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ORIGINAL ARTICLE

Genetic Impact of a Butyrophilin-like 2 (BTNL2) Gene Variation on Specific IgE Responsiveness to *Dermatophagoides farinae* (Der f) in Japanese

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ABSTRACT

Background: *Dermatophagoides farinae* (Der f) is one of the most frequently implicated allergens in several allergic diseases. Several genome-wide screens have identified a linkage between chromosome 6p21 and mite-specific IgE responsiveness. Butyrophilin-like 2 (BTNL2) is a member of the immunoglobulin superfamily and, on the basis of its homology to B7-1, has been implicated as a costimulatory molecule involved in T-cell activation. BTNL2 resides in the HLA region on chromosome 6p21, and significant associations between BTNL2 gene polymorphisms and several inflammatory diseases have been reported.

Objective: The aim of this study was to examine whether BTNL2 gene polymorphisms are associated with specific IgE responses to Der f.

Methods: Three single nucleotide polymorphisms (SNPs), including 2 coding SNPs and 1 intron SNP, were studied. One of the coding SNPs was the rs2076530 A > G, which has a functional consequence. A total of 863 unrelated Japanese subjects (447 positive and 416 negative for IgE to Der f) were recruited for a case-control study.

Results: Controlling for gender, age, smoking, and the presence of asthma, multiple logistic regression analyses showed that homozygosity of the rs2076530 A allele, which has been reported to be a risk allele for sarcoidosis, was associated with a risk of sensitization towards Der f (Odds ratio; 1.55, $p = 0.0060$).

Conclusions: Although an association which may be due to the linkage disequilibrium with other genes in 6p 21 needs to be ruled out, the present findings suggest that the BTNL2 gene might be one of the candidate genes that is responsible for the pathogenesis of Der f-specific IgE responsiveness.

KEY WORDS

BTNL2, Dermatophagoides farinae (Der f), specific IgE

INTRODUCTION

Dermatophagoides farinae (Der f) is one of the most frequently implicated allergens in several allergic diseases. Antibody responsiveness to complex allergens such as Der f is considered to be a multifactorial trait because it is controlled by both genetic and environmental factors.[1,2] In genome-wide screening, chromosome 6p21 has been shown to be linked to mite-specific IgE responsiveness, where the HLA class II gene location suggests evidence for being a strong candidate for the trait.[3-5] A number of studies have shown that specific IgE production in response to individual allergens is associated with particular HLA class II alleles, exclusively for low-molecular-weight allergens such as *Ambrosia artemisifolia V* (Amb a 5)

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Table 1 Primers for direct sequencing

Name of primer	Primer sequence
6813 Forward	5'TGAGTGCAAC CTGGGATACT G 3'
6813 Reverse	5'TCTCCCACCT GACAGGAAGC 3'
11084 Forward	5'CAGACTGACACACATCATGGAATG 3'
11084 Reverse	5'ACAGTGGGAAATGATGCTGC 3'
12155 Forward	5'AGTGAAATTGCTGCCCACTC 3'
12155 Reverse	5'TCGTCTTTCCTTCCATGTCC 3'

and *Lolium Perenne pollen III* (Lol p 3) (molecular weight, about 5 kD).[6-8] However, in the case of very complex allergens such as Der f (molecular weight, 25 kD), genetic regulation of the immune response by HLA genes is known to be quite complex; it involves multiple epitopes and agretopes,[9,10] which inevitably dilutes any association between particular HLA class II alleles and specific IgE responses to a given complex allergen. Marsh *et al.* postulated the existence of an HLA-associated hyperresponsiveness state controlled by an additional genetic factor responsible for the overall expression of the immune response.[11] Therefore, although a particular HLA class II allele is responsible for the development of IgE responses to Der f, susceptibility may be related more to generalized hyperresponsiveness than to specific responses to Der f.

BTNL2, also known as "butyrophilin-like 2" and "BTL-2", is a butyrophilin gene that belongs to the immunoglobulin gene superfamily. On the basis of its amino acid homology to B7 (CD80 and CD86) proteins, BTNL2 has been proposed to play a role as a costimulatory receptor involved in modulation of T-cell responses.[12,13] Recent *in vivo* studies using BTNL2-Immunogloburin (Ig) have demonstrated that BTNL2 binds to a putative receptor on activated T cells and functions to inhibit the proliferation of T cells,[14,15] suggesting that this gene plays an inhibitory role in T cell activation.

