

Mutation screening of interferon regulatory factor 1 gene (IRF-1) as a candidate gene for atopy/asthma

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Summary

Background IL-4 gene cluster on chromosome 5 contains several candidate genes for atopy and asthma. Several independent studies have shown evidence for linkage between the markers flanking IL-4 gene cluster and asthma and/or asthma-related traits. Interferon regulatory factor 1 (IRF-1) is located approximately 300 kb telomeric to IL-4 and recent study reveals that IRF-1 deficiency results in an elevated production of Th2-related cytokines and a compensatory decrease in the expression of native cell- and Th1-related cytokines.

Objective To determine if there are any mutations associated with the development of atopy and asthma present in the coding exons and 5' flanking region of the IRF-1 gene.

Methods and results We have screened the promoter and coding regions of the IRF-1 gene in atopic asthmatics and controls by SSCP method. We found three novel nuclear variants (the –300G/T and 4396 A/G polymorphisms and the 6355G>A rare variant) in the IRF-1 gene. No variants causing amino acid alterations of IRF-1 were detected. The –300G/T polymorphism was in nearly complete linkage disequilibrium with the 4396 A/G polymorphism. An association between the 4396 A>G polymorphism and atopy/asthma was examined by transmission disequilibrium test in 81 asthmatic families. Either of 4396 A or 4396G alleles was not significantly preferentially transmitted to atopy- or asthma-affected children.

Conclusion The IRF-1 gene is less likely to play a substantial role in the development of atopy and asthma in the Japanese population.

Keywords: Asthma, atopy, genetic, IL-4 gene cluster, IRF-1, polymorphism

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Introduction

Atopy, expressed clinically as asthma, atopic dermatitis, and/or rhinoconjunctivitis, is characterized by a genetic predisposition for generating immunoglobulin (Ig) E antibody against common environmental allergens. Since family and twin studies have demonstrated an involvement of genetic components in the development of atopy and asthma [1,2], much work has been done to find the responsible gene(s). Using the candidate gene approach, linkage of

atopy, asthma and/or asthma-associated phenotypes to chromosome 5q31.1-q33 [the interleukin 4 (IL-4) gene cluster] [3,4], 6p21 (HLA loci and *TNF*) [5], 7q35 (*TCRB*) [6], 11q13 (*FCER1B*) [7–11], 12q15-q24 (*IFNG* and others) [12,13], 14q11 (*TCRA*) [14,15] and 16p12 (*IL4R*) [16,17] has been described. Several other loci linked to atopy and asthma have also been suggested through genome-wide linkage studies [18,19].

It is well known that asthmatic patients often show increased levels of Th2-related cytokines such as IL-4 and IL-5 and decreased levels of Th1-related cytokines like interferon-gamma (IFN γ) in stimulated peripheral blood mononuclear cells and bronchoalveolar lavage fluid [20,21].

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Thus, an imbalance in the Th1/Th2 response has been suggested as a cause of asthma. IL-4 gene cluster on chromosome 5 comprises of IL-3, IL-4, IL-5, IL-13, interferon regulatory factor 1 (IRF-1), CDC25, and GM-CSF, which are considered to be important for Th1 and Th2 immune responses. IRF-1 was originally identified by its ability to induce the transcription of interferon and interferon-induced gene [22] and regulate an array of genes involved in cell growth, differentiation and death. Recent studies have shown that IRF-1 is involved in the regulation of inducible nitric oxide synthases (iNOS) [23,24] which induce inflammation in asthmatic lung [25]. A knockout mouse study reveals that IRF-1 deficiency results in an elevated production of Th2-related cytokines and a compensatory decrease in the expression of naive cell- and Th1-related cytokines [26]. Since several independent studies have shown the evidences for linkage between the markers flanking IL-4 gene cluster and asthma and/or asthma-related traits [3,4,27], the IRF-1 gene seems to be a good candidate gene for asthma and atopic diseases. In this study, we have screened for mutations in the promoter and coding regions of the IRF-1 gene in atopic asthmatics and controls. We found three novel nuclear variants in the IRF-1 gene and conducted association study in a Japanese families ascertained through asthmatic children.

Materials and methods

Subjects

Probands of the families studied were asthmatic children visiting the Paediatric Allergy Clinic of the University Hospital of Tsukuba. A full verbal and written explanation of the study was given to all family members interviewed, and 81 families gave us informed consent and participated in this study. Informed consent for subjects younger than school age was given by their parents. The mean numbers of atopic and asthmatic children per family are 2.1 and 1.8. The mean age of the probands and their siblings was 11.2 years (range: 3–29); the mean age of the parents was 41.1 years (28–72). The families examined in this study are, in part, the same ones that participated in our previous study [4].

