

Haplotype analysis of a 100 kb region spanning TNF–LTA identifies a polymorphism in the LTA promoter region that is associated with atopic asthma susceptibility in Japan

O. Migita*, E. Noguchi*†, M. Koga*, Z. Jian*, M. Shibasaki‡, T. Migita§, S. Ito*, K. Ichikawa¶, A. Matsui† and T. Arinami*

Departments of *Medical Genetics and †Pediatrics, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba City, Ibaraki, Japan, ‡Department of Pediatrics, Tsukuba College of Technology, Tsukuba City, Ibaraki, Japan, §Tsukuba Memorial Hospital, Tsukuba City, Ibaraki, Japan and ¶Department of Pediatrics, Tsukuba Medical Center Hospital, Tsukuba City, Ibaraki, Japan

Summary

Background The tumour necrosis factor (*TNF*) gene family, which includes *TNF*, *LTA*, and *LTB*, is located consecutively on human chromosome 6p21 region, which has been linked to asthma by several genome-wide screens. (*LTA*, lymphotoxin- α ; *LTB*, lymphotoxin- β).

Objective The aim of the present study was to determine whether genes on 6q21 are related to development of atopic asthma.

Methods We screened for mutations in the coding and promoter regions of genes in the *TNF–LTA* region, including *BAT1*, *NFKB1L1*, *LTA*, *TNF*, *LTB*, *AIF*, and *BAT2*, and conducted a transmission disequilibrium test of 41 polymorphisms in 137 families identified through pro-bands with childhood-onset atopic asthma. (*BAT1*, HLA-B-associated transcript 1; *NFKB1L1*, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor-like 1; *AIF*, allograft inflammatory factor 1).

Results Haplotypes of the *LTA/TNF* linkage disequilibrium block were associated significantly with asthma (global $P = 0.0097$). Transmission patterns of the common haplotypes to asthmatic offspring were predicted by a single-nucleotide polymorphism in the *LTA* promoter region. The G allele of the *LTA* –753G/A polymorphism was transmitted preferentially to asthma-affected individuals ($P = 0.001$). Luciferase reporter assays with constructs containing the 5' and 3' flanking regions of the *LTA* gene showed 30–50% lower transcriptional activity when the –753A allele was present than that of other haplotypes.

Conclusion Our results suggest that *LTA* is one of the genes that contributes to susceptibility to atopic asthma, and that the association of the *TNF/LTA* haplotypes to asthma may be defined by the polymorphism in the *LTA* promoter region in the Japanese population.

Keywords asthma, chromosome 6, lymphotoxin- α , transmitted disequilibrium test

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Introduction

Asthma is a chronic inflammatory disorder of the airways that involves many cells and cellular elements, particularly mast cells, eosinophils, T lymphocytes, and macrophages [1, 2]. Asthma is thought to have genetic components. Familial aggregation of asthma was first described in 1650, and Cooke and Vanderveer [3] confirmed the familial nature of atopic diseases in 1916. Although numerous studies have been conducted, the exact mechanisms that underlie the familial basis of asthma remain unclear. It is likely that

interactions between genes and environmental factors are associated with asthma susceptibility.

Tumour necrosis factor (TNF) is a pro-inflammatory cytokine that participates in the inflammatory reaction in patients with asthma [4, 5]. *TNF*, lymphotoxin- α (*LTA*), and lymphotoxin- β (*LTB*), which are members of the *TNF* gene family, are located consecutively on human chromosome 6p21, which has been linked to asthma by several genome-wide linkage studies [6–10]. In our genome-wide screen for loci associated with mite-sensitive atopic asthma, we found evidence for linkage of marker on 6p22–p21 to atopic asthma, with a maximum lod score of 2.01 [9].

