Pediatr Neonatol 2008;49(2):30–34

ORIGINAL ARTICLE

Association of a Lymphotoxin-α Gene Polymorphism and Atopic Asthma in Taiwanese Children

Szu-Chao Huang[1], Wen-Jun Wu[2], Hai-Lun Sun[1,3], Ko-Huang Lue[1,3]*, Chia-Hsiu Hsu[1], Pei-Fen Liao[1], Min-Sho Ku[1]

[1]*Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Chung Shan Medical University Hospital, Taichung, Taiwan*
[2]*Institute of Medical and Molecular Toxicology, Chung Shan Medical University, Taichung, Taiwan*
[3]*Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan*

Received: Jul 16, 2007
Revised: Feb 22, 2008
Accepted: Mar 25, 2008

KEY WORDS:
atopic asthma;
gene polymorphism;
lymphotoxin-α gene

Background: The lymphotoxin-α (LT-α) gene is located on chromosome 6 (6p21.1-6p21.3) and it may regulate tumor necrosis factor (TNF) production. TNF is a potent cytokine in the airway inflammatory response. Polymorphisms of TNF-associated genes have been related to asthma. This study investigated an LT-α-NcoI polymorphism in the first intron of the LT-α gene (LT-α-NcoI*1 allele, as a variant type; and LT-α-NcoI*2 allele), which may predispose individuals to asthma and atopy.
Methods: Polymerase chain reaction-based assays were performed to determine LT-α-NcoI genotypes among our subjects. A genetic case control analysis was then performed on 114 atopic asthmatic and 155 non-asthmatic unrelated children.
Results: There was a statistically higher frequency of LT-α-NcoI*1 allele carriers (1/1+1/2) in the subjects with atopic asthma than in controls (OR=1.923; 95% CI=1.061–3.484; p=0.031).
Conclusion: The results indicate that LT-α-NcoI*1 may be a risk factor for atopic asthma in Taiwanese children.

1. Introduction

Asthma is an airway inflammatory disease characterized by reversible airway obstruction and increased airway responsiveness.[1,2] The etiology of the disease is multifactorial, with environmental and genetic components. Genetic components have been mentioned by segregation analysis.[3–5] More than 90% of children with asthma are associated with atopy, which involves increased total serum IgE, specific IgE response to allergens or skin prick test reactivity or both.[6,7] Also, patients with asthma and atopy have higher peripheral serum eosinophil counts, and eosinophils are increased in the lung tissue and bronchoalveolar fluid of asthmatics.[8] Mature eosinophils are important effectors of allergic inflammation, releasing proinflammatory proteins from their granules such as major basic protein, peroxidase and cationic protein.[9]

The airway inflammatory response of asthma is now known to be associated with the release of multiple inflammatory mediators and cytokines.

*Corresponding author. Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Chung Shan Medical University Hospital, No. 110, Section 1, Chien-Kuo North Road, Taichung 402, Taiwan.
E-mail: cshy095@csh.org.tw

Tumor necrosis factor (TNF) is a powerful proinflammatory cytokine that participates in the airway inflammatory response in patients with atopic asthma.[10] The lymphotoxin-α (LT-α) gene (or TNF-β gene), which encodes TNF-β production, is located within the class III human major histocompatibility complex (MHC) on chromosome 6 (6p21.1-6p21.3).[11] A previous study suggested an important functional role for genes on 6p21.1 in the pathogenesis of atopic asthma.[12] Also, the level of TNF secretion seems to be associated with allele 1 of a NcoI polymorphism in the first intron of the LT-α gene (or TNF-β gene).[13] In addition, the LT-α gene may regulate TNF gene expression.[14] Studies have shown that a number of cytokine gene single-nucleotide polymorphisms (SNP) may affect their transcription and influence the level of cytokine production.[15,16] Thus, the LT-α gene is considered a candidate gene for association with asthma.[17,18]

The present study was performed to determine the prevalence of the LT-α NcoI genotype in atopic asthmatic and healthy children in Taiwan, and to determine whether the variants contribute to the risk of atopic asthma.

2. Methods

A total of 269 independent subjects were collected from two populations in the Taichung area for this study. The first population comprised 114 children with atopic asthma recruited from the Department of Pediatrics, Chung Shan Medical University Hospital. The community controls comprised 155 non-asthmatic students selected from three elementary schools in Taichung. The case and control groups were aged from 7 to 14 years.