The BTNL2 gene resides in the HLA region on chromosome 6p21, and significant associations between BTNL2 gene polymorphisms and several inflammatory diseases have been reported.[13,16-18] A loss-of-function allele at rs2076530 in the BTNL2 gene has been implicated as a risk factor for sarcoidosis in two populations.[13,19,20]

Given the possible role of BTNL2 in the inhibition of T cell activation, as well as its chromosomal location, we hypothesized that it was a possible candidate gene for Der f-specific IgE responsiveness. As part of our search for genetic factors that contribute to susceptibility to atopy and asthma,[3,21-23] the current study focused on the BTNL2 gene. In particular, the potentially functional BTNL2 gene polymorphism (rs2076530) was correlated with the development of Der f-specific IgE responses.

METHODS

STUDY POPULATION

A total of 863 unrelated Japanese subjects, including 424 asthma patients and 439 healthy volunteers, were recruited. Total serum IgE levels (IU/ml) were measured in all subjects, and specific IgE responses to 10 common inhaled allergens, including Der f, grass pollen, animal danders, and molds were also assessed. An increase in specific IgE antibody levels (IgE CAP RAST > 0.35 UA/ml, or MAST > 1.0 lumicount) was considered to indicate a positive response. Subjects who showed a positive specific IgE response to Der f (n = 447) included 259 asthma patients and 188 healthy volunteers, while subjects who did not demonstrate a specific IgE response to Der f (n = 416) included 165 asthma patients and 251 healthy volunteers. All asthma patients (n = 424) were recruited from the pulmonary clinic of the First Department of Medicine at the Hokkaido University Hospital. Asthma was defined based on the presence of recurrent episodes of at least two of three symptoms (cough, wheeze, or dyspnea) that were associated with demonstrable reversible airflow limitation and/or increased airway responsiveness to methacholine, as previously described.[21-23] Individuals who visited our clinic for annual routine physical examinations and students at the Hokkaido University's School of Medicine were recruited as healthy controls (n = 446) if they had no history of asthma or any other chronic pulmonary diseases. Atopy was defined as a positive response to at least 1 of the 10 common inhaled allergens, as previously described.[21-23] All subjects were unrelated Japanese who gave their written informed consent for enrollment in the study and all associated procedures. The study was approved by the ethics committee at the Hokkaido University School of Medicine.

GENOTYPING

Six single nucleotide polymorphisms (SNPs) in the BTNL2 gene (6813 A > G, 11084 A > G, 12155 C > T, 12159 G > A, 12197 C > A, and 12198 A > G) were selected from previous reports[13] and the public database, SNPper.[24] Nucleotide numbering was measured from the first nucleotide of the transcription start site. The seven SNPs included the rs2076530 polymorphism (11084 A > G) in exon 5, which led to an alternative splice site and resulted in an early stop codon and a truncated protein, and four polymorphisms in the coding region (exon6) that caused the following amino acid substitutions: 12155 C > T (Pro285Leu); 12159 G > A (Met286Ile); 12197 C > A (Pro299Gln); and 12198 A > G (Pro299Gln). Initially, 25 control subjects (i.e., 50 haploid genomes) were genotyped by direct sequencing for each polymorphism. Since all of the polymorphisms had a minor allele frequency of > 0.01 and the four SNPs in exon 6

Table 2 Primers for allele-specific PCR

Name of primer	Primer sequence*
6813 Forward for A	5'AGCACACCTTTCAGCCCACA 3'
6813 Forward for G	5'AGCACACCTTTCAGCCCACG 3'
6813 Reverse	5'TGTGTTTGTTACCCTGATTTTCCTC 3'
11084 Forward	5'CAGGAGGCCAGTTTGGATCTG 3'
11084 Reverse for A	5'GCAGGTATTGAATACAAAATATCTATCTATCTAGAATTCTTACT 3'
11084 Reverse for G	5'GCAGGTATTGAATACAAAATATCTATCTATCTAGAATTCTTACC 3'
12155 Forward for C	5'AAATGCAGCCGATGTGCTC 3'
12155 Reverse for C	5'CCTTCCATGTCCCTCCAT 3'
12155 Forward for T	5'GGAGAAATGCAGCTGATATGCTC 3'
12155 Reverse for T	5'CCTTCCATGTCCCTCCAC 3'

*The nucleotide corresponding to each SNP is underlined.