Each family member was questioned regarding allergic symptoms and underwent a physical examination by paediatricians. Asthma was diagnosed in subjects according to the criteria of the National Institutes of Health, USA, with minor modifications [28]. Patients had to show the two following characteristics: (1) two or more episodes of wheezing and shortness of breath during the past year and (2) reversibility of the wheezing and dyspnea, either spontaneously by bronchodilator treatment. Since wheezing is often associated with viral respiratory infection in young

children [29], subjects more than 3-year-old were evaluated for the asthma phenotype. Young adult patients included in this study have been suffering from chronic asthma since childhood. The diagnosis of asthma in this population was confirmed by physicians or paediatricians. Total serum IgE levels and specific IgE levels to house dust mite, *Dermatophagoides farinae* (Df), were determined by the Pharmacia CAP System (Uppsala, Sweden). Atopy was defined by the presence of either or both of the following criteria: a total serum IgE level more than one standard deviation above the geometric mean for the normal Japanese population and/or raised specific serum IgE levels to Df (RAST score >2). By this definition, 170 atopy-affected, 146 asthma-affected children and their parents were subjected to an association study.

We selected controls who met all of the following criteria: (1) no symptoms or history of allergic diseases (2) no detectable dust mite specific IgE antibody, and (3) total serum IgE levels below the general population mean for their ages. The mean age of the controls was 11 years (range: 9–14).

Molecular methods

DNA was extracted from peripheral blood leucocytes collected in EDTA. The 10 exons and the promoter region of the IRF-1 gene were amplified from genomic DNA of 24 asthmatics and 24 controls. Asthmatics for the mutation study were randomly selected from the asthmatic probands of the families.

SSCP and sequencing

The regions of all exons and exon-intron junctions and the 5' flanking region of 486 bp were amplified by PCR using the primers listed in Table 1. The primers of all exons were designed according to the published sequence of the IRF-1 gene (Genbank accession number: L05072). The primers of the promoter region were designed according to the sequence described before [30]. Primers and amplified sizes are shown in Table 1. The PCR reaction cocktail contained 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.0–1.5 mM MgCl₂; 0.1% Triton X-100; 200 µM each of deoxynucleotide triphosphates (dNTPs); 0.6 U Taq DNA polymerase (Promega Ltd, Madison, USA); 5 µM each of primers, and approximately 40 ng of template DNA in a total volume of 10 µL. PCR was performed in a 96-well plate using a programmable thermal cycler (GeneAmp PCR system 9600; Perkin Elmer Corp., New Jersey, USA).

Single strand conformational polymorphism (SSCP) analysis was done for all PCR products (each exon and the promoter region in each individual). Two microlitre of PCR product was mixed with equal volume of formamide

Table 1. IRF-1 primer oligonucleotides

Sense (5'-3') (nucleotide numbers*)	Size (bp)	Antisense (5'-3') (nucleotide numbers*)
IRF-1 promoter		
GAGCCAGGCTGCCAGTCG (-486-469)	248	CCCAATCCACCCCTGCTC (-256-239)
GCCCTCCTCCGGGCAAGC (-276-259)	263	CGCCACCGAGCAATCCAA (-31-14)
GGCCAGGGCTGGGGAATC (-60-43)	221	GAGGGCGGAGCGCGACTC (144-161)
IRF-1 coding region		
Exon 1		
TGCCTTCTTCCCTCTTCCAC (121-140)	120	CTCAAAGGCGTACTCACCTC (221-240)
Exon 2		
GACTGGAGCCTCTGTGTTCT (1255-1274)	226	CTTGTCCTGTTATCACTTCC (1461-1480)
Exon 3		
CAAAGTCTGTGGTCTCTGAC (2682-2701)	216	CGTGAGTGCCTCCTCTTGTC (2878-2897)
Exon 4		
ATGGCCCTGACGATCCTCTT (3592-3611)	216	CCTTTTCTCTGGTTCTTGGT (3788-3807)
Exon 5		
GTGCGAGTGTACCGGATGCT (3758-3777)	233	CCTGGCTGCTTAGGACCACA (3971-3990)
Exon 6		
GGGCCTAAGCTGCTTTCTCC (4045-4064)	198	CTGACCTGTGGGGTCTCCTG (4223-4242)
Exon 7		
GAAAGCACACAACCTTGACC (4344-4363)	262	TCAGTGCCAGGTGGAGTTCT (4586-4605)
Exon 8		
TGACTGCCAGCCAACCTCTC (5002-5021)	220	ACTTGTGGGACAATGCAAGC (5202-5221)
Exon 9		
TTGATAGCCTCTTGCTCTTC (6219-6238)	214	CGTGTTCTCCCTCATCTAA (6413-6432)
Exon 10		
GGGGGAAGCACACCTGATGC (6608-6627)	224	GCTTGCCTAGAGGAATAAGA (6812-6831)

*Nucleotide numbers are according to Genbank accession number L05072.

