

Gene–gene synergistic effect on atopic asthma: tumour necrosis factor- α -308 and lymphotoxin- α -NcoI in Taiwan's children

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Summary

Background Asthma is now known to be an inflammatory response caused by the release of inflammatory mediators and cytokines. Tumour necrosis factor (TNF) is a potent cytokine in the inflammation response of the airway, and the polymorphisms of TNF genes have been associated with asthma.

Objective This study investigated two variants, TNF- α -308*2 and lymphotoxin (LT)- α -NcoI*1, which may predispose individuals to asthma and atopy pathogenesis.

Methods PCR-based assays were performed to determine LT- α -NcoI*1 and TNF- α -308*2 genotypes among our subjects, with 128 atopic asthmatics and 51 non-atopic asthmatics, 55 atopic controls, and 78 non-atopic controls in this genetic case–control study.

Results The TNF- α -308*2 polymorphism increased in subjects with atopic asthma vs. non-atopic controls after adjusting for age distribution (adjusted odds ratios, AOR = 2.73, 95% confidence interval, CI = 1.16–6.64), but was not associated with non-atopic asthma (AOR = 2.40, 95% CI = 0.81–7.09). LT- α -NcoI*1 did not show an independent association with either atopic asthma or any one phenotype of specific IgE. The synergistic effect between these two genes was conducted, and the interaction between TNF- α -308*2 and LT- α -NcoI*1 polymorphisms was seen for atopic asthma (OR = 2.59, 95% CI = 1.10–6.10) when compared with all controls.

Conclusion We have concluded that TNF- α -308 may be a risk factor for atopic asthma, whereas the LT- α -NcoI polymorphism may modify risk to atopic asthma with TNF- α -308.

Keywords atopic asthma, LT, polymorphism, TNF

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Introduction

Asthma and atopy are familial syndromes with both environmental and genetic factors involved in their aetiology. Most asthma is associated with atopy, which involves increased total serum IgE, specific IgE response to allergens or skin prick test reactivity, or both [1]. Genetic components have been reported by segregation analysis [2–4] and twin studies [5, 6]. Genome-wide search studies have demonstrated that many candidate regions contribute to asthma or atopy [7–9].

Asthma is now known to be an inflammatory response involving the release of multiple inflammatory mediators and cytokines. Tumour necrosis factor- α (TNF- α) is a potent pro-inflammatory cytokine that is found in increased levels in bronchoalveolar lavage fluid (BALF) in atopic asthmatics [10, 11]. Lymphotoxin (LT) produced by activated lympho-

cytes share many of the biological activities of TNF- α [12]. TNF- α and LT- α (or TNF- β) genes are located on chromosome 6p21.1–21.3, within the class III region of human major histocompatibility complex [13]. Two biallelic polymorphisms are examined here: one is a TNF- α -308 variant with a G $\rightarrow$ A transition at position –308 of TNF- α gene promotor [14], and the other is an LT- α -NcoI variant with NcoI restriction site in the first intron of LT- α gene [15]. TNF- α -308*2 polymorphism is associated with increased TNF- α secretion and promotor activity [16], and LT- α -NcoI*1 allele regulates a significantly higher amount of TNF- β production [15].

Some association studies have suggested that individuals with TNF- α -308*2 (allele 2) are at a higher risk to develop asthma or atopy [17–20], but there have been several negative studies in Chinese and Italian families [21, 22], and in infants [23]. TNF- α -308*2 may even have a protective effect [24]. Inconsistent results have also been found with regard to the association between LT- α -NcoI*1 and asthma [19, 21, 23, 25, 26]. Ethnic diversity and phenotypical heterogeneity may pose considerable confounding factors. The present study was undertaken to understand the prevalence of TNF- α -308*2

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and LT- α -NcoI*1 in asthmatic and healthy children in Taiwan, and to determine whether the variants contribute to risk of asthma and atopy.

Methods

Subjects

Whole blood and questionnaires were collected from two populations for this study. The first population comprised 191 asthmatic children recruited from the Department of Paediatric and Internal Medicine in the Memorial Hospital of Kaohsiung Medical University. In choosing the community control population, the basis for selection was proximity of residence. The community controls comprised 138 non-asthmatic students selected from two junior high schools in Kaohsiung, Taiwan. ISAAC videos and written questionnaires were used to assess the health status of the students' respiratory systems, as has been described previously [27]. The case and control groups were aged from 5 to 18 years, living in the Kaohsiung area, and all non-smokers. The ethnic makeup of all case and control subjects in this study was Fukien Taiwanese. The subjects in our study were assumed to have the same ethno-geographic origin. Questionnaires included demographics (age, sex, ethnicity, education), life styles (cigarette smoking and alcohol use at least once a week, persisting regularly for 1 year), and medical histories (histories of asthma, allergic rhinitis, and atopic dermatitis, as diagnosed by a doctor).