Table 3 Basic characteristics of the study subjects

		Positive IgE to Der f (n = 447)		Negative IgE to Der (n = 416)		P value
		Asthma	Control	Asthma	Control	
Number of subjects		259	188	165	251	
Gender (female/male)		129/130	60/128	103/62	95/156	< .001*
Age (years, median, range)		33, 16–76	27, 19–69	57, 18–81	43, 18–72	< .001*
Current smoker (%)		24.1	23.7	21.2	30.9	< .001*
Serum total IgE levels (log IU/ml, SD)		2.60 (0.60)	2.16 (0.54)	2.01 (0.60)	1.62 (0.58)	< .001*
Three SNPs genotyped						
6813A > G rs3817963	AA	143	102	83	120	0.20†
	AG	102	67	67	109	
	GG	14	19	15	22	
11084A > G rs2076530	AA	110	86	61	89	0.035†
	GA	101	64	78	109	
	GG	48	38	26	53	
12155C > T rs28362678	CC	130	99	85	130	0.97†
	CT	112	77	70	103	
	TT	17	12	10	18	

*One-way ANOVA or χ^2 test as appropriate.

†P value from a genotype-based χ^2 test of 2×3 data (positive IgE to Der f/negative IgE to Der f).

were in complete linkage disequilibrium, three SNPs (6813 A > G, 11084 A > G, and 12155 C > T) were subjected to further analyses. The sequences of the primers used for direct sequencing are shown in Table 1. Alleles were identified using an assay combining kinetic (real-time quantitative) polymerase chain reaction (PCR) with allele-specific amplification, as described elsewhere.[22,23] Primers were designed using Primer Express software (PE Applied Biosystems, Foster City, CA, USA) to specifically amplify each SNP allele in separate PCRs. The sequences of the primers used for allele-specific PCR are shown in Table 2. Real-time PCR was performed using Syber Green I Master Mix (Applied Biosystems) and an ABI PRISM TM 7700 Sequence Detection System (Applied Biosystems).

DATA ANALYSIS

All three SNPs were tested for conformation with Hardy-Weinberg expectations (HWE) using Haploview software, version 3.2.[25] Statistical analyses were based on the calculation of odds ratios to provide estimates of the relative risk of sensitization to Der f. Logistic regression analysis was used to estimate odds ratios adjusted by gender, age, and smoking status (current, ex, or never). The linkage disequilibrium (LD) structure was examined using Haploview software.[25] For haplotype analysis, we used the Haplo. score program, which adjusts for covariates and calculates p values for each haplotype.[23,26]

RESULTS

The characteristics of the subjects with specific IgE

Table 4 Genetic impact on sensitization to Der f of three single nucleotide polymorphisms in the BTNL2 gene

SNP	Genotype	Odds Ratio (95% CI)*	
		Unadjusted	Adjusted†
6813A > G (rs3817963)	AA	Reference	Reference
	AG	0.80 (0.60 – 1.06)	0.77 (0.56 – 1.06)
	GG	0.74 (0.45 – 1.22)	0.68 (0.38 – 1.16)
	AA	Reference	Reference
	AG or GG	0.81 (0.50 – 1.33)	0.75 (0.56 – 1.02)
	AA or AG	Reference	Reference
	GG	0.78 (0.45 – 1.33)	0.78 (0.45 – 1.33)
11084A > G (rs2076530)	GG	Reference	Reference
	GA	0.84 (0.58 – 1.21)	0.76 (0.50 – 1.15)
	AA	1.23 (0.84 – 1.77)	1.30 (0.85 – 2.00)
	GG	Reference	Reference
	GA or AA	1.01 (0.72 – 1.41)	0.99 (0.67 – 1.45)
	GG or GA	Reference	Reference
	AA	1.38 (1.05 – 1.81)‡	1.55 (1.13 – 2.10)§
12155C > T (rs28362678)	CC	Reference	Reference
	CT	1.03 (0.78 – 1.36)	0.85 (0.62 – 1.16)
	TT	0.91 (0.53 – 1.57)	0.72 (0.38 – 1.34)
	CC	Reference	Reference
	CT or TT	1.01 (0.78 – 1.32)	0.83 (0.61 – 1.12)
	CC or CT	Reference	Reference
	TT	0.90 (0.53 – 1.52)	0.77 (0.42 – 1.41)

*Odds Ratios were calculated using logistic regression analysis.

†The adjusted value was calculated after adjusting for gender, age, smoking status, and asthmatic status.