(90% v/v) dye containing bromophenol blue and xylene cyanol. The samples were boiled for 3–5 min, quenched on ice and separated by electrophoresis on 12.5% homogenous Pharmacia Phast Gel (Pharmacia, Uppsala, Sweden) using PhastSystem (Pharmacia, Piscataway, NJ, USA). The gels were run for 2–3 h at 15°C, then visualized using silver staining. The PCR products showing different SSCP patterns were subjected to the sequence analysis. The sequencing were performed using a thermal cycle sequencing kit (US Biochemical Corp., Cleveland, OH, USA) on ABI PRISM 310 DNA Sequencer (Perkin Elmer-Cetus, Oak Brook, IL, USA).

We have found one mutation in the promoter (G to T exchange at position 300 upstream from exon 1, -300G>T) and two silent mutations in exon 7 (A to G exchange at position 4396, 4396 A>G) and in exon 9 (G to A exchange at position 6355, 6355G>A). To genotype

these variants using PCR-restriction fragment length polymorphism method (RFLP), each region of interest was amplified by PCR with a primer pairs 5'-TCGCTGCG-TTCGGGAGA and 5'-TAGCGGCGAAGGGGAAGTACA for the promoter, 5'-GAAAGCACACAACCTTGACC and 5'-TCAGTGCCAGGTGGAGTTCT for exon 7 and 5'-TGTCTATGGAGACTTTAGCTGTGA and 5'-TAAGGC-ATGGCAGCCGTAGC for exon 9 polymorphisms. The original sequence of positions 6331–6354 in the IRF-1 gene for the upstream primer of exon 9 is TGTCTATGGAGACTTTAGCTGTAA. The mismatch '5'-CTGTCTATG-GAGACTTTAGCTGTGA' was introduced in the upstream primer in order to obtain the *Mbo* II restriction site. The PCR product sizes for the promoter, exon 7 and exon 9 polymorphisms were 270 bp, 262 bp and 200 bp, respectively. The -300G>T, 4396 A>G and 6355G>A mutations were identified by the gain of *Bst*NI (the G allele,

210 bp, 60 bp; the T allele, 170 bp, 60 bp, 40 bp), loss of *Nla* III (the A allele 115 bp, 94 bp, 53 bp; the G allele, 147 bp, 115 bp), and gain of *Mbo* II (the G allele, 200 bp; the A allele, 165 and 35 bp) restriction sites, respectively. Digested PCR fragments were electrophoresed by agarose gel electrophoresis and visualized by ethidium bromide staining and ultraviolet transillumination. The accuracy of genotyping by these RFLPs has been confirmed by the direct sequence of PCR products from three individuals with 4396 A/A, 4396 A/G, and 4396G/G genotypes determined by RFLP and confirmed the accuracy of our RFLP.

Statistical analyses

Transmission disequilibrium test of the polymorphisms in the IRF-1 genes was performed using the ASTDT routine in the Genome Analysis System software (GAS ver 2.0: A. Young, University of Oxford, 1993–95). Linkage disequilibrium statistics (D) and statistical significance of linkage disequilibrium were computed using the ASSOCIAT software program [31]. Standardized disequilibrium statistics (D') were calculated as described [32]. An association between total serum IgE levels and genotypes was analysed by ANOVA.

Results

We have found three novel variants (–300G>T, 4396 A>G and 6355G>A) in the IRF-1 gene. The –300G/T and 4396 A/G were polymorphic. The 6355G>A variant was very rare, appeared only one in 168 unrelated samples genotyped. The –300G/T polymorphism was in nearly complete linkage disequilibrium with the 4396 A/G polymorphism ($n = 162$, $D = 0.22$, $D' = 0.98$, $P < 0.0001$).

We examined an association between the IRF-1 gene polymorphism and atopy/asthma by genotyping the 4396 A/G polymorphism because the –300G/T was in nearly complete linkage disequilibrium with the 4396 A/G and the 6355G/A was very rare in our population.

