes had been previously obtained (23, 24, 27).

Data Analysis

The genotype frequencies, allele carriage frequency (i.e., number of individuals carrying the allele either in both [homozygous] or only one [heterozygous] chromosome), and frequency of an allele in the chromosomal pool of the population (allelic frequency) were determined by direct counting. The genotypes and carriage, allele, and haplotype frequencies for the TNF-α and LT-α genes in the IPF population were compared with the frequencies in the white British control population reported by Fanning and coworkers (24). The allelic frequencies for the TNF-RII gene in the IPF population were compared with the frequencies in the white British control population previously described by Pantelidis and colleagues (23). The polymorphisms in the IL-6 gene in the IPF group were compared with those in the 100 white control subjects examined in our study. All frequencies in the IPF population were compared with the control population by using a 2×2 contingency table and Woolf–Haldane analysis. A value of $p < 0.05$ was considered significant.

Associations between alleles and haplotypes in each locus were also explored, using the chi-square test for independence (KnowledgeSEEKER; Angoss Software, Guildford, UK). For the TNF-α/LT-α haplotypes and the TNF-RII alleles, the IPF group allele or haplotype associations were compared with those previously reported for the British control population (23, 24). For the IL-6 alleles we report for both the white British normal control and the IPF group the significance value for the chi-square test for independence and a standardized Δ value (Δs), which was calculated as:

$$\Delta s = \frac{\Delta}{\Delta max} = \frac{Pab - (PaPb)}{Pa(1 - Pb)}$$

where *Pab* is the observed frequency of haplotype ab, *Pa* is the frequency of allele a, and *Pb* is the frequency of allele b.

TABLE 2. ALLELIC FREQUENCIES OF TUMOR NECROSIS FACTOR-α LYMPHOTOXIN-α POLYMORPHISMS IN THE IDIOPATHIC PULMONARY FIBROSIS AND CONTROL GROUPS

Polymorphism		IPF (*n* = 74)		UK Controls (*n* = 201)	
TNF-α					
1		102	(70.8)	262	(65.2)
2		25	(17.4)	84	(20.9)
3		13	(9.0)	32	(8)
4		4	(2.8)	24	(6)
+488	G	131	(91)	362	(90)
	A	13	(9)	40	(10)
−238	G	140	(97.2)	378	(94)
	A	4	(2.8)	24	(6)
−308	G	119	(82.6)	318	(79)
	A	25	(17.4)	84	(21)
LT-α					
1		57	(38.5)	153	(38.1)
2		59	(39.9)	139	(34.4)
3		32	(21.6)	109	(27.1)
4		0	(0)	1	(0.2)
+249	A	89	(60.1)	263	(65)
	G	59	(39.9)	139	(35)
+365	G	91	(61.5)	248	(62)
	C	57	(38.5)	154	(38)
+720	C	89	(60.1)	262	(65)
	A	59	(39.9)	140	(35)

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; LT-α = lymphotoxin-α; TNF-α = tumor necrosis factor-α; UK = United Kingdom.

Numbers represent allele frequencies, with percentages given in parentheses. The UK control data were taken from Fanning and colleagues (24).

All 12 polymorphisms were examined in the same cohort of 100 white British control subjects and all 74 IPF patients, permitting the investigation of differences in the cocarriage of combinations of alleles located on different genes and chromosomes in these two groups. For the interchromosomal allelic associations, we report: (*1*) the significance value for the chi-square test for independence; (*2*) the significance value corrected for the number of alleles examined in this study (according to the formula $pc = 1 - (1-p)^n$, where *pc* is the corrected value, *p* the uncorrected value, and n the number of alleles) and; (*3*) a standardized Δ value.

Genetic influences on the rapidity of disease progression were evaluated through stepwise linear regression (STATA Corporation, College Station, TX). Relationships between alleles or allelic combinations and $D_{L_{CO}}$ levels were examined after adjusting for the duration of dyspnea; in this way, reduction in $D_{L_{CO}}$ for a given duration of disease was quantified, and thus the genetic determinants of the rapidity of deterioration were identified. Smoking status was also included as a covariant in all models. $D_{L_{CO}}$ levels were transformed logarithmically (zero skewness logarithmic transformation) before analysis. Tests for heteroscedasticity and omitted variables were used to ensure that the assumptions of multiple linear regression were not violated.

