Tumour necrosis factor family genes in a phenotype of COPD associated with emphysema

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ABSTRACT: Genetic factors are believed to play a role in the individual susceptibility to chronic obstructive pulmonary disease (COPD). Tumour necrosis factor (TNF) family genes have been widely investigated but inconsistent results may lie either in the genetic heterogeneity of populations or in the poor phenotype definition. A genetic study was performed using a narrower phenotype of COPD.

The authors studied 86 healthy smokers and 63 COPD subjects who were enrolled based on irreversible airflow obstruction (forced expiratory volume in one second/forced vital capacity <70% predicted) and a diffusing capacity for carbon monoxide <50% predicted (moderate-to-severe COPD associated with pulmonary emphysema). The following polymorphisms were investigated: TNF-308, the biallelic polymorphism located in the first intron of the lymphotoxin-α gene, and exon 1 and exon 6 of the TNF receptor 1 and 2 genes, respectively.

No significant deviations were found concerning the four polymorphisms studied between the two populations.

The authors confirm that the tumour necrosis factor family genes, at least for the polymorphisms investigated, are not major genetic risk factors for chronic obstructive pulmonary disease in Caucasians, either defined in terms of emphysema (this study) or airflow obstruction (previous studies). Nevertheless, the authors would like to emphasise the importance of narrowing the phenotype in the search for genetic risk factors in chronic obstructive pulmonary disease.
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Chronic obstructive pulmonary disease (COPD), a worldwide leading cause of mortality and morbidity, is a complex trait arising from the effects of environmental risk factors, mostly tobacco smoking, on susceptible individuals. In an attempt to further understand the role of genetic factors accounting for individual COPD susceptibility, a number of gene polymorphisms have been investigated in the last few years, including α_1-antitrypsin-deficient variants in an intermediate status, microsomal epoxide hydrolase, glutathione-S-transferase, tumour necrosis factor (TNF) gene complex, and haemoxygenase. However, many of the associations found with COPD are controversial, since they have not been replicated in different populations [1–3].

Studies of TNF gene complex, coding for cytokines relevant to the pathophysiology of COPD [2], are the most abundant reports on this issue available in the literature, and they are a peculiar example of such inconsistent results. It was previously reported that the frequency of allele 2 (TNF-308*2), of the biallelic polymorphism located within the promoter region of the TNF-α gene (TNF-308), associated with higher levels of TNF production [4], was significantly higher in the Taiwanese with chronic bronchitis [5] and in the Japanese with COPD [6] than in appropriate controls. In marked contrast, the association with COPD was rejected by

similarly planned case-control studies in Italian [7] and British [8] populations and by a longitudinal study focused on decline of lung function within the Lung Health Study in Northern Americans [9].

A likely explanation for these inconsistent results lies in genetic heterogeneity among different populations, a feature that is well known in other ubiquitous disorders, such as sarcoidosis [10]. However, another critical point in planning a genetic investigation on a complex trait is the phenotype choice, and poor definition of the phenotype and/or heterogeneity of the condition are likely to reduce the power to find significant association [11]. COPD is a widely heterogeneous condition [12, 13], including pulmonary emphysema, chronic bronchitis, and bronchial hyperreactivity, often in combination [14]. Therefore, it cannot be ruled out that an incomplete definition of the phenotype underlies inconsistency in results of genetic studies on COPD [15] and those dealing with the TNF gene in particular.

Working on this assumption and given the lack of association of allele 2 of the TNF-308 (TNF-308*2) with COPD in another Japanese study [16], and the findings of KEATINGS et al. [17] who identified a COPD phenotype, i.e. poor prognosis possibly associated with the same allele in the British population, the present authors decided to re-evaluate

the role of the TNF family genes in the Italian COPD population. In order to do this, a new COPD population was recruited, characterised by narrower functional inclusion criteria: irreversible airflow obstruction and a diffusing capacity <50% predicted, the latter of which was assumed to be a surrogate marker for pulmonary emphysema. The authors evaluated the frequency of the TNF-308 polymorphism and that of the biallelic polymorphism (LtαNcoI) located in the first intron of the lymphotoxin-α gene (Lt-α, previously referred to as TNF-β), in which allele 1 (LtαNcoI*1) is associated with higher levels of TNF production [18], and compared the results with those obtained in appropriate controls. The investigation was also expanded in the two populations by adding an investigation of two biallelic polymorphisms of the TNF receptors 1 and 2 (TNFR1 and TNFR2, also referred to as p55 TNFR and p75 TNFR, respectively), which are type-I membrane ligands on the cell surface for both TNF-α and Lt-α [19]. TNFR1 and TNFR2 genes are also thought to be candidate genes involved in mediating the numerous TNF-α and Lt-α effector functions [20].