The number of the 4396 A/4396 A, 4396 A/4396G and 4396G/4396G genotypes in the parents were 71 (44%), 75 (46%) and 16 (10%), respectively, which did not deviate from expected values based on Hardy–Weinberg equilibrium ($P = 0.55$, chi-square = 0.35, d.f. = 1). Mean total serum IgE levels of parents with the 4396 A/4396 A, 4396 A/4396G and 4396G/4396G were 311 ± 64 , 252 ± 49 and 299 ± 98 IU/mL, respectively, and no significant differences were observed between them (ANOVA, $P > 0.1$).

Under a hypothesis of no linkage disequilibrium between the 4396 A or 4396G allele and atopy/asthma, the expected numbers of transmissions of 4396 A and 4396G alleles

Table 2. Transmission disequilibrium test for asthma and atopy

Phenotype	Allele	Transmitted	Not transmitted	P-value
Atopy	4396A	62	72	0.87
	4396G	76	62	0.13
Asthma	4396A	52	65	0.87
	4396G	65	52	0.13

are equal. Using TDT analysis, either of 4396 A or 4396G alleles was not significantly preferentially transmitted to atopy- ($P = 0.13$) or asthma- ($P = 0.13$) affected children (Table 2).

Discussion

The present study detected two novel polymorphisms and one rare variant in the IRF-1 gene. These variants are not likely to alter the structure of IRF-1. The two polymorphisms are in nearly complete linkage disequilibrium. We did not observe significant correlation between the 4396 A/G polymorphism and total serum IgE levels or atopy/asthma. Therefore, we concluded that the polymorphisms detected in this study are not likely to be functional for asthma-related phenotypes.

We must note the limitations of this study. First, the detection power of an association (linkage disequilibrium) between the 4396 A/G polymorphism and atopy/asthma by TDT using 81 families is not high. The detection power drops to nearly 0.5, if the risk for atopy/asthma by carrying a putative risk allele is 1.5 times greater than that by not carrying the allele. Therefore, this study did not exclude minor contributions of the polymorphisms detected in this study for atopy/asthma. Second, the power to detect a mutation by SSCP is not perfect [33]. The possibility remains that undetected mutations in the IRF-1 gene might be related to the cause of asthma and atopy. Third, we screened only Japanese subjects. It remains possible that nuclear variants of the IRF-1 gene play a substantial role in the development of atopy and asthma in other ethnic population.

Marsh *et al.* [3] first reported significant linkage of total serum IgE antibody concentrations to IL4 or a nearby gene in 5q31.1 in 11 Caucasian Amish families. Subsequently linkage has been suggested in some populations [4,27] but not in other populations [18,34]. Therefore, mutations in the IRF-1 gene contributing atopy/asthma are more likely to exist in the populations in whom linkage between the IL-4 gene cluster and atopy/asthma is suggested. We have shown the evidence for linkage

between the markers flanking IL-4 gene cluster and asthma/atopy in the Japanese families [4] and suggested that IL-4 promoter -590C/T polymorphism may be associated with the development of asthma in Japanese children [35]. However, we did not detect possibly functional variants in the IRF-1 gene, which is located approximately 300 kb telomeric to IL-4. There was no significant linkage disequilibrium between the -590C/T polymorphism in the IL-4 gene and the 4396 A/G polymorphism in the IRF-1 gene in our population ($n = 179$, $D' = -0.05$, $P = 0.64$). Therefore, an association of atopy/asthma was suggested for the IL4 gene but not for the IRF1 gene in our Japanese families. Further studies in different populations are needed to elucidate the role of IRF-1 in the development of asthma and atopy.

