Association of IFN-γ and IFN regulatory factor 1 polymorphisms with childhood atopic asthma

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Background: IFN-γ and related molecules play important roles in the differentiation and function of T_H2 cells.
Objective: We sought to determine whether *IFNG* and related genes contribute to any susceptibility to atopic asthma, a representative T_H2-dominant disorder.
Methods: We investigated the association of *IFNG* (CA repeat polymorphism within the first intron), *IRF1* (GT repeat polymorphism within the intron 7), *IFNGR1* (Val 14 Met), and *IFNGR2* (Gln 64 Arg) gene polymorphisms with atopic asthma in the Japanese child population.
Results: A significant association (*P* = .0018) was observed between *IFNG* gene polymorphism and atopic asthma. The tendency was more prominent in patients with age of onset of 3 years or younger (*P* = .0004) or patients with a family history of allergic diseases (*P* = .0038). Furthermore, there was a significant association between *IRF1* gene whole-allele distribution and atopic asthma (*P* = .044). The tendency was more prominent in patients with onset at 3 years of age or less (*P* = .0058). On the other hand, *IFNGR1* and *IFNGR2* gene polymorphisms showed no association with atopic asthma.
Conclusion: These results suggested that among *IFNG* and related genes, *IFNG* and *IRF1* genes confer genetic susceptibility to atopic asthma in Japanese children. (J Allergy Clin Immunol 2001;107:499-504.)

Key words: *IFN-γ, IFN regulatory factor 1, IFN-γ receptor, atopic asthma, polymorphism*

Atopic asthma occurs in genetically susceptible individuals in the presence of environmental factors. Allergic responses involving IgE-dependent mast cell degranulation and eosinophil accumulation in the sites of inflammation are considered to be due to the development and activation of T_H2 cells.[1,2] IL-4 is a representative cytokine secreted from T_H2 cells and induces class switching to IgE synthesis,[3] whereas IFN-γ, a major cytokine from T_H1 cells, directly suppresses IgE synthesis and inhibits the development toward T_H2 cells.[4-6] IFN-γ exerts its various biologic effects through its binding to the cell-surface ligand-binding chain IFN-γ receptor 1 in combination with its signaling chain, IFN-γ receptor 2.[7,8] Receptor binding of IFN-γ activates intracellular signaling pathway, which induces expression of various genes, including the *IRF1* gene.[9] IFN regulatory factor 1 (IRF-1) is a transcription factor that also functions as a determinant for T_H1 response.[10-12]

Abbreviations used
IRF-1: IFN regulatory factor 1
SNP: Single nucleotide polymorphism

In subjects with atopic disease, the reduction of IFN-γ secretion was demonstrated.[13,14] Infants whose cord blood cells had shown diminished IFN-γ production in vitro were susceptible to the subsequent development of atopy.[15] In addition, chromosome loci of the *IFNG*, *IFNGR1*, *IFNGR2*, and *IRF1* genes were linked to atopy and asthma in genome-wide searches (12q24.1 for *IFNG*, 6q23-24 for *IFNGR1*, 21q22 for *IFNGR2*, and 5q31.1 for *IRF1*).[8,16-19] Furthermore, a positive association was observed between *IFNG* gene polymorphism and T_H1-dominant autoimmune disorders[20,21] and between *IFNGR1* and *IFNGR2* gene polymorphisms and a disorder in T_H1/T_H2 balance, systemic lupus erythematosus.[22,23] Thus *IFNG* and its associated genes may be responsible for an inherited predisposition to atopic asthma. Therefore we analyzed the polymorphisms of the 4 genes, a CA repeat polymorphism within the first intron of the *IFNG* gene, a GT repeat polymorphism within intron 7 of the *IRF1* gene, and single nucleotide polymorphisms (SNPs) in codon 14 Val/Met in the *IFNGR1* gene and codon 64 Gln/Arg in the *IFNGR2* gene, in patients with atopic asthma and control subjects in the Japanese population to determine a possible association between any of the 4 genes and atopic asthma.

METHODS

The study population consisted of 158 patients with atopic asthma and 218 normal children. For the association studies of *IFNGR1* and *IFNGR2* genes, 118 patients and 118 control subjects were enrolled. Atopic asthma was defined as having both bronchial asth-

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ma and one of the following findings: serum IgE concentrations above normal range and a positive RAST result (score >2) in response to one or more inhalant allergens. The mean ± SD age at the onset of atopic asthma was 2.9 ± 3.3 years (range, 0.5–13 years). Informed consent was obtained from patients, their parents, or both. Genomic DNA was extracted from peripheral blood by using the extraction kit QIAamp Blood Kit (QIAGEN, Tokyo, Japan).

We amplified the region containing a CA repeat polymorphism within the first intron of the *IFNG* gene by using the PCR method with a primer pair as follows: forward, 5′- GCTGTCATAATAATATTCAGAC-3′; and reverse, 5′- CGAGCTTTAAAAGATAGTTCC-3′. The 5′ end of the forward primer was fluorescence labeled with 6-carboxyfluorescein dye. PCR was used in a volume of 25 μL containing 20 ng of genomic DNA, 10 pmol of each primer, 0.625 U of Taq polymerase (Promega, Madison, Wis), and 0.2 mmol/L of each deoxynucleotide triphosphate. The PCR profile was as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. Genotyping was performed in a mixture of amplified products and an internal size standard by ABI PRISM 310 genetic analyzer (Perkin-Elmer, Foster City, Calif).