Subjects and methods

All individuals gave their consent prior to entering the study, which was approved by the Local Ethical Committees of the Institutions involved.

Design of the study

The investigation was designed as a case-control association study with candidate genes, a powerful approach for finding genetic determinants of a complex disorder such as COPD [15, 21].

Subjects and inclusion criteria

A total of 149 subjects, divided in two groups, with all Caucasians of Italian descent, were investigated. The first group of subjects consisted of 63 consecutive male patients with history of COPD, diagnosed according to the American Thoracic Society (ATS) guidelines [22] and characterised by significant impairment in diffusing capacity for carbon monoxide (D_L,CO), a functional abnormality known to be associated with pulmonary emphysema [23, 24]. These patients were recruited in two clinical centres, 33 in Pavia and 30 in Gussago, both located in Northern Italy, based on a common protocol requiring forced expiratory volume in one second (FEV1) <50% pred, <12% reversible, FEV1/forced vital capacity (FVC) <70% pred, and D_L,CO <50% pred. To narrow the phenotype, patients fulfilling the first three criteria, but with a D_L,CO >50% were excluded from the study.

The second group consisted of 86 healthy male current or former smokers who served as a control group. They were recruited from the clinical staff and from a cohort of blood donors. A periodic medical survey (including medical examination, questionnaire, blood and urine chemistry, chest radiography, and pulmonary function tests) excluded any diseases.

Pulmonary function tests

Lung volumes were measured by water-sealed spirometers. Measurements were performed according to the European

Community for Steel and Coal statements [25] and the ATS recommendations [26]. The best FVC measurement was recorded, as was the FEV1, and the FEV1/FVC calculated. D_L,CO was determined using the single-breath method and corrected for haemoglobin content, as described previously [25, 26]. Since correction of D_L,CO for alveolar volume did not influence the results of the analysis, only uncorrected D_L,CO values are reported.

Genetic analysis

Genomic deoxyribonucleic acid (DNA) was extracted from whole blood by standard methods. For detection of TNF-308 and LtαNcoI polymorphisms in COPD and healthy control populations, the same primers [18] and conditions adopted in previous investigations [7] were used. For the TNFR1 exon 1 polymorphism, the polymerase chain reaction (PCR) conditions were as follows: PCR amplifications were carried out in a total volume of 25 μl containing 100 ng genomic DNA, 0.5 unit Thermus Aqiaticus (Taq) DNA polymerase (Laboratoires Eurobio, Les Ulis Cèdex, France), 0.5 μM of each PCR primer, 1.5 mM MgCl$_2$, 200 μM of each dNTP, 67 mM Tris-HCl pH 8.8, 16 mM (NH$_4$)SO$_4$, and 0.01% Tween. Amplification was performed using a Gene Amp system 2400 (Perkin Elmer,Norwalk, CT, USA) with the following conditions: initial incubation at 95°C for 5 min, followed by 35 cycles each of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C, and a final incubation at 72°C for 5 min. The primers used were forward 5′ GAGCCCAAATGGGGGAGTGA-GAGG 3′ and reverse 5′ ACCAGGCCCGGGCAGGAGAG 3′ [27]. The size of the PCR fragment product was 183 base pairs (bp). Restriction fragment length polymorphism (RFLP) analysis identified a single-nucelotide polymorphism (SNP) at position 36 (A→G), which creates a recognition site for the restriction enzyme *MspA1 I* (New England Biolabs, Beverly, MA, USA) when the G allele, but not the A allele, is present [27]. The G allele digestion generates restriction fragments of 108 and 75 bp from the PCR products (fig. 1).

For the TNFR2 exon 6 polymorphism, the PCR conditions were the same as above. Primers used were: forward 5′ ACTCTCCTATCCTGCCTGCT 3′ and reverse 5′ TTCT-GGAGTTGGCTGCGTTGT 3′ [27]. The size of the PCR fragment generated was 242 base pairs. RFLP analysis identified an SNP at position 196 (T→G), which creates a recognition site for the restriction enzyme *Nla III* (New England Biolabs) when the T allele, but not the G allele, is present. This polymorphism results in an amino acid substitution (Met→Arg). The 242 base pair PCR product is uncleaved in the 196 G allele (also called the R allele), and cleaved into two fragments of 133 and 109 bp in the 196 T allele (also called the M allele) [28] (fig. 1).