Whole blood samples and questionnaires were collected when subjects were enrolled. Blood samples were obtained by venipuncture. Serum eosinophil counts, serum total IgE and specific IgE levels were examined for each subject. Specific IgE was detected using the Cap system (Pharmacia, Uppsala, Sweden). Questionnaires included demographics, lifestyles, and medical histories. Atopy, as defined in our study, was diagnosed as total IgE concentrations greater than 200 IU/mL and a positive specific IgE titer against one or more of *Bomia tropicalis, Dermatophagoides microrcers, Dermatophagoides pteronyssinus, Blatella germanica,* cat dander, dog dander, *Aspergillus fumigatus, Candida albicans,* and *Penicillin notatum*.

2.1. Mutation analysis

Genomic DNA was extracted from the peripheral blood of study participants using the standard method. Polymerase chain reaction (PCR) of the LT-α-NcoI polymorphisms was carried out using the LT-α-NcoI primers 5′-CCGTGCTT CGTGCTTTGGACTA-3′ and 5′-AGAGCTGGTGGGGA CATGTCTG-3′. Genomic DNA, 100 ng, extracted from venous blood was added to a 20-μL reaction mixture containing 10 pmol of each primer with 2 mM of each dNTP (Gibco-BRL, USA), 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCL_2$, 50 mM KCL, 0.1% Triton X-100 and 10 units of Taq DNA polymerase (DyNAzyme II, Finland). Amplification conditions were 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 66°C for 1 minute, and 72°C for 1 minute. Following amplification, 8 μL of PCR products were digested with 1 μL of NcoI endonuclease at 37°C for 16 hours. The resultant products were analyzed on 2% agarose gel with ethidium bromide.

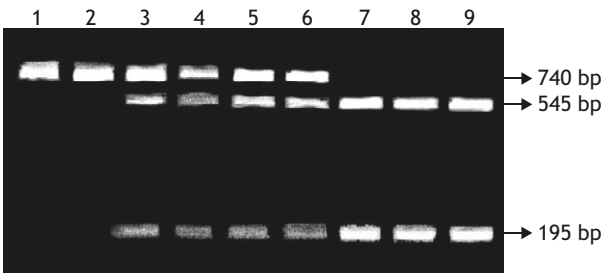

Figure 1 Results of electrophoresis of nine cases. Lanes 1 and 2 (allele 2/allele 2) manifested a single 740 bp band; lanes 3–6 (allele 1/allele 2) manifested 195 bp, 545 bp and 740 bp bands; lanes 7–9 (allele 1/allele 1) manifested 195 bp and 545 bp bands.

The NcoI fragments of the LT-α-containing allele 1 were identified by 195 bp and 545 bp fragments (LT-α-NcoI*1) as a variant type, and allele 2 by a single 740 bp band (LT-α-NcoI*2).[13] Figure 1 shows the results of electrophoresis.

2.2. Statistical analysis

All statistical analyses were performed with SPSS statistical software (SPSS Inc., Chicago, IL, USA). Pearson's χ^2 test was applied to analyze the genotypic difference in frequency and male/female ratio between the two groups. Comparisons of age, serum total IgE, and serum eosinophil count between the atopic asthma and control groups were analyzed with Student's *t* test; 95% confidence intervals were adopted and $p < 0.05$ were taken to indicate statistically significant difference.

3. Results

In this study, there were more boys in the atopic asthma group, but there was no statistically significant difference in the male/female ratio between

Table 1 Male ratio, age, serum IgE and eosinophil count in atopic asthma and control groups

	Male ratio	Mean age (± SD)	Mean serum IgE (± SD)	Mean eosinophil count (± SD)
Atopic asthma	52.6% (60/114)	9.9±4.1 yr	895±420 IU/mL	521±310 cells/mm^3
Control	49.0% (76/155)	9.2±2.53 yr	82±55 IU/mL	173±92 cells/mm^3
p	0.56	0.02*	<0.01*	<0.01*

*Comparison of age, serum IgE and eosinophil count between atopic asthma and control groups was analyzed using Student's *t* test.

Table 2 Genotypes of LT-α-NcoI polymorphism in control and atopic asthmatic children

	Wild type (2/2)	Heterozygous carrier type (1/2)	Homozygous carrier type (1/1)	*p*	χ^2
Atopic asthma	20 (17.5%)	69 (60.5%)	25 (22%)	0.025*	7.417
Control	45 (29%)	69 (44.5%)	41 (26.5%)		

*Pearson's χ^2 test was applied to analyze the genotypic difference in frequency between the two groups.