‡$p = 0.021$, §$p = 0.0060$.

responses to Der f (n = 447) and of the subjects without specific IgE responses to Der f (n = 416) are shown in Table 3. The median age of the subjects with Der f-specific IgE responses was significantly lower than that of the subjects without Der f-specific IgE responses ($p < 0.001$). There were more asthma patients in the positive Der f-specific IgE group ($p < 0.001$), and their total serum IgE levels were higher than those of subjects without Der f-specific IgE ($p < 0.001$). The three SNPs investigated (6813 A > G, 11084 A > G, and 12155 C > T) were within the HWE in subjects without Der f-specific IgE ($p > 0.05$). A significant association between 11084 A > G [rs2076530] and Der f sensitization was found when the analysis was adjusted for age, gender, smoking status, and asthmatic status (Table 4). The OR for the AA homozygotes of the 11084 A > G polymorphism was 1.55 compared with the other genotypes, GG homozygotes, or GA heterozygotes (95% CI, 1.13–2.10; $p = 0.0060$).

Pair-wise linkage disequilibrium (LD) values for the three SNPs are shown in Figure 1. The frequency of the BTNL2 haplotypes is shown in the haplotype tables (Table 5). The 6813G/11084G/12155C (GGC) haplotype was associated with a significantly lower risk of sensitization to Der f ($p = 0.019$) as judged by haplotype-specific scores (Table 5).

In the case-control study, associations between asthma-related phenotypes, such as total serum IgE levels, atopy, asthma, and the polymorphisms of BTNL2, were also investigated. No significant associations between the genotypes of the three SNPs and total serum IgE levels, atopy, or asthma were found (data not shown).

DISCUSSION

In the present study, a significant association between BTNL2 gene polymorphisms and specific IgE responses to Der f was identified in a Japanese population.

In genome-wide screening, chromosome 6p21 has been shown to be linked with mite-specific IgE responsiveness, and a strong association with the HLA class II gene has been reported.[3-5] Chromosome 6p21 contains several important genes involved in immunological responses, and there is extensive linkage disequilibrium (LD) within this region.[27,28] Therefore, it is still unclear whether the HLA genes deter-

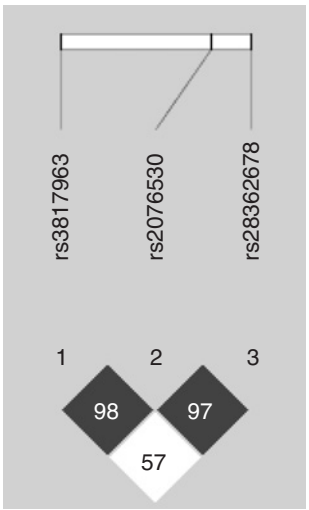

Fig. 1 Location and linkage disequilibrium (LD) map structure of single-nucleotide polymorphisms (SNPs) in BTNL2. Haploview plot shows pairwise LD (D' values) for 3 SNPs based on the genotypes of the 863 individuals in the case-control study. Each square plots the level of LD (D' values) between a pair of SNPs.

mine susceptibility directly, or whether associations may be due to other genes within this region. The association between HLA class II antigens and the IgE response was first noted in patients sensitized to small, well-defined antigens, such as the ragweed allergens Amb a 5 and the rye grass allergens Lol p 3.[6-8] Several recent studies have also demonstrated HLA class II associations in industrial asthma, where the triggering molecules are simple, low molecular weight molecules such as isocyanates and acid anhydrides.[29,30] On the other hand, high molecular weight allergens such as Der f presumably express multiple epitopes or sequence motifs that can each be presented in association with different HLA class II polymorphic molecules expressed on the surface of antigen-presenting cells.[9,10] This phenomenon inevitably dilutes any association between particular HLA class II alleles and specific IgE responses to a given allergen in a population, particularly when whole allergen extracts are used. The results of this study support previous observations that non-HLA genes may be of importance in controlling specific IgE-responsiveness to complex allergens such as Der f,[31-33] and we demonstrated that BTNL2 is a possible candidate gene.

The rs2076530 is located within a donor splice site and the G to A nucleotide transition results in the recruitment of an alternative splice site located four base pairs upstream of the affected donor site. The exclusion of the four base pairs from the spliced mRNA transcript derived from the A allele produces a frameshift and a premature stop codon in the following downstream exon. It has been hypothesized that the truncating polymorphism results in impairment of a potential T-cell downregulatory function of BTNL2, possibly by mis-localization from the membrane, leading to dysregulated T-helper cell activation.[13] A loss-of-function allele at rs2076530 in the BTNL2 gene has been implicated as a risk factor for sarcoidosis,[13,19,20] a disorder that probably results from an exaggerated T-cell response to airborne antigens. In the present study, we observed an increased prevalence of the same dysfunctional allele in subjects with a positive Der f-specific IgE response, indicating that BTNL2 might inhibit the production of Der f-specific IgE.