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References

- Sibbald B, Horn ME, Gregg I. A family study of the genetic basis of asthma and wheezy bronchitis. *Arch Dis Child* 1980; 55:354-7.
- Duffy DL, Martin NG, Battistutta D, Hopper JL, Mathews JD. Genetics of asthma and hay fever in Australian twins. *Am Rev Respir Dis* 1990; 142:1351-8.
- Marsh DG, Neely JD, Breazeale DR et al. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 1994; 264: 1152-6.
- Noguchi E, Shibasaki M, Arinami T et al. Evidence for linkage between asthma/atopy in childhood and chromosome 5q31-q33 in a Japanese population. *Am J Respir Crit Care Med* 1997; 156:1390-3.
- Moffatt MF, Cookson WO. Tumour necrosis factor haplotypes and asthma. *Hum Mol Genet* 1997; 6:551-4.
- Noguchi E, Shibasaki M, Arinami T et al. Evidence for linkage between the development of asthma in childhood and the T-cell receptor beta chain gene in Japanese. *Genomics* 1998; 47: 121-4.
- Cookson WO, Sharp PA, Faux JA, Hopkin JM. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. *Lancet* 1989; 1:1292-5.
- Cookson WO, Young RP, Sandford AJ et al. Maternal inheritance of atopic IgE responsiveness on chromosome 11q. *Lancet* 1992; 340:381-4.
- Shirakawa T, Li A, Dubowitz M et al. Association between atopy and variants of the beta subunit of the high-affinity immunoglobulin E receptor. *Nat Genet* 1994; 7:125-9.
- Hill MR, James AL, Faux JA et al. Fc epsilon RI-beta polymorphism and risk of atopy in a general population sample. *BMJ* 1995; 311:776-9.
- Van Herwerden L, Harrap SB, Wong ZY et al. Linkage of high-affinity IgE receptor gene with bronchial hyper-reactivity, even in absence of atopy. *Lancet* 1995; 346: 1262-5.
- Barnes KC, Neely JD, Duffy DL et al. Linkage of asthma and total serum IgE concentration to markers on chromosome 12q: evidence from Afro-Caribbean and Caucasian populations. *Genomics* 1996; 37:41-50.
- Nickel R, Wahn U, Hizawa N et al. Evidence for linkage of chromosome 12q15-q24.1 markers to high total serum IgE concentrations in children of the German Multicenter Allergy Study. *Genomics* 1997; 46:159-62.
- Moffatt MF, Hill MR, Cornelis F et al. Genetic linkage of T-cell receptor alpha/delta complex to specific IgE responses. *Lancet* 1994; 343:1597-600.
- Moffatt MF, Schou C, Faux JA, Cookson WO. Germline TCR-A restriction of immunoglobulin E responses to allergen. *Immunogenet* 1997; 46:226-30.
- Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA. The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. *N Engl J Med* 1997; 337:1720-5.
- Mitsuyasu H, Izuhara K, Mao XQ et al. Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. *Nat Genet* 1998; 19:119-20.
- Daniels SE, Bhattacharya S, James A et al. A genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996; 383:247-50.
- The collaborative study of genetics of asthma. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. *Nat Genet* 1997; 15:389-92.
- Tang ML, Coleman J, Kemp AS. Interleukin-4 and interferon-gamma production in atopic and non-atopic children with asthma. *Clin Exp Allergy* 1995; 25:515-21.
- Ying S, Durham SR, Corrigan CJ, Hamid Q, Kay AB. Phenotype of cells expressing mRNA for TH2-type (interleukin 4 and interleukin 5) and TH1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am J Respir Cell Mol Biol* 1995; 12:477-87.
- Miyamoto M, Fujita T, Kimura Y et al. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell* 1988; 54: 903-13.
- Kamijo R, Harada H, Matsuyama T et al. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 1994; 263:1612-5.
- Martin E, Nathan C, Xie QW. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J Exp Med* 1994; 180:977-84.
- Barnes PJ, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995; 16:128-30.
- McElligott DL, Phillips JA, Stillman CA, Koch RJ, Mosier DE, Hobbs MV. CD4+ T cells from IRF-1-deficient mice exhibit

- altered patterns of cytokine expression and cell subset homeostasis. *J Immunol* 1997; 159:4180–6.
- 27 Mansur AH, Bishop DT, Markham AF, Britton J, Morrison JF. Association study of asthma and atopy traits and chromosome 5q cytokine cluster markers. *Clin Exp Allergy* 1998; 28:141–50.
- 28 National Heart Lung and Blood Institute, National Institutes of Health. Guidelines for the diagnosis, management of asthma. Washington DC: Government Printing Office, 1995.
- 29 Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Med Associates *N Engl J Med* 1995; 332:133–8.
- 30 Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. *Mol Cell Biol* 1994; 14:1500–9.
- 31 Terwilliger JD, Ott J. Handbook of Human Genetic Linkage The Johns Hopkins University Press, 1994.
- 32 Lewontin RC. On measures of genetic disequilibrium. *Genetics* 1988; 120:849–52.
- 33 Cotton RG. Current methods of mutation detection. *Mut Res* 1993; 285:125–44.
- 34 Laitinen T, Kauppi P, Ignatius J et al. Genetic control of serum IgE levels and asthma: linkage and linkage disequilibrium studies in an isolated population. *Hum Mol Genet* 1997; 6:2069–76.
- 35 Noguchi E, Shibasaki M, Arinami T et al. Association of asthma and the interleukin 4 promoter gene in Japanese. *Clin Exp Allergy* 1998; 28:449–53.