Table 3 Proportions of LT-α-NcoI*1 carriers in control and atopic asthmatic children

	Wild type (2/2)	Variant type (1/1+1/2)	*p*	OR (95% CI)
Atopic asthma	20 (17.5%)	94 (82.5%)	0.031*	1.923 (1.061–3.484)
Control	45 (29%)	110 (71%)		

*Pearson's χ^2 test was applied to analyze the difference in LT-α-NcoI*1 carriers between the two groups.

the two groups ($p=0.56$). The mean age of all subjects was 9.55±1.83 years (range, 7–14 years). The age of the children in the atopic asthma group seemed to be slightly higher than those of the control group ($p=0.02$). There were significant differences in serum total IgE and eosinophil counts between the atopic asthma and control groups ($p<0.01$) (Table 1).

Table 2 shows the genotypic frequencies of the LT-α-NcoI polymorphism in atopic asthmatic children and controls. The genotypic distributions were significantly different between the two groups ($p=0.025$). Table 3 shows the proportions of polymorphic LT-α-NcoI*1 carriers in the atopic asthma and control groups. LT-α-NcoI*1 carriers (homozygous and heterozygous) were present at a statistically higher frequency in the atopic asthma group than in the control group (OR=1.923; 95% CI=1.061–3.484, $p=0.031$).

4. Discussion

The LT-α gene, which encodes TNF-β secretion, is located within the class III region of human major histocompatibility complex.[11] In our study, we examined an LT-α-NcoI polymorphism of the LT-α gene. The polymorphic NcoI site is linked with an amino acid substitution of TNF-β protein at position 26, where the LT-α-NcoI*1 allele reveals asparagine and the LT-α-NcoI*2 allele reveals threonine.[13]

LT-α (TNF-β) is mainly produced by activated lymphocytes.[19] It has been identified in two molecular forms: a secreted form consisting of an $LT\alpha_3$ homotrimer that binds to TNF receptors and a membrane-associated heterotrimeric form with $LT\alpha\beta_2$ that binds to the LT-β receptor. The major effect of lymphotoxin is its cytotoxicity against sensitive target cells. It shares many of the biological activities of TNF-α and has roles in lymphocyte homing.[20] B cell proliferation and IgE synthesis also need the participation of LT-α.[21,22] In addition, LT-α is known to be involved in the pathogenesis of various diseases, including bacteria and malaria infection.[23,24] Polymorphisms in the LT-α gene and the gene encoding its interacting protein, galectin-2, have been shown to be associated with myocardial infarction.[25,26]

In our study, the frequency of LT-α-NcoI*1 carriers was statistically higher in atopic asthmatic children than in controls, although the data showed only borderline significance (OR=1.923; 95% CI=1.061–3.484; $p=0.031$). There are several related studies in which carriers with LT-α-NcoI*1 were at a higher risk to develop asthma. In a Busselton study (Australia), asthma was significantly more common in carriers

with LT-α-NcoI*1.[27] Almost all of the asthmatics in that study were atopic. The results of the Busselton study resembled ours. But the Busselton study employed a simple definition of asthma based on a standard questionnaire. Answers to the question, "Have you had an attack of asthma on more than one occasion?" depended greatly on the previous medical care and education of individuals. In our study, the diagnosis of asthma was made by pediatric immunologists using the Global Initiative for Asthma (GINA) guidelines. Additionally, the severity of asthma in asthmatics was mild persisted or moderate persisted. Our diagnosis of asthma was more precise than that in the Busselton study. Moreover, asthma may have its onset at any age, but 90% of asthmatic children have their first symptoms before the age of 5 years. In our study, the case and control groups were aged from 7 to 14 years. Thus, we might have little chance to have ongoing asthma in our control group.

In another Taiwanese study (in Kaohsiung), LT-α-NcoI polymorphism was not associated with asthma alone.[28] That result is not consistent with our data. The asthmatic children in our study were all atopic, but the subjects in the Kaohsiung study included both atopic and non-atopic asthmatic patients. The pathogenesis and modes of inheritance of atopic and non-atopic asthma are different. The chronic allergic inflammation of the airway and rapid secretion of the inflammatory airway are found in atopic asthma but not in non-atopic asthma.[29] Interestingly, in the Kaohsiung study, when the atopic patients were selected from the asthmatic group, there was a significant association between the TNF-α-308*2/LT-α-NcoI*1 haplotype and atopic asthma. It seems that the effects of the LT-α-NcoI gene polymorphism play a more important role in atopic individuals than in non-atopic individuals. Also, an Italian study indicated that LT-α-NcoI polymorphism was associated with atopy.[30]

Studies of cytokine gene polymorphisms, especially SNP, need a larger population to obtain a convincing outcome. Besides, studies of SNP in different areas, even in the same race, are also referable.[12,27] Although there were similar studies about the association between LT-α-NcoI polymorphism and asthma in Tainan and Kaohsiung,[28,31] our samples were mainly recruited from the Taichung area. In this way, our data might increase the credibility of the association between LT-α-NcoI polymorphism and atopic asthma in the Taiwanese population.