For the present study we report all interchromosomal allelic associations, as well as the major genotype associations with disease progression, as assessed by $D_{L_{CO}}$ impairment adjusted for duration of dyspnea, in which a value of $p < 0.05$ was observed, on the grounds that significance at this level merits further exploration in independent studies. This is because we cannot exclude the possibility that the observed genetic associations were due to a type 1 error, since the size of our study sample, although large for IPF, was relatively small compared with samples in common disease population studies.

TABLE 3. ALLELE FREQUENCIES OF THE TUMOR NECROSIS FACTOR-RECEPTOR 2 POLYMORPHISMS IN THE IDIOPATHIC PULMONARY FIBROSIS AND CONTROL GROUPS

Polymorphisms	IPF (*n* = 74)		UK Controls (*n* = 192)	
TNF-RII (exon 6) − 676				
T	122	(82.4)	297	(77.3)
G	26	(17.6)	87	(22.7)
TNF-RII (exon 10) − 1663				
G	73	(49.3)	197	(51.3)
A	75	(50.6)	187	(48.7)
TNF-RII (exon 10) − 1668				
T	143	(96.6)	366	(95.3)
G	5	(3.4)	18	(4.7)
TNF-RII (exon 10) − 1690				
T	87	(58.8)	246	(64.1)
C	61	(41.2)	138	(35.9)

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; TNF-RII = tumor necrosis factor-receptor 2; UK = United Kingdom.

Numbers represent allele frequencies, with percentages given in parentheses. The UK control data were taken from Pantelidis and coworkers (23).

RESULTS

Relative Frequency Comparisons

Tables 2 to 4 summarize the allele frequencies in the IPF and control populations. Direct comparisons between genotype, allele carriage, and allelic frequencies in the IPF and control populations did not reveal significant frequency differences between the two groups. In the IPF group the TNF-2 haplotype was observed more frequently in females (58.8%) than in males (21.1%) ($p = 0.002$).

Locus-Specific Allelic Association

We assessed whether there were differences in allele association between polymorphisms at different loci for the IPF and control groups. In the normal white British population, a tight linkage disequilibrium between the TNF-2/LTα-2 haplotypes ($p = 1.2 \times 10^{-16}$) has been described (24). The same association was observed in the IPF group in our study (TNF-2/LTα-2 haplotype ($p = 9 \times 10^{-6}$).

In the TNF-RII gene, we have previously reported the association between the TNF-RII(1,663A) and TNF-RII(1,690C) alleles ($p < 0.0001$) for a normal white British control population (23), and we observed the same association in the IPF group ($p = 0.009$) in the current study.

In both the IPF and control groups a strong linkage disequilibrium was observed in the IL-6 gene between the IL-6(intron 4G)/IL-6(−174C) alleles (normal: $p = 7 \times 10^{-11}$, $\Delta s = 0.55$; IPF: $p = 5 \times 10^{-13}$, $\Delta s = 0.82$) and the IL-6(intron 4A)/IL-6(−174G) alleles (normal: $p = 3 \times 10^{-14}$, $\Delta s = 0.75$; IPF: $p = 4 \times 10^{-15}$, $\Delta s = 0.87$).

Interchromosomal Allelic Association

The genetic component in complex-trait diseases such as IPF is likely to involve the interaction of alleles in multiple genes. In the present study we determined whether the frequency of

TABLE 4. ALLELE FREQUENCIES FOR THE TWO INTERLEUKIN-6 GENE POLYMORPHISMS IN PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS AND WHITE BRITISH CONTROL SUBJECTS

Polymorphism	Allele	IPF (*n* = 74)		UK Controls (*n* = 100)	
IL-6 (intron 4)	G	83	(56.1)	116	(58)
	C	65	(43.9)	84	(42)
IL-6 (−174)	A	82	(55.4)	108	(54)
	G	66	(44.6)	92	(46)

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; IL-6 = interleukin-6; UK = United Kingdom.

Numbers represent allele frequencies, with percentages given in parentheses.

cooccurrence of alleles on the four genes examined differed between the IPF and normal control populations. Interestingly, we observed a strong association between carriage of the IL-6(intron 4G) allele and the TNF-RII(1690C) allele in patients with IPF ($p = 0.00093$, $pc = 0.0184$, $\Delta s = 0.43$) but not in the control subjects.

