

Atopic Asthma and TNF-308 Alleles: Linkage Disequilibrium and Association Analyses

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ABSTRACT: The association of a tumor necrosis factor –308 allele (TNF2) to asthma has been reported in some studies but not in others. The aim of this study was to test this association in a population recruited on the basis of allergy to *Parietaria*. In the study population, asthma was positively associated to HLA-DRB1*03 ($p = 0.01$) and to the haplotype TNF2/DRB1*03 ($p = 0.02$). In the parent subgroup, the proportion of asthmatics was increased in patients with TNF2 ($p = 0.01$), but the primary association of asthma was to the haplotype TNF2/DRB1*1104 ($p = 0.005$). The study population was subdivided according to prick skin test (ST) positivity to *Lolium*, *Parietaria*, and *D. pteronyssinus*. Asthma was associated to HLA-DRB1*03 and to the haplotype TNF2/DRB1*03 ($p = 0.0015$ and 0.0001 , respectively) in patients ST positive to *Lolium*, and to the haplotype TNF2/DRB1*1104 ($p = 0.025$) in patients ST positive to *Parietaria*. The transmission disequilibrium test detected excess transmission of

HLA-DRB1*03 and of the haplotype TNF2/DRB1*03 ($p = 0.03$ and 0.04 , respectively) to siblings with asthma and ST positivity to *Lolium* and of HLA-DRB1*1104 and of the haplotype TNF2/DRB1*1104 ($p = 0.04$ and 0.015 , respectively) to siblings with asthma and ST positivity to *Parietaria*. Taken together, these observations indicate that the haplotypes TNF2/DRB1*03 and TNF2/*B1*1104 contain alleles controlling atopic asthma in patients with sensitization to *Lolium* and *Parietaria*, respectively. This suggests that the association of asthma to TNF2 reflects linkage disequilibrium with genes influencing specific immune response. *Human Immunology* 64, 359–365 (2003). © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Science Inc.

KEYWORDS: TNF- α ; asthma

ABBREVIATIONS

TNF tumor necrosis factor
HLA human leukocyte antigen
ST prick skin test

BBS borate buffered saline
TDT transmission disequilibrium test
DARIA double antibody radioimmunoassay

INTRODUCTION

In the human population, the phenotypes of atopy and asthma are partially overlapping. Atopy is a common condition, characterized by the presence in serum of antibodies of IgE isotype specific for environmental antigens (allergens) as documented by the results of immu-

noassays and/or by the presence of cutaneous sensitization to allergenic extracts (positivity to prick skin test [ST]). Atopy can occur in healthy patients or be accompanied by clinical symptoms (rhinitis, asthma, conjunctivitis). Asthma is defined by clinical symptoms and is conventionally subdivided in the subtypes of extrinsic (or atopic), and intrinsic (nonatopic), according to the atopic phenotype of the patient. It is controversial whether these subtypes, which demonstrate distinct clinical and biologic features, share common immunopathologic mechanisms [1, 2]. Susceptibility to develop atopy and/or asthma is genetically controlled and candidate genes have been identified at numerous chromosomal regions [3, 4].

The human leukocyte antigen (HLA) region (chromosome 6p21) contains genes that control the individual's

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ability to mount antibody responses to many allergens (including *Lolium*, olive, ragweed, and *Parietaria* allergens) [5–10]. More recently, some genomic studies have reported the linkage of HLA region markers to asthma. These include a genomic screen of Northern American populations with different ethnic backgrounds, reporting the linkage of 6p21 markers to asthma in the European American group [11], and a genomic screen in Northern American Hutterites reporting association of HLA region to asthma but not linkage, suggesting the involvement of a minor susceptibility locus [12]. No linkage was detected in a large European study on genetics of mite sensitization [13].

A candidate for contributing to the control of asthma is the tumour necrosis factor (TNF) gene, located at the telomeric end of HLA class III region. The encoded protein is a proinflammatory cytokine, released from many activated cells [14]. TNF may have multiple proinflammatory roles in the pathogenesis of asthma, including aggravation of airway hyperreactivity, activation of mast cells, macrophages and eosinophils enhancing effects on IL-4-induced germ-line epsilon RNA synthesis in B cells [15–18], and upregulation of release of IL-9 by cultured human peripheral blood eosinophils from asthmatic patients [19]. In the lung, TNF is synthesized and stored primarily in mast cells and alveolar macrophages [20].