Phenotype analysis

Blood samples were collected by venipuncture. Serum total IgE and specific IgE levels were examined for each subject. Specific IgE was detected by MAST system (Multiple Allergens Simultaneous Test, Immunosystems, USA) or Cap system (Pharmacia, Uppsala, Sweden). Total serum IgE was measured using a Microparticle Enzyme Immunoassay (MEIA) (IMX System Abbot, Japan). A high total IgE was taken to be greater than 200 IU/mL. Atopy, defined in our study, may be diagnosed as total IgE concentrations greater than 200 IU/mL, a positive specific IgE titre against one or more of house dust (HD), *Dermatophagoides pteronyssinus* and cockroaches, or a combination of the two features. In this study, 128 atopic asthmatics, 51 non-atopic

asthmatics, 55 atopic controls, and 78 non-atopic controls were specified.

Mutation analysis

Genomic DNA was extracted from the peripheral blood of study participants using the standard method. PCR of the TNF- α -308 (cachectin) and LT- α -NcoI (lymphotoxin) polymorphisms was carried out using the TNF- α primer 5'-AGGCAATAGGTTTTGAGGGCCAT, 5'-TCCTCCCTGC TCCGATTCCG [14] and LT- α -NcoI primer 5'-CCGTGCTT CGTGCTTTGGACTA-3', and 5'-AGAGCTGGTGGGA CATGTCTG-3' [15]. Two hundred ng of genomic DNA extracted from venous blood was added to a 100 μ L reaction mixture containing 10 pmol of each primer with 2.5 mM of each dNTP, 67 mmol/L Tris-HCl, pH 8.8, 16.6 mmol/L $[(\text{NH}_4)_2\text{SO}_4]$, and 2.5 U Taq DNA polymerase (PROMEGA Corporation). Amplification conditions were 95 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min for both markers. Following amplification, 8 μ L of PCR products of two genes were digested with 1 U of NcoI endonuclease at 37 °C for 16 h. The NcoI fragments of LT- α -containing allele 1 were identified by 200 and 550 bp fragments (LT- α -NcoI*1) as a variant type, and allele 2 by a single 750 bp band (LT- α -NcoI*2) [15]. TNF- α fragments containing allele 1 were identified by 20 and 87 bp fragments (TNF- α -308*1), and allele 2 was identified as a variant type by a single 107 bp band (TNF- α -308*2). The resultant products were analysed on 3.5% agarose gel with ethidium bromide (1.5 μ g/mL).

Statistical analysis

The Monte Carlo simulation tests were performed to evaluate the differences in allele frequencies of TNF- α and LT- α for asthmatic children and controls in the genetic case-control study. The Monte Carlo approach was used to correct false-positive rates for multiple testing by the CLUMP package [28] (shown in Table 1). A goodness of fit χ^2 test was used to test for Hardy-Weinberg equilibrium (HWE) by comparing the observed number of subjects for each genotype with the expected number of subjects assuming HWE (Table 1). Logistic regression models were used to evaluate the associations between different asthma phenotypes (atopic asthma and atopy) and TNF genes polymorphisms. Adjusted odds ratios (AOR) and 95% confidence interval (CI) for each

Table 1. Genotypes of TNF- α -308 and LT- α -NcoI and children's asthma

	1/1	1/2	2/2	χ^2	P^*	HWE†	P
<i>TNF-α-308</i>							
Asthma							
Yes	140 (73.3%)	49 (25.7%)	2 (1.0%)	7.98	0.018	0.95	0.62
No	111 (86.0%)	18 (14.0%)	0 (0%)			0.09	0.77
<i>LT-α-NcoI</i>							
Asthma							
Yes	29 (16.1%)	93 (51.7%)	58 (32.2%)	5.62	0.06	0.43	0.81
No	8 (6.8%)	66 (56.4%)	43 (36.8%)			6.63	0.036

*The significance for TNF polymorphism and asthma was assessed using a Monte Carlo approach in the genetic case-control association study. †Hardy-Weinberg equilibrium tests were performed in case and control groups.