It has been shown that *TNF* polymorphisms and haplotypes are associated with asthma [11–18]. The –308G/A polymorphism in the promoter of *TNF* was reported to be associated with asthma [11, 14–16] and increased bronchial hyper-responsiveness [13]. Because the –308G/A polymorphism is rare in the Japanese population [17, 19], we conducted

Correspondence: Emiko Noguchi, Department of Medical Genetics, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Laboratory of Advanced Research D, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8577, Japan.
E-mail: enoguchi@md.tsukuba.ac.jp

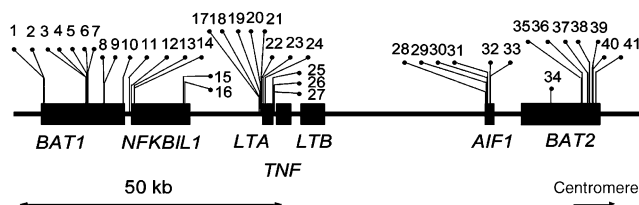


Fig. 1. Physical map of the genes around tumour necrosis factor (*TNF*). Boxes indicate positions of the genes. The positions of identified single-nucleotide polymorphisms are marked by lines. Polymorphism numbers are shown in Table 1.

an association study of asthma with other common *TNF* polymorphisms (−1031T/C, −863C/A, and −857C/T) and found that the −857C/T polymorphism and −1031T/−863C/−857C haplotype were associated with asthma [17].

It is possible that *TNF* haplotypes associated with asthma are in linkage disequilibrium (LD) with other functional polymorphisms that influence asthma susceptibility. In the present study, we screened for mutations in the coding and promoter regions of the HLA-B-associated transcript 1 (*BAT1*), nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor-like 1 (*NFKBIL1*), *LTA*, *LTB*, allograft inflammatory factor 1 (*AIF*), and HLA-B-associated transcript 2 (*BAT2*) genes, which are located within the 100 kb around *TNF* (Fig. 1). We conducted a transmission disequilibrium test (TDT) of 41 polymorphisms in 137 asthmatic families, and found that haplotypes of the *LTA/TNF* and a polymorphism in *LTA* are significantly associated with asthma.

Materials and methods

Pro-bands were children with asthma who visited the Pediatric Allergy Clinic of the University Hospital of Tsukuba (Tsukuba City, Ibaraki, Japan). A full verbal and written explanation of the study was given to all family members interviewed, and 137 families (466 members) gave informed consent and participated in this study. The mean age of the pro-bands and their siblings was 11.2 years (range, 3–29 years); the mean age of the parents was 41.1 years (28–72 years). We used two panels of families. One was the screening panel, comprising 92 families (286 persons), and the other was the genome-scan panel, composed of 45 asthmatic families (190 persons), who were also used for our genome-wide study [9]. Asthma was diagnosed in subjects according to the criteria of the National Institutes of Health (Bethesda, MD, USA) with minor modifications [20]. Patients had to fulfil the following two criteria: (1) two or more episodes of wheezing and shortness of breath during the past year and (2) reversibility of the wheezing and dyspnoea, either spontaneously or by bronchodilator treatment. Methacholine challenge was not carried out because of the young age of the children with asthma in these families; however, the diagnoses of asthma in this population were confirmed clinically by participating physicians or paediatricians who had treated the children for more than 2 years. Because wheezing is often associated with viral respiratory infection in young children, we excluded children younger than 3 years old [21]. The young adult patients included in this study had

suffered from chronic asthma since childhood. Total serum IgE levels and IgE levels specific for *Dermatophagoides farinae* (Df) were determined with the Pharmacia CAP System (Pfizer, New York, NY, USA). To achieve homogeneity of the patients with asthma, we selected families having at least one child with asthma with Df-RAST scores greater than 3. This study was approved by the Committee of Ethics of the University of Tsukuba. No ethnic minorities in Japan were included in this study.

Genomic DNA was extracted from peripheral blood leucocytes. The promoter, 5'-untranslated region (UTR), 3'-UTR, and coding regions of the *BAT1*, *NFKBIL1*, *LTA*, *LTB*, and *BAT2* genes were amplified by PCR from DNAs of 32 subjects with asthma, and the amplified products were subjected to direct sequencing with the Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 DNA Genetic Analyzer (Applied Biosystems). Polymorphisms were genotyped by restriction fragment length polymorphism, SNaPshot (Applied Biosystems), or direct sequencing.