A diallelic polymorphism at –308 of the TNF promoter involves a guanine to adenosine substitution (TNF1 and TNF2, respectively) [21]. The association of TNF2 to increased gene expression has been reported by Wilson *et al.* [22], but not by others [23, reviewed in 24]. TNF polymorphisms are associated to certain diseases related to inflammation [25–28], but it is often difficult to distinguish their contribution from that of alleles at other HLA region loci because of linkage disequilibrium [29, 30].

A positive association of asthma to TNF2 has been reported in some studies [31–37] but not in others [38–40]. It has been hypothesized that the effect of TNF on asthma is independent from that of the HLA class II genes controlling antibody responses to allergens. In support of the hypothesis that TNF effect on asthma is not mediated by IgE antibodies, Moffatt *et al.* [33, 34] reported that although TNF2 and the haplotype TNF2/HLA-DRB1*02 are associated with asthma, the locus DRB1 is associated with high levels of serum total IgE and of mite and cat allergens specific IgE antibodies, but not to asthma [35].

Polygenic control, genetic heterogeneity, and the effect of environmental and lifestyle factors hinder the identification of susceptibility genes for allergic as for other common diseases [41]. The results of recent studies in populations selected for sensitization to mite [10–13] suggest that one strategy that may increase the resolution

power of genetic analysis is the selection of study populations based on sensitization to specific allergens. In southern Europe, a major cause of allergy is the pollen of *Parietaria*. Sensitization to this pollen is responsible for 36% to 77% of adults pollenosis, the proportion of patients with asthma ranging between 50% and 62% [42]. Previously, in a case-control study, we reported the association between specific HLA-DR-B1* alleles and antibody response to the major *Parietaria* allergen, Par o 1 [8]. The association was confirmed in a second family-based independent population, recruited on the basis of one sibling's allergy to *Parietaria* [5]. The present study utilizes this population to investigate the association between TNF-308 markers and atopic asthma.

MATERIALS AND METHODS

Study Population

The study population (239 patients) has been previously described [5]. Participants were analyzed by ST for reactivity towards 18 glycerinated extracts of common inhalant allergens (Hollister-Stier Laboratories, Spokane, WA, USA). ST positivity was defined by a wheal diameter > 3 mm more than the negative control. Atopy was defined as ST positivity to at least one of the allergenic extract tested. As the results of tests performed with *P. judaica* and *P. officinalis* pollen extracts were in each case concordant, participants were classified as *Parietaria* ST positive/negative.

The study population was comprised of 239 individuals: 70 subjects (29%) had asthma, 161 subjects had atopy (67%), and 65 subjects had (27%) asthma and atopy. Arithmetic mean (median) of age was 34 ± 17 (31) years. Arithmetic mean (median) of serum total IgE levels was 195 ± 353 (88) IU/ml. In the parents and siblings subsets, arithmetic means (medians) of age were 49 ± 9 years (51) and 22 ± 11 (21) years, respectively. Arithmetic means (medians) of serum total IgE level were 117 ± 141 (70) and 273 ± 471 (121) IU/ml, respectively. Arithmetic means (medians) of age at onset of asthma were 26 ± 19.2 years (29) and 10 ± 8.5 years (8), respectively. Table 1 reports the prevalence of ST positivity to specific allergens in patients with asthma and with atopy.

Informed consent was obtained from all participants. Clinical history was recorded by a physician in a standard questionnaire. Asthma was defined as a positive response to one or more of the questions: have you had an attack of asthma on more than one occasion?; have you had attacks of shortness of breath with wheezing on more than one occasion?; and has asthma been diagnosed by a physician?

TABLE 1 Study population: skin test reactivity to allergens

	Phenotype	With atopy to Der ^a number (%)	With atopy to Lol ^b number (%)	With atopy to Par ^c number (%)
Study population Number = 239	With asthma (<i>n</i> = 70)	40 (57)	28 (40)	52 (73)
	With atopy (<i>n</i> = 162)	83 (51)	65 (40)	117 (72)
Parents Number = 105	With asthma (<i>n</i> = 16)	6 (38)	6 (38)	12 (75)
	With atopy (<i>n</i> = 36)	12 (34)	16 (46)	24 (67)
Siblings Number = 134	With asthma (<i>n</i> = 54)	33 (63)	22 (41)	39 (72)
	With atopy (<i>n</i> = 145)	71 (49)	60 (41)	93 (64)

Number of subjects with HLA-DRB1* alleles B1*03, B1*1101, and B1*1104, and with TNF-308 allele 2. Haplotypes were determined by pedigree analysis. The study population is subdivided into parents and siblings.