Allelic Associations with Disease Progression

Using percent predicted $D_{L_{CO}}$ values as a measure of disease progression (by taking into account time since the onset of dyspnea, and adjusting for the confounding effect of smoking status), we examined whether polymorphic variants at the genotype or carriage level were independent determinants of $D_{L_{CO}}$ levels. When examined in isolation, the only genotype significantly linked to disease progression was IL-6(intron 4GG), which was independently associated with lower $D_{L_{CO}}$ levels ($p = 0.035$, $R^2 = 0.18$) after controlling for disease duration and smoking status (both of which were independently associated with lower $D_{L_{CO}}$ levels ($p < 0.02$)). Carriage of either the IL-6(intron 4G) or IL-6(−174C) allele was also independently associated with lower $D_{L_{CO}}$ levels, but these trends were not statistically significant (both $p = 0.07$).

DISCUSSION

It is now well established that there is a strong link between the overexpression of lower respiratory tract proinflammatory mediators, including TNF-α, LT-α, and IL-6, and the development of IPF. A number of lines of evidence also support the involvement of a genetic component as a determinant of susceptibility to development and progression of IPF. Polymorphisms in the TNF-α, and LT-α genes and the gene for their receptor, TNF-RII, as well as in the IL-6 gene, are therefore good candidates in relation to the development and progression of IPF. In the present study, we found that the genotype, carriage, and allele frequencies did not differ between a normal white British control population and a population with IPF. However, we did observe a significant increase in the frequency of the TNF-2 haplotype in females with IPF as compared with males with IPF. Interestingly, a similar gender association was observed in a recent study of the distribution of TNF-α haplotypes in ulcerative colitis. There, the TNF-2 haplotype was found to be more frequent in women with extensive rather than distal colitis (16). Whether this indicates that carriage of the TNF-2 haplotype predisposes women to more severe forms of a chronic inflammatory response needs to be assessed.

TNF-α is one of the early cytokines that has been consistently found in animal models of pulmonary fibrosis to play a cardinal role in the pathogenesis of this disease. This makes the TNF-α gene a primary candidate gene for susceptibility to IPF. Interestingly, none of the three TNF-α polymorphisms examined in the present study, nor the haplotypes defined from these polymorphisms, were found to be significantly associated with IPF. In a recent study, Whyte and colleagues also examined the TNF-α −308 polymorphism in a white British IPF population and reported the absence of an association between this polymorphism and IPF in this population, although they did report an association between the TNF-α −308 allele 2 and IPF in an Italian cohort (28). However, the TNF-α −308 allele 2 is in strong linkage disequilibrium with other polymorphisms in this region of chromosome 6, such as the DRB1(*)03 allele (29), and since a number of polymorphisms exist in the TNF-α gene other than those examined here (30), further analysis is needed to clarify this association.

However, the genetic component that contributes to the manifestation of complex-trait diseases such as IPF is likely to involve an interaction between multiple alleles located on different genes and chromosomes. In the present study we observed in the IPF population an increased frequency of co-carriage of the IL6(intron 4G) allele located on chromosome 7p21–p14, and the TNF-RII(1690C) allele located on chromosome 1p36.2, which may denote susceptibility to IPF. The size of our IPF population sample, although large for IPF, was not large enough to achieve the statistical power required to absolutely confirm this type of complex analysis. However, we report here the observed interchromosomal association with a value of $p > 0.05$ on the grounds that it merits further exploration in independent studies of additional IPF populations. If the association between the IL6(intron 4G) and TNF-RII (1690C) alleles in IPF is independently confirmed, it will raise the intriguing question of whether the association is primary or indicative of an association with polymorphisms in the same or other genes near these IL-6 and TNF-RII alleles. The former would imply a yet unidentified functional role for the two polymorphic loci in these two genes.

Significantly, the IL-6(intron 4GG) genotype was the only genotype found to be independently associated with lower $D_{L_{CO}}$ levels (after controlling for the duration of dyspnea and smoking status). Although, the functional role of IL-6(intron 4G) is not known, we have found that this allele is in tight linkage disequilibrium with the IL-6(−174C) allele, which in a luciferase reporter vector system has been shown to be associated with lower levels of expression than the alternative IL-6(−174G) allele (31). Therefore, these associations would suggest that greater reduction in $D_{L_{CO}}$ for a given duration of disease might be associated with a genetic predisposition to lower levels of IL-6 production.

In conclusion, the present study is the first to link IPF susceptibility to the carriage of a combination of alleles on different genes, and to suggest that genetic variations within proinflammatory mediators may affect disease progression in IPF. The biologic significance of these genetic associations now requires further evaluation.

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