To clarify the terminology with respect to the polymorphisms, we numbered the polymorphisms according to a published nomenclature recommendation [22]. The A of the ATG of the initiator methionine codon was denoted as nucleotide +1, and all single-nucleotide polymorphisms (SNPs) were numbered relative to this translation initiation site. The *TNF* −1031T/C, *TNF* −863C/A, and *TNF* −857C/T, described previously [17, 23, 24] are referred to, hereafter, as *TNF* −1211T/C, *TNF* −1043C/A, and *TNF* −1037C/T, respectively.

We calculated pairwise disequilibrium coefficients for all marker pairs. *D'*-values were calculated with SIMWALK2 (version 2.83, http://watson.hgen.pitt.edu/register/soft_doc.html) [25] and the Haploview package (Haploview 3.0, <http://www.broad.mit.edu/personal/jcbarret/haplo/index.php>). Haplotype blocks were generated by the default algorithm adapted from Gabriel et al. [26] SIMWALK2 was also used to generate haplotypes from the genotype data of our families. TDT was performed with the ASPEX package (version 1.88, <http://aspe.sourceforge.net/>). *P*-values were corrected for multiple testing with the method described by Nyholt [22], and *P*-values less than 0.002 were considered statistically significant. Haplotype TDT was performed with the UNPHASED program (version 2.403, <http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/>) [27].

We generated luciferase reporter gene constructs of the promoter region of *LTA* from −842 to −384 relative to the translation initiation site (−415 to +44 relative to the transcription initiation site) that differed only at position −753 (G vs. A) (Fig. 3). A fragment of the *LTA* promoter was amplified with primers 5'-GAAGATCTTCGGTAGTC-CAAAGCACGAAG-3' and 5'-GAAGATCTTCAAGCCTGTACTCAGCCAAGG-3'. Products were digested with *Bgl*II (New England Biolabs, Beverly, MA, USA) overnight at 37 °C and then subcloned into a *Bgl*II-digested pGL3-basic vector (Promega, Madison, WI, USA). We generated constructs containing the *LTA* promoter region upstream of the *luciferase* gene and the 3' flanking region of *LTA*, which included the *TNF* −1211T/C, *TNF* −1043C/A, and *TNF* −1037C/T sites (*TNF* −1031T/C, *TNF* −863C/A, and *TNF* −857C/T, respectively, in previous papers [17, 23, 24])

downstream of the *luciferase* gene (Fig. 3). We generated three constructs: one with *LTA* -753G/*TNF* 1037C, one with *LTA* -753A/*TNF* -1037T, and one with *LTA* -753A/*TNF* -1037C. A fragment of the *LTA* 3' flanking region (-80 to +1066 relative to the translation termination site of *LTA*) was amplified by PCR with primers 5'-TCCAG-GATCCGAGGTCCTGGAGGCTCTTTC-3' and 5'-CCAG-GGATCCCAGCTATCCACCCACACAGA-3'. The amplified product was digested with *Bam*HI (New England Biolabs) and then subcloned into *Bam*HI-digested pGL3-basic vectors containing the *LTA* promoter fragments. The orientation and accuracy of the inserted fragments were confirmed by direct sequencing.

K562 cells (Cell Resource Center for Biomedical Research, Tohoku University, Miyagi, Japan) were cultured in RPMI 1640 (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich Co.) at 37 °C with 5% CO₂. Approximately 1×10^6 cells were cotransfected with 4 µg of test construct and 150 ng of pRL-TK (Promega) by lipofection (TransFast, Promega) according to the manufacturer's instructions. Cells were incubated at 37 °C for 48 h. Cells were disrupted by the addition of 500-µL lysis buffer (Promega). Twenty microlitres of each lysate was used for luciferase assay with the Dual-Luciferase Reporter Assay System (Promega). The expression efficiency was measured with a TD-20/20 luminometer (Turner Designs Instruments, Sunnyvale, CA, USA). The firefly luciferase values were normalized to the *Renilla* luciferase values of pRL-TK, which were determined at the same time. Reporter activity is presented as the mean of five or six independent values. The difference in transcriptional activity was analysed with one-way ANOVA and the Tukey-Kramer honest significant difference test.