^a, ^b, ^c Patients with prick skin test positivity to ^a *D. pteronyssinus* (Der), ^b *Lolium perenne* (Lol), or ^c *Parietaria* (Par) allergenic extracts.

Collection and Storage of Blood Samples

At least 25 ml of blood were drawn from each patient in 0.1-M EDTA (pH 7.4). Cells were separated from plasma by centrifugation, aliquoted, and stored at -70°C . Sera were recovered from plasma by addition of 2 ml of a 2.4% solution of CaCl_2 to 1 ml of blood at 37°C , aliquoted and stored at -20°C .

Determination of Levels of Total Serum IgE and of Par o I Specific IgG and IgE Antibodies

Total serum IgE level was determined by radioimmunoassay (Pharmacia CAP system; Pharmacia, Upsala, Sweden), following the manufacturer's instruction. The measuring range was 2–2,000 kU/l.

The levels of IgG and IgE antibodies specific for the major allergen Par o I were determined by the double antibody radioimmunoassay (DARIA), using purified radiolabeled Par o I as previously described [5, 43].

Determination of Genotypes

HLA-DRB1* typing was achieved as previously reported [5, 7]. TNF*308 alleles were determined as already described [40]. Extended haplotypes were determined by pedigree analysis.

Statistical Analyses

Association of alleles to phenotypes was analyzed by the χ^2 test (Yates corrected), and by the Mann-Whitney rank test. The transmission disequilibrium test (TDT), which originally was introduced to test for linkage between a genetic marker and a disease-susceptibility locus in the presence of association, has become a standard procedure in studies of association between genetic markers and a

disease [44, 45]. The *p* values were not corrected for type 1 error.

RESULTS

Association of TNF-308 alleles and TNF/HLA-DRB1* Haplotypes to ST Reactivity to *D. Pteronyssinus*, *Lolium Perenne*, and *Parietaria*

Atopics were subdivided according to ST reactivity to *D. pteronyssinus*, *Lolium perenne*, and *Parietaria* and the association between these phenotypes, TNF alleles, and selected TNF/HLA-DRB1* haplotypes was investigated (Table 2).

No association of TNF-308 alleles with responsiveness to specific allergens was observed. The association of HLA-DRB1*03 to responsiveness to *Lolium* allergens has been reported by other groups [7]. The positive and negative association of ST positivity to *Parietaria* to HLA-DRB1*1104 and DRB1*03, respectively, confirmed the previous observation that, in the same population, these alleles were associated with high levels of Par o I specific antibody [5].

The haplotype TNF2/HLA-DRB1*1104 was increased in patients with atopy to *Parietaria* compared with patients without this atopy (14% and 2%, respectively), but these differences did not reach statistical significance. The haplotype TNF2/HLA-DRB1*03 was increased in patients with atopy to *Lolium* compared with patients without this atopy (11% and 3%, respectively) and was negatively associated to atopy to *Parietaria* (*p* = 0.0001).

TABLE 2 Atopics: association of alleles and haplotypes to specific atopy

Phenotype	Number of patients with allele/haplotype (%)						
	DRB1*03	DRB1*1101	DRB1*1104	TNF2	TNF2/B1*03	TNF2/B1*1101	TNF2/B1*1104
With atopy to Der. (<i>n</i> = 83)	14 (17)	20 (25)	18 (21)	34 (42)	6 (7)	5 (6)	7 (11)
Without atopy to Der. (<i>n</i> = 78)	13 (17)	15 (21)	27 (35)	37 (49)	4 (3)	4 (3)	9 (12)
With atopy to Lol (<i>n</i> = 66)	16 (24)	13 (20)	18 (27)	30 (46)	5 (11)	5 (8)	7 (11)
Without atopy to Lol. (<i>n</i> = 95)	11 (12)	22 (23)	27 (28)	43 (45)	5 (3)	4 (4)	9 (10)
	<i>p</i> = 0.05(–)						
With atopy to Par. (<i>n</i> = 116)	13 (11)	25 (21)	43 (36)	52 (44)	1 (1)	7 (6)	16 (14)
Without atopy to Par. (<i>n</i> = 45)	14 (31)	10 (22)	3 (7)	22 (49)	9 (20)	2 (4)	1 (2)
	<i>p</i> = 0.003(–)		<i>p</i> = 0.000(–)		<i>p</i> = 0.0001(–)		

Atopics (161 patients) subdivided according to prick skin test reactivity (atopy) to *D. pteronyssinus* (Der), *Lolium perenne* (Lol), or *Parietaria* (Par) allergenic extracts. Number of patients with HLA-DRB1* alleles B1*03, B1*1101, B1*1104, and with TNF-308 allele 2. Haplotypes were determined by pedigree analysis.