LT-α-NcoI*1 allele leads to a higher TNF-β response.[13] It is conceivable that an allelically varying TNF-β response of activated T lymphocytes might contribute to the slow and self-protruding inflammatory mechanisms of local immunological reactions. As we know, TNF-β is categorized as a Th1 cytokine. In contrast to the data that Th2-mediated inflammation is dominant in allergen-induced asthma, several papers have shown, surprisingly, that the addition of allergen-specific Th1 cells and cytokines could exacerbate airway inflammation.[32,33] From our results, the total frequency of the LT-α-NcoI*1 allele in the atopic asthma group (52.2%) was not statistically higher than that in the control group (48.7%; $p=0.434$, data not shown). But probably due to the increment of heterozygous carriers (allele1/allele2), our results still showed that the frequency of whole LT-α-NcoI*1 carriers (allele1/allele2; allele1/allele1) was statistically higher in subjects with atopic asthma than in controls. Our results may be due to the interactions between LT-α-NcoI*1 carriers and TNF-β production. It is possible that increasing frequency of LT-α-NcoI*1 carriers, whether homozygous or heterozygous carriers, resulted in a higher TNF-β response, which might disturb the Th1/Th2 balance and exacerbate the airway inflammatory reactions of atopic asthmatic children. Besides, some articles indicated that intron 1 of the LT-α gene, where the NcoI polymorphism is found, contains regulatory elements that affect TNF-α gene expression.[12,14] TNF-α has been previously shown to be involved in the pathogenesis of atopic asthma.[34,35]

Several negative studies were also reported.[36,37] In a Japanese study, no association was found between the LT-α-NcoI polymorphism and asthma, but another functional polymorphism, LT-α-753G/A, was found for the development of clinical asthma.[37,38] It was concluded that polymorphisms of the TNF gene family on chromosome 6p21.1, including TNF-α, LT-α and LT-β genes, play an important role in the pathogenesis of atopic asthma. Also, the authors stated that the minor contribution of the LT-α-NcoI polymorphism in the first intron of the LT-α gene to asthma susceptibility could not be excluded.

Asthma is a polygenetic disorder in which several candidate genes are involved. Each candidate gene may modify airway inflammation but each one may have only a puny effect. Although our sample size (a total of 269 independent cases) was similar (even slightly larger) to those associated studies in Taiwan, more study is required to corroborate our results.

In conclusion, we did find a statistical association between atopic asthma and LT-α-NcoI*1 carriers in Taiwanese children, even though LT-α-NcoI polymorphism may have only a minor effect on atopic asthma.

References

1. Robison DS, Hamid Q, Ying S, et al. Predominant Th2-like bronchoalveolar T lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298–304.

2. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease and asthma. *Am Rev Respir Dis* 1988;136:225–44.
3. Holberg CJ, Elston RC, Halonen M, et al. Segregation analysis of physician-diagnosed asthma in Hispanic and non-Hispanic white families. *Am J Respir Crit Care Med* 1996;154:140–50.
4. European Community Respiratory Health Survey Group. Gene for asthma? An analysis of the European Community Respiratory Health Survey. *Am J Respir Crit Care Med* 1997;156:1773–80.
5. Wang TN, Ko YC, Wang TH, Li Cheng SH, Lin YC. Segregation analysis of asthma: a recessive major gene component for asthma in relation to history of atopic diseases. *Am J Med Genet* 2000;93:373–80.
6. Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 1991;325:1067–71.
7. Shibasaki M, Tajima K, Morikawa A, Mitsuhashi M, Sumazaki R, Tokuyama K. Relation between frequency of asthma and IgE antibody levels against *Dermatophagoides farinae* and total serum IgE levels in school children. *J Allergy Clin Immunol* 1988;82:86–94.
8. Denburg JA. Bone marrow in atopy and asthma: hematopoietic mechanisms in allergic inflammation. *Immunol Today* 1999;20:111–3.
9. Busse WW, Lemarke RF Jr. Asthma. *N Engl J Med* 2001; 344:350–62.
10. Ying S, Robinson DS, Varney V, et al. TNF α mRNA expression in allergic inflammation. *Clin Exp Allergy* 1991;21:745–50.
11. Carroll MC, Katzman P, Alicot EM, et al. Linkage map of the human major histocompatibility complex including the tumour necrosis factor genes. *Proc Natl Acad Sci USA* 1987; 84:8535–9.
12. Albuquerque RV, Hayden CM, Palmer LJ, et al. Association of polymorphisms within the tumour necrosis factor (TNF) genes and childhood asthma. *Clin Exp Allergy* 1998;28: 578–84.
13. Messer G, Spengler U, Jung MC, et al. Polymorphic structure of the tumour necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-beta gene correlates with a variant amino acid in position 26 and a reduced level of TNF-beta production. *J Exp Med* 1991;173:209–19.
14. Stuber F, Udalova IA, Book M, et al. -308 Tumor necrosis factor polymorphism is not associated with survival in severe sepsis and is unrelated to lipopolysaccaride inducibility of the human TNF promoter. *J Inflamm* 1996;46:42–50.
15. Bidwell J, Keen L, Gallagher G, et al. Cytokine gene polymorphism in human disease. *Genes Immun* 2001;2:61–70.
16. Hoffmann SC, Stanley EM, Cox ED, et al. Association of cytokine polymorphism inheritance and in vitro cytokine production in anti-CD3/CD28-stimulated peripheral blood lymphocytes. *Transplantation* 2001;72:1444–50.
17. Daniels SE, Bhattacharrya S, James A, et al. A genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996;383:247–50.
18. Wjst M, Fischer G, Immervoll T, et al. A genome-wide search for linkage to asthma. German Asthma Genetics Group. *Genomics* 1999;58:1–8.
19. Ruddle NH, Waksman BH. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. III. Analysis of mechanism. *J Exp Med* 1968;128:1267–79.
20. Gajewska BU, Alvarez D, Vidric M, et al. Generation of experimental allergic airways inflammation in the absence of draining lymph nodes. *J Clin Invest* 2001;108: 577–83.
21. Worm M, Ebermayer K, Henz B. Lymphotoxin-alpha is an important autocrine factor for CD40 interleukin-4-mediated B cell activation in normal and atopic donors. *Immunology* 1998;94:395–402.
22. Kang HS, Blink SE, Chin RK, et al. Lymphotoxin is required for maintaining physiological levels of serum IgE that minimizes Th1-mediated airway inflammation. *J Exp Med* 2003; 198:1643–52.
23. Roach DR, Briscoe H, Saunders B, et al. Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection. *J Exp Med* 2001;193:239–46.
24. Engwerda CR, Mynott TL, Sawhney S, et al. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. *J Exp Med* 2002;195:1371–7.
25. Ozaki K, Ohnishi Y, Iida A, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 2002;32:650–4.
26. Ozaki K, Inoue K, Sato H, et al. Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-alpha secretion *in vitro*. *Nature* 2004;429: 72–5.
27. Moffatt MF, Cookson WO. Tumour necrosis factor haplotypes and asthma. *Hum Mol Genet* 1997;6:551–4.
28. Wang TN, Chen WY, Wang TH, et al. Gene-gene synergistic effect on atopic asthma: TNF-α-308 and lymphotoxin-α-NcoI in Taiwan's children. *Clin Exp Allergy* 2004;34:184–8.
29. Mochizuki H, Shigeta M, Tokuyama K, et al. Difference in airway reactivity in children with atopic vs. non-atopic asthma. *Chest* 1999;116:619–24.
30. Trabetti E, Patuzzo C, Malerba G, et al. Association of a lymphotoxin alpha gene polymorphism and atopy in Italian families. *J Med Genet* 1999;36:323–5.
31. Lin YC, Lu CC, Su HJ, et al. The association between tumor necrosis factor, HLA-DR alleles, and IgE-mediated asthma in Taiwanese adolescents. *Allergy* 2002;57:831–4.
32. Hansen G, Berry G, Dekruyff RH, et al. Allergen-specific Th1 cells fails to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175–83.
33. Randolph DA, Carruthers CJ, Szabo SJ, et al. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J Immunol* 1999;162:2375–83.
34. Virchow JC, Walker C, Hafner D, et al. T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am J Respir Crit Care Med* 1995;151:960–8.
35. Broide DH, Lotz M, Cuomo AJ, et al. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992;89: 958–67.
36. Malerba G, Trabetti E, Patuzzo C, et al. Candidate genes and a genome-wide search in Italian families with atopic asthmatic children. *Clin Exp Allergy* 1999;29:27–30.
37. Noguchi E, Yokouchi Y, Shibasaki M, et al. Association between TNFA polymorphism and the development of asthma in the Japanese population. *Am J Respir Crit Care Med* 2002;166: 43–6.
38. Mogita O, Noguchi E, Shibasaki M, et al. Haplotype analysis of a 100 kb region spanning TNF-LTA polymorphism in the LTA promoter region that is associated with atopic asthma susceptibility in Japan. *Clin Exp Allergy* 2005;35:790–6.