Results

There were 41 polymorphisms in *BAT1*, *NFKB1L1*, *LTA*, *TNF*, *AIF1*, and *BAT2* (Table 1). We numbered these polymorphisms according to the nomenclature recommendation, published previously [28]. No polymorphisms in *LTB* were identified in 32 individuals analysed. The allele frequencies of polymorphisms are shown in Table 1. Among these 41 polymorphisms, eight were in complete LD ($r^2 = 1$) with other polymorphisms. Therefore, we genotyped 33 polymorphisms on the screening panel to explore the association of individual SNPs with asthma. Polymorphisms showing *P*-values less than 0.1 on the screening panel were subsequently genotyped in the genome-scan panel.

The LD map constructed with 41 SNPs is shown in Fig. 2. There were six haplotype blocks in this region, and polymorphisms in *LTA* and *TNF* showed haplotype-block structure (Block4, Fig. 2).

The results of TDT in the asthmatic families are shown in Table 1. The G allele of the -753G/A polymorphisms of *LTA* (numbered relative to the translation initiation site) was transmitted preferentially to asthma-affected offspring (G allele transmissions rate 45/71 = 0.63; $P = 0.02$) in the screening panel. In the genome-scan panel, *LTA* -753G was also transmitted preferentially to asthma-affected children (gen-

ome-scan panel, transmission rate 43/67 = 0.64, $P = 0.0055$; combined, transmission rate 88/138 = 0.64, $P = 0.001$). The C allele of *BAT2* +5320C/T and the G allele of *BAT1* IVS5 +180G/T polymorphisms were transmitted preferentially to asthmatic offspring in the screening panel ($P = 0.052$ and 0.082, respectively), but these associations were not confirmed in the genome-scan panel.

Because polymorphisms in *LTA* and *TNF* showed a haplotype-block structure, we constructed *LTA*/*TNF* haplotypes with 11 polymorphisms (*LTA* -754C/T, *LTA* -753G/A, *LTA* -449G/A, *LTA* -379A/C, *LTA* IVS1 +91A/G, *LTA* IVS1 -82C/G, *LTA* +37T/C, *LTA* +179C/A, *TNF* -1211T/C, *TNF* -1043C/A, *TNF* -1037C/T) and conducted haplotype TDTs in asthmatic families. Because of LD, there were only four common haplotypes with frequencies higher than 0.1. Minor alleles of *LTA* -754C/T, *LTA* +37T/C, *TNF* -1211T/C, and *TNF* -1043C/A were not included in these four common haplotypes. These common haplotypes were significantly associated with asthma (PDTPhase, global $P = 0.0097$). The common haplotypes CGACGG-TATCC and CGGAAGTCTCC were transmitted preferentially to asthma-affected offspring, whereas haplotypes of CAGAACTCTCC and CAGAACTCTCT were transmitted less frequently to affected children (Table 2). Among SNPs constituting these haplotypes, we found that the *LTA* -753G/A polymorphism might act as a tag to distinguish the transmission pattern (Table 2).

To investigate the possibility that the *LTA* -753G/A polymorphism may be associated with altered different transcriptional regulation of *LTA*, we generated luciferase reporter gene constructs of the promoter region from -842 to -384 relative to the translation initiation site (-415 to +44 relative to the transcription initiation site) and differed only at position -753 (G vs. A) (Fig. 3). The constructs did not show significantly different transcriptional activities (Fig. 4). As shown in Fig. 1, *TNF* and *LTA* are adjacent to each other and separated by 2049 bp of intergenic sequence on 6q21. Therefore, the *LTA* 3' flanking region corresponds to the *TNF* promoter region. Because allelic and haplotypic associations of the *TNF* promoter region with asthma have been reported [17], we generated constructs containing the *LTA* promoter region upstream of the luciferase gene and the 3' flanking region of *LTA*, which included the *TNF* -1211T/C, *TNF* -1043C/A, and *TNF* -1037C/T sites, downstream of the luciferase gene (Fig. 3). The *TNF* -1211T/C, -1043C/A, and -1037C/T polymorphisms are located 838, 1006, and 1012 bp downstream of *LTA*, respectively. Because only major alleles of the *TNF* -1211T/C and -1043C/A polymorphisms were included in the common haplotypes, we generated three constructs: one with *LTA* -753G/*TNF* -1037C, one with *LTA* -753A/*TNF* -1037T, and one with *LTA* -753A/*TNF* -1037C. These fragments of the *LTA* 3' flanking region were into pGL3-basic vectors containing the *LTA* promoter fragments. The constructs with *LTA* -753A and either *TNF* haplotype showed nearly 30–50% lower transcriptional activity than the construct with *LTA* -753G (Fig. 4). In contrast, in the presence of *LTA* -753A, constructs with *TNF* -1037C showed approximately 30% lower transcriptional activity than those with *TNF* -1037T (Fig. 4). The difference in transcriptional activity was analysed with one-way ANOVA