Association of Alleles and Haplotypes to Antibody Levels Specific for the Major *Parietaria* Allergen

By the Mann-Whitney rank test in the population there was a positive association between the haplotype TNF2/HLA-DRB1*1104 and the level of IgG antibodies (*p* = 0.01) to the major allergen Par o I. In the parent's subgroup, there was a positive association between TNF2 and TNF2/HLA-DRB1*1104 and the levels of Par o I specific antibodies of IgG (*p* = 0.02 and 0.0005, respectively), and IgE isotype (*p* = 0.01 and 0.01, respectively).

Association of Alleles and Haplotypes to Asthma

The association of HLA-DRB1*, TNF-308 alleles, and of the haplotypes TNF2/HLA-DRB1*03 and TNF2/HLA-DRB1*1104 to asthma are analyzed in Table 3.

In the study population, the proportions of HLA-DRB1*03 and of TNF2/HLA-DRB1*03 were significantly increased in subjects with asthma as compared to subjects without asthma (*p* = 0.01 and 0.02, respectively). The proportion of TNF2 was increased in asthmatics,

but the difference was not statistically significant. In the parents, asthma was associated to TNF2 (*p* = 0.01), but the primary association was to the haplotype TNF2/HLA-DRB1*1104 (*p* = 0.005). In the siblings there was a modest association of DRB1*03 to asthma.

In Table 4 the proportions of alleles and haplotypes are compared between asthmatics subdivided according to specific atopic sensitisation and nonasthmatics. DRB1*03 and TNF2/DRB1*03 were significantly increased in asthmatics with atopy to *Lolium* (*p* = 0.0015 and 0.0001, respectively) and TNF2/DRB1*1104 in asthmatics with atopy to *Parietaria* (*p* = 0.025).

Transmission Disequilibrium Test

Transmission disequilibrium test is based on unequal probability of meiotic transmission of different marker alleles from parents to the affected offspring and may offer greater power to identify genes with small to moderate effect than traditional family based linkage methods [44, 45]. The meiotic transmission of HLA-DRB1* and TNF alleles and of the haplotypes TNF/HLA-

TABLE 3 Study population: association of alleles and haplotypes to asthma

Phenotype	Number of patients with allele/haplotype (%)						
	DRB1*03	DRB1*1101	DRB1*1104	TNF2	TNF2/B1*03	TNF2/B1*1101	TNF*2/B1*1104
Study population							
With asthma (<i>n</i> = 70)	17 (24)	20 (29)	21 (30)	37 (53)	8 (11)	3 (4)	11 (16)
Without asthma (<i>n</i> = 169)	18 (13)	28 (17)	51 (30)	73 (43)	5 (3)	5 (3)	13 (7)
	<i>p</i> = 0.01				<i>p</i> = 0.02		
Parents							
With asthma (<i>n</i> = 16)	5 (31)	5 (31)	8 (50)	13 (81)	3 (19)	0	6 (38)
Without asthma (<i>n</i> = 89)	11 (12)	15 (17)	28 (31)	39 (44)	3 (3)	3 (3)	8 (9)
				<i>p</i> = 0.01			<i>p</i> = 0.005
Siblings							
With asthma (<i>n</i> = 54)	12 (22)	15 (28)	13 (24)	24 (44)	5 (9)	3 (6)	5 (9)
Without asthma (<i>n</i> = 80)	7 (9)	13 (16)	23 (28)	34 (42)	2 (3)	2 (3)	6 (1)
	<i>p</i> = 0.05						

Number of subjects with HLA-DRB1* alleles B1*03, B1*1101, B1*1104, and with TNF-308 allele 2. Haplotypes were determined by pedigree analysis.

TABLE 4 Study population: association of alleles and haplotypes to atopic asthma

Phenotype	Number of subjects with allele/haplotype (%)						
	DRB1*03	DRB1*1101	DRB1*1104	TNF2	TNF2/ B1*03	TNF2/B1*1101	TNF2/ B1*1104
Without asthma ($n = 169$)	18 (13)	28 (17)	51 (30)	73 (43)	5 (3)	5 (3)	13 (8)
With asthma and atopy to Lol. ($n = 28$)	10 (36)	7 (25)	8 (29)	15 (54)	6 (21)	2 (7)	4 (14)
	$p = 0.0015$				$p = 0.0001$		
With asthma and atopy to Par. ($n = 50$)	9 (18)	13 (27)	19 (37)	24 (47)	1 (2)	3 (6)	10 (20)
							$p = 0.025$
With asthma and atopy to Der. ($n = 42$)	10 (24)	11 (26)	10 (24)	21 (50)	6 (14)	3 (7)	5 (12)

Asthmatics subdivided according to prick skin test reactivity (atopy) to *D. pteronyssinus* (Der), *Lolium perenne* (Lol), or *Parietaria* (Par) allergenic extracts.