The interaction between dendritic cells and T cells during allergen sensitization largely depends on a coordinated process involving the main pathway of HLA class II/antigen peptide complex to antigen-specific T cell receptor (TCR) and the additional pathway involving costimulatory molecules, including BTNL2. Therefore, HLA class II alleles and BTNL2 alleles might have cross-relationships, which might result in an association between the BTNL2 gene and the Der f-specific IgE response.

Even though the functional background of BTNL2 and rs2076530 has been reported, the significance of genetic variants of the BTNL2 gene is complicated because of extensive linkage disequilibrium (LD) in chromosome 6p21. Indeed, it has been reported that the effect of BTNL2 on susceptibility to multiple sclerosis,[16] rheumatoid arthritis,[17] systemic lupus erythematosus,[17] and Graves' disease[18] is secondary and is simply due to linkage disequilibrium with some MHC class II alleles. In addition to the HLA gene, chromosome 6p21 contains several important genes, such as BTNL2, lymphotaxin-α (*LTA*), transporter, ATP-binding cassette (*TAP*), tumor necrosis factor-α (*TNFA*), retinoid X receptor B (*RXRB*), which are involved in immunological and inflammatory responses (Fig. 2). Therefore, it is necessary to ascertain whether the association that was identified in the present study is attributable to direct involvement of the BTNL2 gene itself in disease pathogenesis or whether this is merely a reflection of linkage disequilibrium with one or more disease genes located on adjacent areas of chromosome 6. Extensive recent analyses have provided wider haplotype blocks across the HLA region and a nearby locus in chromosome 6p21.[27,28] Further extensive analyses using this information will provide the answer and address the limitations of our studies.

In conclusion, given that non-HLA genes in the chromosome 6p21 region may be important for the development of specific IgE responses to high molecular weight allergens, the functional relevance of the BTNL2 polymorphisms, along with its chromosomal location, strongly suggest that BTNL2 is an excellent candidate gene for the development of Der f-specific IgE responsiveness. Further studies are needed to clarify the pathological mechanisms by

Table 5 Association analysis of haplotypes in BTNL2 gene with sensitization to Der f

Haplotype	6813	11084	12155	Haplotype Frequency*		Haplotype-specific Score	*p* value
				Sensitized to Der f	Not sensitized to Der f		
1	G	G	C	0.076	0.121	− 2.352	0.019
2	G	G	T	0.183	0.173	− 0.552	0.562
3	A	G	T	0.086	0.097	− 0.531	0.582
4	A	G	C	0.030	0.019	0.912	0.355
5	A	A	C	0.618	0.587	1.645	0.098

*The frequency of each haplotype in 863 subjects was estimated using the Haplo.Stats program.[26]

Haplotypes with frequencies less than 1% are not listed.

Note that haplotype-specific scores give effect estimates, negative haplotype-specific scores are associated with a protective effect, and positive haplotype-specific scores are associated with an increased risk.

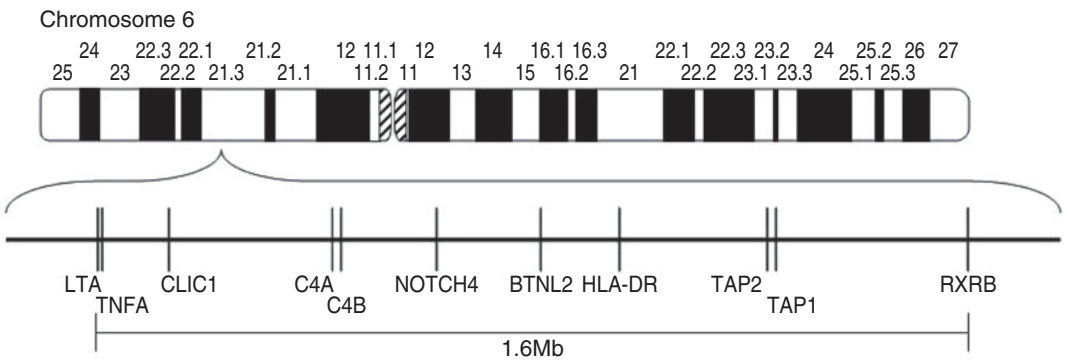

Fig. 2 Gene map and location of several important genes involved in immunological and inflammatory responses at 6p21. LTA, lymphotaxin-α; TNFA, tumor necrosis factor-α; CLIC, chloride intracellular channel; C4, complement component 4; TAP, transporter, ATP-binding cassette; RXRB, retinoid X receptor B.

which BTNL2 contributes to the development of Der f-specific IgE production.

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