Table 1. Single nucleotide polymorphism in genes around *TNF*, including *BAT1*, *NFKBIL1*, *LTA*, *LTB*, *AIF*, and *BAT2* genes

	Gene	dbSNP#	Polymorphism	Minor allele frequency*	P-value (TDT)†
1	<i>BAT1</i>	rs3093978	IVS12 +59T/G	0.11	0.73
2	<i>BAT1</i>	rs2516478	IVS11 – 34C/T	0.13	0.67
3	<i>BAT1</i>	rs1129640	+354A/G	0.11	1.00
4	<i>BAT1</i>	rs933208	IVS5 – 16C/A	0.32	0.17
5	<i>BAT1</i>	rs 2071596	IVS5 – 59C/A	0.39	0.48
6	<i>BAT1</i>	rs2516393	IVS5 +180G/T	0.09	0.59‡
7	<i>BAT1</i>	rs2523512	IVS5 +123C/T	0.13	1.00
8	<i>BAT1</i>	–	– 1451G/C	0.44	
9	<i>BAT1</i>	rs2239528	IVS2 – 179C/T	0.33	0.13
10	<i>BAT1</i>	rs2523502	IVS2 +23A/T	0.37	0.92
11	<i>NFKBIL1</i>	rs3219186	– 421insT	0.10	0.6
12	<i>NFKBIL1</i>	rs2071592	– 62T/A	0.47	0.55
13	<i>NFKBIL1</i>	rs2239708	IVS1 – 178A/T	0.11	0.15
14	<i>NFKBIL1</i>	rs2071591	IVS1 – 142G/A	0.41	0.55
15	<i>NFKBIL1</i>	rs2239707	IVS2 – 86T/C	0.38	0.77
16	<i>NFKBIL1</i>	rs2230365	+377C/T S126S	0.29	0.37
17	<i>LTA</i>	rs2844482	– 754C/T	0.13	0.63
18	<i>LTA</i>	rs2071590	– 753G/A	0.26	0.001‡
19	<i>LTA</i>	rs1800683	– 449G/A	0.40	0.41
20	<i>LTA</i>	rs2239704	– 379A/C	0.47	0.44
21	<i>LTA</i>	rs909253	IVS1 +91A/G	0.40	0.32
22	<i>LTA</i>	rs746868	IVS1 – 82C/G	0.45	0.36
23	<i>LTA</i>	rs2857713	+37T/C	0.17	0.55
24	<i>LTA</i>	rs1041981	+179C/A	0.38	0.92
25	<i>TNF</i>	rs1799964	– 1211T/C (– 1031)	0.14	0.13‡
26	<i>TNF</i>	rs1800630	– 1043C/A (– 863)	0.15	0.14‡
27	<i>TNF</i>	rs1799724	– 1037C/T (– 857)	0.13	0.0071‡
28	<i>AIF</i>	rs2844475	IVS1 +25T/C	0.44	
29	<i>AIF</i>	rs4711274	IVS1 +35C/T	0.38	
30	<i>AIF</i>	–	IVS1 – 74C/T	0.06	
31	<i>AIF</i>	rs2736182	+40G/A	0.28	0.80
32	<i>AIF</i>	rs2259571	IVS3 – 54A/C	0.42	
33	<i>AIF</i>	rs2269475	IVS4 +9G/A	0.05	0.29
34	<i>BAT2</i>	rs2736171	IVS12 – 55C/T	0.42	
35	<i>BAT2</i>	rs2242659	IVS21 +72C/T	0.44	1.00
36	<i>BAT2</i>	rs2242658	IVS21 +79A/G	0.36	
37	<i>BAT2</i>	rs2242657	IVS24 – 40A/G	0.28	0.20
38	<i>BAT2</i>	rs1046089	+5219C/T	0.36	0.13
39	<i>BAT2</i>	–	+5320C/T	0.09	0.17‡
40	<i>BAT2</i>	rs2261033	IVS28 +65C/T	0.18	0.35
41	<i>BAT2</i>	rs13716	+5649C/G	0.42	

Numbers in parenthesis of the Polymorphisms were ones from previous studies [17, 22, 23].