Table 2. Association between polymorphisms of TNF genes and asthma and atopy in children by logistic regression models

	LT- α -NcoI				TNF- α -308			
	Variant % (1/1+1/2)	Wild % (2/2)	AOR*	95%CI	Variant % (1/2+2/2)	Wild % (1/1)	AOR	95% CI
Child asthma	123 (67.6)	59 (32.4)	1.21	0.67–2.20	51 (26.4)	142 (73.6)	2.17‡	1.29–4.33
Child control	74 (63.2)	43 (36.2)	1		18 (14.0)	111 (86.0)	1	
Atopy†	110 (66.7)	55 (33.3)	0.77	0.40–1.49	44 (25.0)	132 (75.0)	2.58‡	1.13–5.91
Non-atopy control	44 (65.7)	23 (34.3)	1		9 (12.2)	65 (87.8)	1	
Atopic* asthma	83 (69.2)	37 (30.8)	0.80	0.38–1.62	35 (27.8)	91 (72.2)	2.73‡	1.16–6.44
Non-atopic asthma	30 (63.8)	17 (36.2)	0.49	0.19–1.27	13 (25.5)	38 (74.5)	2.40	0.81–7.09
Atopic* control	27 (60.0)	18 (40.0)	0.74	0.34–1.63	9 (18.0)	41 (82.0)	1.58	0.58–4.32
Nonatopic control	44 (65.7)	23 (34.3)	1		9 (12.2)	65 (87.8)	1	

*AOR: Adjusted odds ratio, which adjusted age distribution by logistic regression models. †Atopic: defined as IgE responsiveness; may be diagnosed as total IgE concentrations was greater than 200 IU/mL, a positive specific IgE titre against one or more of house dust (HD), *Dermatophagoides pteronyssinus*, and cockroaches, or a combination of the two features. ‡ $P < 0.05$.

Table 3. Synergistic effects between TNF-308 and LT-NcoI genes on atopic asthma compared with all control children by logistic regression models

TNF- α -308/LT- α -NcoI	Atopic asthma		OR (95% CI)
	Yes	No	
Variant/variant	26 (70.3)	11 (29.7)	2.59* (1.10–6.10)
Variant/normal	6 (50.0)	6 (50.0)	1.10 (0.32–3.76)
Normal/variant	56 (49.6)	57 (50.4)	1.08 (0.59–1.98)
Normal/normal	31 (47.7)	34 (52.3)	1

* $P < 0.05$

were calculated using multiple logistic regression after adjusting for age distribution (Tables 2 and 3).

Result

Table 1 shows the genotypic frequencies for the TNF- α -308 and LT- α -NcoI polymorphisms in asthmatic children and controls aged less than 18 years. The frequency of TNF- α -308*2 was significantly different between asthmatic and non-asthmatic children ($P = 0.018$), but not for LT- α -NcoI polymorphisms. HWE were observed in the asthma and control groups for TNF- α -308, respectively, and in the asthma group for LT- α -NcoI genotypes. In this study, both asthmatic and non-asthmatic male children were 132/196 (67.3%) and 87/138 (63.0%) ($P > 0.05$). The mean ages between two groups were statistically different (mean ages = 9.5 ± 3.3 and 13.3 ± 2.5 for asthmatics and controls, respectively, $P < 0.05$). To control the confounding factor, we adjusted for age distribution by multiple logistic regressions.

After controlling age distribution, the rare 2 allele at the TNF- α -308 marker was significantly associated with all asthmatic children (AOR = 2.17, 95% CI = 1.29–4.33, $P = 0.03$), atopic asthma (AOR = 2.73, 95% CI = 1.16–6.64), and atopy response (AOR = 2.59, 95% CI = 1.13–5.91), but not associated with non-atopic asthma (AOR = 2.40, 95% CI = 0.81–7.09) (Table 2).

We conducted further analysis examining the interaction between these two genes on the risk of atopic asthma by logistic regression models (Table 3). Compared with all

controls with TNF- α -308*1/LT- α -NcoI*2 wild genotypes, the odd ratio was 1.08 on atopic asthma, for those individuals with TNF- α -308*1 and LT- α -NcoI*1 genotypes. Also, the risk was not significant for those who were TNF- α -308*2 and LT- α -NcoI*2 genotypes [OR = 1.10, 95% CI = 0.32–3.76 on atopic asthma]. There was a significant synergistic effect for both variant genotypes of TNF- α -308*2 and LT- α -NcoI*1 on atopic asthma (OR = 2.59, 95% CI = 1.10–6.10) when compared with all control children. But there was no significant synergistic effect on atopy response when combining these two candidate polymorphisms (OR = 2.71, 95% CI = 0.88–8.35, $P = 0.08$, data not shown).

Discussion

A recessive inheritance pattern of asthma in Taiwan was reported by our group [4]. In this study, we tested for an association between markers in two candidate genes, LT- α -NcoI and TNF- α -308, on the risk of asthma and other associated phenotypes. Significant associations between atopic asthma and TNF- α -308*2 polymorphism are suggested.