Number of patients with HLA-DRB1* alleles B1*03, B1*1101, B1*1104, and with TNF-308 allele 2. Haplotypes were determined by pedigree analysis.

DRB1*1104 and TNF2/HLA-DRB1*03 are analyzed in Table 5. There was excess transmission of HLA-DRB1*03 and of the haplotype TNF2/HLA-DRB1*03 to siblings with asthma and atopy to *Lolium* ($p = 0.03$ and 0.04 , respectively) and of DRB1*1104 and TNF2/DRB1*1104 to siblings with asthma and atopy to *Parietaria* ($p = 0.04$ and 0.015 , respectively).

DISCUSSION

The results of this study indicate that TNF2 is transmitted to asthmatics with the DRB1* alleles associated with atopy, and suggests that haplotypes TNF2/DRB1*03 and TNF2/DRB1*1104 contain functional alleles controlling asthma and atopy to *Lolium* and *Parietaria*, respectively. The modest association of asthma to TNF2 observed in the parents subgroup appears to be secondary to association to TNF2/DRB1*1104 (Table 3). These observations do not support the hypothesis that HLA region genes control asthma independently from atopy, in partial disagreement with the conclusions of studies from other groups [33–35]. It is perhaps relevant to this discrepancy that, in these studies, the analysis of specific atopic sensitization had been limited to mite and cat allergens [35].

In the study population, asthma was positively associated to HLA-DRB1*03 and to the haplotype TNF2/

DRB1*03 (Table 3), but, as depicted in Table 4, the major contribution to these associations was from patients with atopy to *Lolium*, whereas asthma was associated to TNF2/DRB1*1104 in subjects with atopy to *Parietaria*.

In the parents asthma was associated with TNF2/DRB1*1104, whereas in the siblings there was a modest association with HLA-DRB1*03, suggesting that *Parietaria* allergens have increased relevance as determinants of asthma in the parents (Table 3). This hypothesis is supported by the results of a previous study in the same population reporting the association of asthma to increased level of IgE antibodies specific for the major *Parietaria* allergen in the parents and to increased level of total serum IgE in the siblings [5]. This difference between parents and siblings may reflect recruitment biases or could be the effect of differential exposure to certain environmental actors (including lifestyle factors).

Transmission Disequilibrium Test analysis revealed that, in atopics, HLA-DRB1*1104 and TNF2/DRB1*1104 were meiotically transmitted with asthma (Table 5; $p = 0.04$ and 0.015). When asthmatics were subdivided according to specific atopic sensitisation, HLA-DRB1*03 and TNF2/DRB1*03 were transmitted with asthma and atopy to *Lolium* ($p = 0.03$ and 0.04) and HLA-DRB1*1104 and TNF2/DRB1*1104 with asthma and atopy to *Parietaria* ($p = 0.04$ and 0.015).

TABLE 5 Transmission disequilibrium test: number of alleles and haplotypes meiotically transmitted (T) or not transmitted (NT) from parents to siblings with asthma and atopy

Allele/Haplotype	Study population T/NT	With atopy to ^a		
		Der. T/NT	Lol. T/NT	Par. T/NT
DRB1*03	9/9	7/7	5/0 ($p = 0.03$)	5/3
DRB1*1104	14/5 ($p = 0.04$)	8/2	7/4	14/5 ($p = 0.04$)
TNF2/DRB1*03	5/4	5/4	4/0 ($p = 0.04$)	0/4 ($p = 0.04$)
TNF2/DRB1*1104	6/0 ($p = 0.015$)	4/1	4/4	6/0 ($p = 0.015$)

^a Atopics subdivided according to prick skin test reactivity (atopy) to *D. pteronyssinus* (Der), *Lolium perenne* (Lol), and *Parietaria* (Par) allergenic extracts.

Taken together, these observations suggest that the association of asthma to TNF2 reflects linkage disequilibrium with genes influencing specific immune response and that allergen-specific IgE antibodies may play a major role in the etiology of atopic asthma.

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