*Frequencies in the parents.

†P-value in the screening panel families.

‡P-values in the screening panel and genome-scan panel families.

TNF, tumor necrosis factor; *BAT1*, HLA-B-associated transcript 1; *NFKBIL1*, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor-like 1; *AIF*, allograft inflammatory factor 1; *LTA*, lymphoxin- α ; *LTB*, lymphotoxin- β ; SNP, single-nucleotide polymorphism; TDT, transmission disequilibrium test.

and the Tukey–Kramer honest significant difference test. One-way ANOVA revealed that three constructs had different transcriptional activity ($P < 0.0001$).

Discussion

Our results indicate that the *LTA/TNF* haplotypes are associated with development of childhood atopic asthma in the Japanese population, and that the best marker for this is the *LTA* – 753G/A polymorphism. Our transcription assay

suggested that the *LTA* – 753G allele, which is associated with asthma susceptibility, increases the transcriptional activity of *LTA*. Although transcriptional activity was not significantly different between constructs with the *LTA* – 753G/A polymorphism, when only the *LTA* promoter sequences were examined by luciferase assay, altered transcription was observed with constructs containing both *LTA* promoter and 3' flanking regions. Furthermore, different transcriptional activities were also observed between constructs that differed at the *TNF* – 1037C/T site. These findings suggest that genomic variations in the 3' flanking region of *LTA* also influence *LTA* expression.

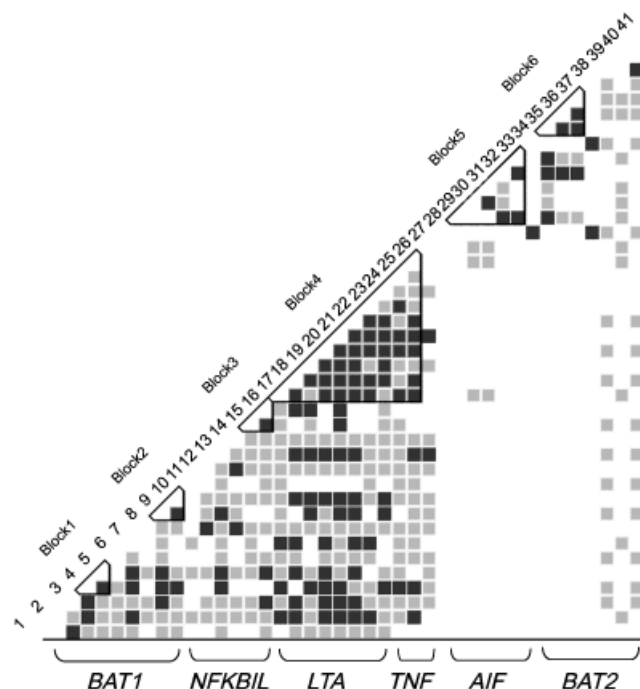


Fig. 2. Haplotype-block structures in the 100-kb region around tumour necrosis factor (*TNF*). The haplotype blocks were generated by Haploview using the default algorithm [24]. Black boxes indicated strong evidence of linkage disequilibrium (LD) between single-nucleotide polymorphisms (SNPs), while white ones showed no or little LD between pairs. Uninformative pairs of SNPs are shown in grey boxes. Numbers (1–41) correspond to the ones in Table 1.