Several association studies of asthma and LT- α -NcoI*1 and TNF- α -308*2 variants have been performed. However, the results are inconsistent and may reflect heterogeneity in ethnic groups and phenotypic definition, as well as considerable complexity of pathogenesis of asthma and atopy. In an Australian study, asthma was significantly higher in subjects with both TNF-308*2 ($P = 0.004$) and LT- α -NcoI*1 ($P = 0.005$) [19]. An association between bronchial hyper-reactivity in asthma and TNF- α -308*2 polymorphism, but no association with LT- α -NcoI allele, has been reported in either a large cohort study [25] or in Spanish atopic patients [26]. The results of the above studies were similar to those of our study, but we found that LT- α -NcoI*1 has a modifying effect on atopic asthma. In sib-pair studies, the distribution of the identical by descent (IBD) alleles at LT- α -NcoI locus, but not for TNF- α -308, was significantly distant from the expected value of skin prick test and atopy [21, 23].

We tested the interactions between LT- α -NcoI and TNF- α -308 on atopic asthma. The synergistic effects of gene and gene are highly significant on atopic asthma (AOR = 2.59,

$P < 0.05$). LT- α -NcoI*1/TNF- α -308*2/HLA-DRB*02 haplotype was associated with asthma, BHR, and the use of steroids [18]. Asthma was significantly associated with LT- α -NcoI*1/TNF- α -308*2 haplotype in an Australian family study [19] and a sib-pair study [21]. The synergistic effects of different cytokines have been suggested by several studies. Cytokine interactions are demonstrated in the activation of eosinophils [29], in perpetuating airway inflammation in asthma [30], and in increasing concentrations of alveolar macrophage [31].

We did not perform HLA typing in this study. Several studies have suggested extensive linkage disequilibrium between the TNF- α gene and HLA. TNF-308*2 was in linkage disequilibrium with A1, B8, and DR3 [14, 19, 32]. In general, linkage disequilibrium occurs over 50–500 kb of DNA [33]. TNF locus was 250 kb centromeric of the HLA-B locus and 350 kb telomeric of the class III cluster [13]. It is worthy of consideration that polymorphisms both within the TNF gene and also the MHC loci may influence TNF production. In a recent study, class 2 and 3 alleles within the MHC were typed to determine linkage disequilibrium with TNF- α -308. However, they did not provide evidence for the independent association of extended TNF2 haplotype with asthma [25]. Although LT- α -NcoI*1/TNF-308*2/HLA-DRB1*2 haplotype was associated with asthma, it was found in only 0.6% of the subjects, and accounted for a very small percentage of the population-attributable risk of asthma [18].

The fundamental mechanism and modes of inheritance of atopic asthma and non-atopic asthma may be different [4]. The presence of chronic allergic inflammation in airway mucosa in atopic asthma has been reported [34], but chronic allergic inflammation and the rapid secretion and/or edema may not be increased in non-atopic asthma [35]. In the present study, TNF- α -308*2 variant was associated with increased risk of atopic asthma, but not associated with non-atopic asthma. The synergistic effect of LT- α -NcoI*1 and TNF- α -308*2 was also a significant predictor of atopic asthma, but not in non-atopic asthmatics. *In vitro* studies have suggested that TNF-308*2 alleles are correlated with increased TNF- α secretion [16]. Increased concentrations of TNF- α secretory have been identified in the airways of atopic asthmatics [31, 36]. Therefore, constitutional variations in TNF secretion might influence inflammatory airway diseases such as atopic asthma.

For complex diseases with modest genetic effects, association studies can play a critical role in the evaluation of candidate genes [37–39]. Spurious association will not occur in this study because the ethnicity of all subjects is Fukien Taiwanese, and are assumed to be a homogenous population. In this study, most of the positive associations showed borderline significance between TNF- α -308*2 and asthma, in Nottingham (OR = 2.12, 95% CI = 1.04–4.32) [25] and UK/Irish (OR = 2.6, 95% CI = 1.1–6.2) [17], which is similar to our result. Asthma is a polygenic disorder in which several candidate genes are involved that may modify the severity of inflammation. TNF- α -308*2 may have only a minor effect on asthma. Therefore, it would be helpful to have the plausibility of combining these two candidate polymorphisms. In this association study of TNF gene and asthma and atopic diseases, we found that TNF- α -308-promoter polymorphism

was associated with atopic asthma in Taiwan, whereas LT- α -NcoI polymorphism does not have an independent effect, but rather modifies atopic asthma.

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