Statistical analysis

Clinical data are presented as mean±SD and differences among study groups were assessed by two-tailed Student′s t-tests. Frequencies of the polymorphisms were compared with Chi-squared test and Fisher′s exact test, and differences considered statistically significant when the p-value was <0.05. Hardy-Weinberg′s equilibrium was assessed by goodness-of-fit Chi-squared test for biallelic markers. Correspondence analysis [29] was used to explore the interactions among the four genes investigated simultaneously. The population used in this study was large enough to have the statistical power to detect an allele that imparted an odds ratio of 2.5 given a 0.10 prevalence for the TNF-308 polymorphism, a 0.80 prevalence
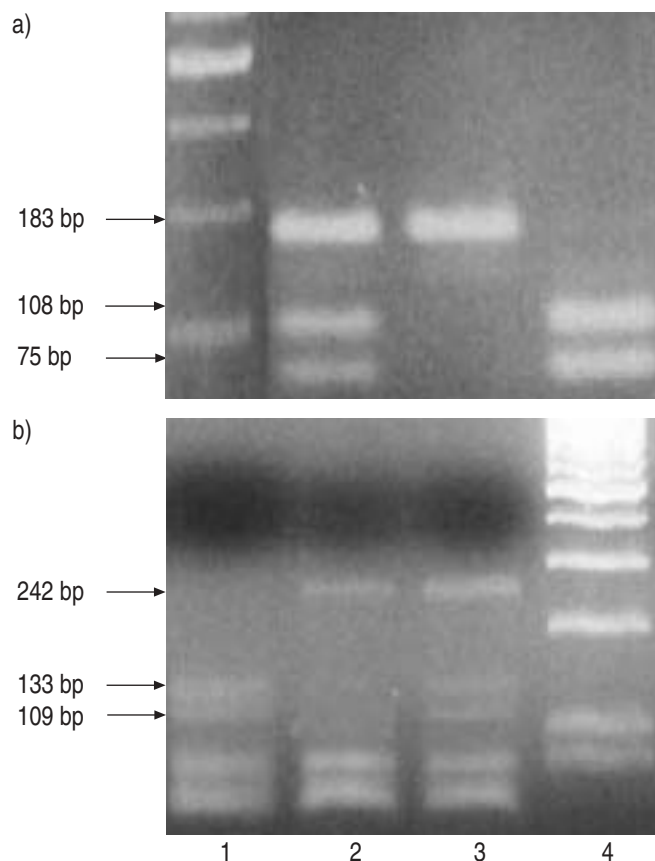
a)

183 bp →

108 bp →
75 bp →

b)

242 bp →

133 bp →
109 bp →

1   2   3   4

Fig. 1.– a) Restriction analysis of tumour necrosis factor receptor (TNFR)1 exon 1 polymorphism resolved in 2% agarose gel electrophoresis of polymerase chain reaction (PCR) amplified genomic deoxyribonucleic acid. A 183 base pair (bp) product (A allele) is restricted by the enzyme *MspA1 I* to fragments of 108 and 75 bp (G allele). Lane 1: molecular weight marker (GeneRuler 100 bp, Fermentas, Vilnius, Lithuana); lane 2: heterozygous AG; lane 3: homozygous AA; lane 4: homozygous GG. b) Restriction analysis of TNFR2 exon 6 polymorphism resolved in 2% agarose gel electrophoresis. Restriction of a 242 bp PCR product (allele R) with the enzyme *Nla III* results in fragments of 133 and 109 bp (allele M). Lane 1: homozygous MM; lane 2: homozygous RR; lane 3: heterozygous RM; lane 4: molecular weight marker.

for the LtαNcoI polymorphism, a 0.40 prevalence for the TNFR1 polymorphism, and a 0.70 prevalence for the TNFR2 polymorphisms in the COPD population.

Results

Characteristics of the study populations

The COPD group included 63 subjects, all males aged 69 ± 8 yrs. Fifty patients in this group were former smokers, and 13 current smokers, with a smoking history of 51 ± 30 pack-yrs. The control group comprised 86 healthy smokers, all males aged 58 ± 12 yrs. Forty-nine subjects of this group were former smokers, and 37 current smokers, with a smoking history of 34 ± 21 pack-yrs.