Table 2. *LTA*-*TNF* common haplotypes and transmission disequilibrium test for atopic asthma

Polymorphism	Haplotype (frequency)			
	1 (0.27)	2 (0.22)	3 (0.21)	4 (0.13)
<i>LTA</i> – 754	C	C	C	C
<i>LTA</i> – 753	G	A	G	A
<i>LTA</i> – 449	A	G	G	G
<i>LTA</i> – 379	C	A	A	A
<i>LTA</i> IVS1 +91	G	A	A	A
<i>LTA</i> IVS1 – 82	G	C	C	C
<i>LTA</i> +37	T	T	T	T
<i>LTA</i> +179	A	C	C	C
<i>TNF</i> – 1211 (– 1037)	T	T	T	T
<i>TNF</i> – 1043 (– 863)	C	C	C	C
<i>TNF</i> – 1037 (– 857)	C	C	C	T
T/NT ratio (<i>P</i> -value)	2.5 (0.041)	0.41 (0.0097)	2.3 (0.14)	0.29 (0.04)

Global *P*-value is 0.0097 (PDTPHASE). T, transmitted; NT, not transmitted; *LTA*, lymphotoxin- α ; *TNF*, tumour necrosis factor.

Numbers in parenthesis of the polymorphisms are ones from previous studies [17, 22, 23].

Although we did not examine *LTA* expression differences in cells that had naturally different haplotypes, Knight et al. [29, 30] have identified functionally important *LTA*/*TNF* haplotypes correlated with allele-specific transcription of *LTA* in lymphoblastoid cell lines. They divided *LTA*/*TNF* haplotypes into three types (A, B, and C), and found that expression of *LTA* in haplotype A was greater than that of haplotype B [29]. Haplotypes A and B in the Knight et al. [29] study correspond to haplotypes 1 and 2, respectively, in the

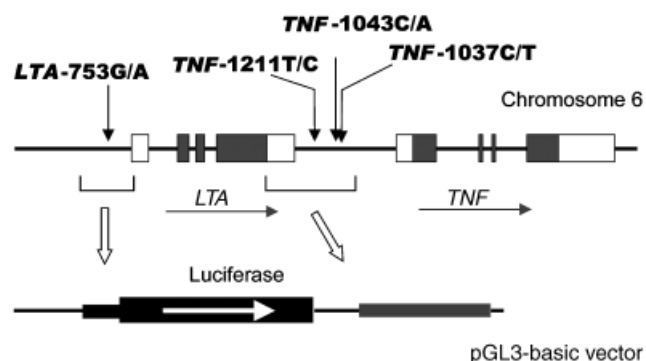


Fig. 3. Structures of vectors and locations of the polymorphisms. Top, genomic structure of tumour necrosis factor (*TNF*) and lymphotoxin- α (*LTA*). Open and closed boxes indicate untranslated region (UTR) and coding region of the genes, respectively. Arrows indicate polymorphic sites. Bottom, structure of pGL3-basic vector with the promoter and 3'UTR fragments of *LTA*. The fragment of the *LTA* 5' region from –415 to +44 relative to the transcription initiation site and that from –80 to 1066 relative to the translation termination site of *LTA* were inserted into pGL3-basic vector.

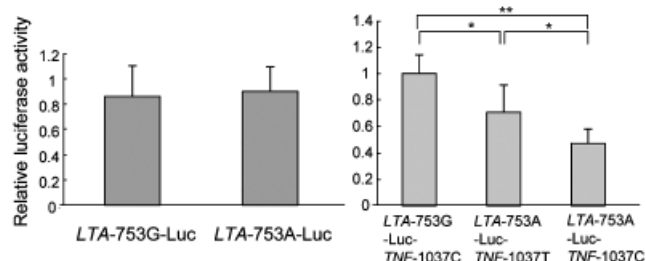


Fig. 4. Comparison of polymorphisms of lymphotoxin- α (*LTA*) analysed by relative luciferase activities in K562 cells. Left, constructs with the *LTA* promoter region. Right, constructs with both the promoter and 3'UTR regions. Results are expressed as relative luciferase activity normalized to those of *Renilla* luciferase (pRL-TK). Standard deviation (SD) is indicated by the error bar. The mean relative activities and $\pm$ SD were calculated in five or six independent transfections. Statistic analysis was performed with Tukey–Kramer's honest significant difference test; ***P* < 0.0001 **P* < 0.05. Luc, Luciferase gene.