Pulmonary function testing data of the study populations are reported in table 1. According to the recently published Global initiative for Chronic Obstructive Lung Disease report [30], COPD patients were characterised, in terms of severity, by moderate-to-severe COPD (Stage II–III). The mean±SD FEV1 reversibility in these subjects was 4 ± 4. The low level of

Table 1.– Functional characteristics of the populations studied

	Patients n	FEV1	FVC	FEV1/FVC	$D_{L,CO}$
COPD	63	31 ± 10	60 ± 13	36 ± 9	37 ± 12
Controls	86	92 ± 8	94 ± 16	95 ± 11	

Data are presented as mean±SD % predicted; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; $D_{L,CO}$: diffusing capacity of carbon monoxide; COPD: chronic obstructive pulmonary disease.

$D_{L,CO}$ in the COPD group ($37\pm12\%$ pred) allowed them to be defined as affected by moderate-to-severe COPD (Stage II–III) associated with pulmonary emphysema [23, 24].

Genetic analysis

All genotypes of control and COPD groups agreed with the Hardy-Weinberg equilibrium. With reference to the TNF-308 polymorphism, the results obtained in the COPD population defined in terms of COPD associated with emphysema were consistent with the authors' previous findings and with those obtained by others in Caucasian subjects defined in terms of airflow obstruction, showing no different distribution of both genotypes and alleles with respect to the distribution in controls (table 2). Table 3 shows the data concerning the investigation of the TNFR1 and TNFR2 polymorphisms. No significant deviations between the two groups were observed concerning either the allele or the genotype frequencies.

Results in the COPD group did not change after stratification by severity of respiratory impairment and correspondence analysis failed to reveal combinations of genotypes in the COPD group with significant deviations with respect to controls (data not shown).

Table 2.– Frequencies of tumour necrosis factor (TNF)-308 polymorphisms and the biallelic polymorphism located in the first intron of the lymphotoxin-α gene (LtαNcoI) in the two populations studied

	Controls	COPD
TNF-308		
Patients n	86	63
Genotype		
1,1	72 (0.84)	54 (0.86)
1,2	14 (0.16)	9 (0.14)
2,2	0	0
Allele		
TNF-308*1	158 (0.92)	117 (0.93)
TNF-308*2	14 (0.08)	9 (0.07)
LtαNcoI		
Patients n	85	59
Genotype		
1,1	4 (0.05)	1 (0.02)
1,2	34 (0.4)	18 (0.3)
2,2	47 (0.55)	40 (0.68)
Allele		
LtαNcoI*1	42 (0.25)	20 (0.17)
LtαNcoI*2	128 (0.75)	98 (0.83)

Data are presented as frequency (%). COPD: chronic obstructive pulmonary disease. One subject among controls and four subjects among COPD were not typed for LtαNcoI because of insufficient deoxyribonucleic acid. All comparisons were nonsignificant.

Table 3. – Frequencies of tumour necrosis factor receptor (TNFR)1 exon 1 nt 36 and TNFR2 exon 6 nt 196 polymorphisms in the two populations studied

	Controls	COPD
TNFR1		
Patients n	81	61
Genotype		
A,A	28 (0.35)	18 (0.30)
A,G	44 (0.54)	33 (0.54)
G,G	9 (0.11)	10 (0.16)
Allele		
A	100 (0.62)	69 (0.56)
G	62 (0.38)	53 (0.44)
TNFR2		
Patients n	81	63
Genotype		
M,M	45 (0.55)	28 (0.45)
M,R	31 (0.38)	31 (0.49)
R,R	5 (0.06)	4 (0.06)
Allele		
M	121 (0.75)	87 (0.69)
R	41 (0.25)	39 (0.31)

Data are presented as frequency (%). COPD: chronic obstructive pulmonary disease. Five subjects among controls and two subjects among COPD were not typed for TNFR1 and/or TNFR2 because of insufficient deoxyribonucleic acid. All comparisons were nonsignificant.

Discussion

Phenotype choice and biological reasonability of candidate genes are crucial prerequisites for a genetic investigation on a complex trait such as COPD [21]. All previously published papers, including the authors' [5–8, 16, 17], dealing with the TNF gene family in COPD, have used FEV1 as an objective parameter to define the phenotype. FEV1 is strongly related to COPD [15] and predictive of all-cause mortality in population-based studies [31]. Nevertheless, FEV1 is relatively nonspecific, reflecting many different aspects of lung pathology in COPD [32], and it does not help provide a better definition of these patients affected by a widely heterogeneous condition [12–14]. In an attempt to narrow the phenotype choice to COPD-associated pulmonary emphysema, a severe reduction of DL,CO was used as an inclusion criterion. FEV1 % pred is weakly related to the extent of emphysema [24, 33, 34], suggesting that flow obstruction in severe COPD is not totally accounted for by the extent of emphysema. Reduction of DL,CO is more strongly correlated with the severity of emphysema, as assessed by high-resolution computed tomography analysis [24], reflecting the reduction of the alveolar-capillary surface, although with some limitations [24, 35].

The biological reasonability of the TNF family as candidate genes in COPD has already been emphasised [2, 36]. TNF-α levels are increased in sputum of COPD patients [37], and weight loss in COPD has been associated with increased levels of circulating TNF-α [38]. The potential implications of the two TNF-α and Lt-α polymorphisms investigated have already been extensively discussed [1–3, 5–9]. In this article, for the first time, the authors have added the genetic study of two other TNF family members, TNFR1 and TNFR2, biologically reasonable candidates involved in mediating a number of TNF-α and LT-α effector functions [19]. The soluble form of TNFR1 has been shown to correlate with leptin plasma concentration, in turn related to lower fat mass, in emphysema, but not in chronic bronchitis [39]. The TNFR2 exon 6 polymorphism investigated in the present paper was previously found to be associated with human narcolepsy in the Japanese [40], and with systemic lupus erythematosus (SLE) [41] and rheumatoid arthritis in British patients [42], but not with SLE in either Spanish or British populations [28] or idiopathic pulmonary fibrosis [43] in British patients.

In the present paper, the authors provide evidence that the two alleles of the TNF-α and Lt-α genes (TNF-308*2 and LtαNcoI*1, respectively), related to higher constitutional production of both cytokines, are not linked to moderate-to-severe COPD associated with emphysema in Italians. When the present study was planned, it was debated whether inconsistent results in different populations with respect to association of TNF-308*2 allele with COPD was due to a bias related to heterogeneity of COPD patients. In fact, the paper by SAKAO et al. [6] reported a positive association with Japanese COPD patients with chronic obstruction in whom a history of chronic bronchitis had been excluded, thus suggesting, even if not clearly stated in the paper, that those patients were mostly affected by emphysema. By contrast, a positive association with the TNF-308*2 allele was found in Taiwanese subjects meeting the criteria for chronic bronchitis [5]. However, it must be underlined that neither study provided any objective verification of the presence (or absence) of emphysema. The negative study reported by HIGHAM et al. [8] in British subjects did not use DL,CO as an inclusion criterion, but reported a mean±SEM of 75±4% pred in their series of 86 COPD patients, a much less severe impairment than that found in the present series (mean±SD 37±12).

TNF receptor polymorphisms were first studied in COPD in the present paper. The data, however, allows rejection of the hypothesis that they are a major genetic risk factor for COPD, at least that defined in terms of pulmonary emphysema.

In this paper, the authors focused attention on biologically reasonable polymorphic sites, such as the TNF-308 polymorphism of the TNF-α gene and the LtαNcoI polymorphism of the Lt-α gene, as well as on a polymorphic site whose gene product is of unknown biological effect, such as TNFR2 exon 6 polymorphism, but that has been reported previously to be associated with a number of disorders [40–42]. However, a number of other polymorphic sites are described within the TNF family genes [43, 44] and, therefore, their involvement in COPD cannot be excluded.

To conclude, these findings seem to confirm that the polymorphisms of tumour necrosis factor family genes related to higher constitutional production of cytokines are not major genetic determinants for chronic obstructive pulmonary disease in Caucasians, thus confirming the previously reported negative data in this ethnic group. In spite of the negative results from the investigation, the authors would like to stress the importance of narrowing the phenotype spectrum in a heterogeneous condition such as chronic obstructive pulmonary disease. This concept is further strengthened by two recent, independent reports [45, 46], which dealt with linkage analysis of quantitative spirometric phenotypes and demonstrated that forced expiratory volume in one second, forced vital capacity, and forced expiratory volume in one second/forced vital capacity are influenced by different loci. Another strategy would be to introduce a different phenotype of chronic obstructive pulmonary disease, such as qualitative and quantitative assessment of pulmonary emphysema by high-resolution computed tomography scan.

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