present study, although some of the polymorphisms genotyped by Knight et al. [29] were not included in our study. Our luciferase assay data were consistent with those from lymphoblastoid cell lines studied by Knight et al. [29]

Because of the effort of the international HapMap project, haplotype data and block structures are available for human genome (HapMap, <http://www.hapmap.org/>). There are differences for SNP typing between our present data and the HapMap. Our initial mutation screen included 32 Japanese asthmatic subjects. We examined the exons, exon–intron junctions, and promoter regions of the genes, because these regions are most likely to contain functional variants. In contrast, currently available data in the HapMap are from the Centre d'Etude du Polymorphisme Humain (CEPH, 30 trios, European origin), Han Chinese (45 individuals, Asian origin), Japanese (45 individuals, Asian origin), and Yoruba (30 trios, African origin) subjects. In the HapMap project, SNPs used for genotyping come from the dbSNP database, which includes a large number of intronic and intergenic polymorphisms. We analysed 41 SNPs in the 100 kb region, whereas 24 SNPs were examined in the HapMap CEPH dataset for the corresponding region. Five SNPs, rs2516393 and rs2239528 in *BAT1*, rs2239708 in *NFKBIL1*, rs2844482 in *LTA*, and rs2242657 in

BAT2, were typed in both the CEPH dataset and our present study. We genotyped 11 SNPs with minor allele frequencies greater than 0.1 in the *LTA/TNF* region, and the haplotype-block structure was examined for this region. In contrast, only three SNPs in the *LTA/TNF* region, rs2844482, rs1800750 and rs3093559, were genotyped in the HapMap, and two of these SNPs (rs1800750 and rs3093559) were not informative because the minor allele frequencies were less than 0.02 in the CEPH trios, and 0 in the Asian populations. Therefore, the haplotype-block structures in the *LTA/TNF* region on HapMap are not comparable with those of the present study.

Our LD data revealed that SNPs in the *LTA/TNF* region have haplotype-block structures, and that haplotypes in this region were associated with asthma. In the previous studies of the *MHC* region, LD was presented in a region between the *TNF* and *HLA* class II loci, such as DQ and DR [31, 32]. Therefore, it is possible that the *LTA* – 753G/A polymorphism and the *LTA/TNF* haplotypes may be markers of causal variants located further away from the *LTA/TNF* region.

In our previous study, the *TNF* – 1037C/T (– 857C/T) polymorphism and *TNF* – 1211T/– 1043C/– 1037C (– 1031T/– 863C/– 857C) haplotype were associated with asthma [17]. The *P*-values of the TDT data for the *TNF* – 1037C/T polymorphism in our previous study [17] and our present study differ slightly because we used different analytical methods and different asthmatic families in each study. Because the haplotypes, which were composed of *TNF* – 1211T/C, – 1043C/A, and – 1037C/T, were not in strong LD with *LTA* – 753G/A ($D' = 0.37$), the association between asthma and *LTA* – 753G/A is not likely to be because of simple LD with *TNF* haplotypes. The present findings indicate that *TNF* allelic and haplotypic associations should be reconsidered in the context of the *LTA*–*TNF* haplotype.

LTA, which is also known as TNF- β , is produced by a variety of cells after mitogenic stimulation and has roles in lymphocyte homing and formation of spleen and lymph nodes [33]. *LTA* is involved in the pathogenesis of various diseases, including bacterial infection [34] and malaria [35]. Worm et al. [36] reported that *LTA* plays a potentially significant role during B cell proliferation and IgE synthesis, and that *LTA* production appears to be regulated differently in B cells from atopic and non-atopic donors. *LTA* polymorphisms have been studied for possible associations with several diseases. A recent study revealed that polymorphisms in *LTA* and the gene encoding its interacting protein, galectin-2, are associated with myocardial infarction [37, 38]. *NcoI* polymorphism in intron 1 of *LTA* (rs909253) has been studied extensively and was reported to be associated with asthma [11, 12] and atopy [39]; however, other groups have reported conflicting results [17, 40]. In the present study, we did not find an association between the *NcoI* polymorphism and asthma. However, we did observe association of a functional *LTA* polymorphism, *LTA* – 753G/A, with asthma, providing further support for the involvement of *LTA* in the development of asthma.

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