The GT repeat polymorphism of intron 7 at the *IRF1* gene was determined by using PCR with the following primer pair: forward, 5′-GGTTTGAGAGGCTGAGTCAC-3′ and reverse, 5′- TCACTGAGAAACGGTCACTTC-3′. Genotyping was done with ABI PRISM 310 genetic analyzer, as described above. The 5′ end of the forward primer was fluorescence labeled with hexachloro-6-carboxyfluorescein dye. PCR was used with the same PCR condition as for the first intron of the *IFNG* gene.

The genotypes for *IFNGR1* Val14Met were defined by using PCR with oligonucleotide primers as follows: forward, 5′-CGGCTTCCCGGACTTGA-3′; and reverse, 5′-CCATCTCAGCCCTGCTCA-3′. The primers were designed on the basis of the sequence data obtained from GenBank U19241. PCR was carried out on Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) in a total volume of 25 μL containing 20 ng of genomic DNA. The PCR profile was as follows: initial denaturation at 94°C for 7 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final 7-minute extension at 72°C. Forty PCR products were purified and analyzed by means of direct sequencing with the ABI PRISM 310 genetic analyzer to verify the Val/Met substitution at codon 14 of *IFNGR1*. Multiple-sample screening was performed with a single-stranded conformation polymorphism. Briefly, 3 μL of PCR products were mixed with 3 μL of deionized formamide, denatured for 5 minutes at 95°C, and loaded onto 12.5% polyacrylamide gel. Gel electrophoresis was carried out at 25 mA for 1.5 hours, keeping a constant temperature of 20°C. Subsequent silver staining revealed variable mobilities of conformational fragments, which corresponded to the Val/Met substitution as defined by the direct sequencing.

The genotypes for *IFNGR2* Gln64Arg polymorphism were determined by using PCR with oligonucleotide primers as follows: forward, 5′-CCCCGCCAGACCCTCTTTCC-3′; and reverse, 5′-ACTGTCGGTGTATTTAAACTGCACT-3′. The PCR condition consisted of an initial denaturation at 94°C for 5 minutes and 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, with a final 5-minute extension at 72°C. The primers were designed on the basis of the sequence data obtained from GenBank NM 005534. The reaction condition was as follows: 2 mmol/L magnesium chloride, 1× PCR buffer, 0.2 mmol/L of each deoxynucleotide triphosphate, 1.25 U of Taq DNA polymerase (Promega), and 12.5 pmol of each primer in a volume of 50 μL containing 20 ng of genomic DNA. Multiple-sample screening was performed with single-stranded conformation polymorphism according to the same procedure as described above, except for the current, time, and temperature for gel electrophoresis, which were 10 mA, 3.5 hours, and 5°C, respectively.

A whole-allele distribution was analyzed by using the χ^2 test with 2×7 or 2×8 contingency table, and differences between allele or genotype frequencies of 2 groups were evaluated by using χ^2 analysis with 2×2 contingency tables. When at least one cell number was not more than 5, the 2-sided Fisher exact test was applied to the χ^2 value. A P value of less than .05 was considered to be statistically significant.

RESULTS

Association study for *IFNG* gene CA repeat polymorphism and atopic asthma

As shown in Table I, there were 7 types of dinucleotide CA repeat polymorphisms, with the repeat number varying from 12 to 18 in the Japanese population. Comparison of the allele frequencies between patients with atopic asthma and control subjects showed a significant difference in the whole-allele distribution (P = .0018). When the frequency of each allele was compared between patients and control subjects, allele 5 was significantly less frequent in patients with atopic asthma (1.3% vs 4.8%, P = .0069), whereas allele 7 showed a higher frequency in patients with atopic asthma (2.8% vs 0.7%, P = .034). In addition, the patient subgroup with onset of atopic asthma at less than 3 years of age or with a positive family history showed a stronger association (whole distribution, P = .0004; allele 5, P = .0076 in a subgroup with onset at <3 years; allele 7, P = .0074 and .0037 in subgroups with onset at <3 years and with a positive family history, respectively; Table I). There were no significant associations between this polymorphism and patient subgroups classified by severity of asthma or serum IgE concentration (data not shown).

Analysis of *IRF1* gene polymorphisms in patients with atopic asthma

Because IRF-1 is an IFN-γ/IFN-αβ–inducible transcription factor that regulates T_H1-type responses,[12] we analyzed *IRF1* gene polymorphism in atopic asthma. There were 8 types of *IRF1* GT repeat polymorphisms, with the repeat number from 11 to 19 in the Japanese population. Table II summarizes the frequencies of *IRF1* gene polymorphism in patients and control subjects. Comparison of the allele frequencies between patients with atopic asthma and control subjects showed a significant difference in the whole-allele distribution (P = .044). In addition, the patients' subgroup with onset of atopic asthma at less than 3 years of age showed a stronger association (P = .0058, Table II). When the frequency of each allele was compared between patients with onset at less than 3 years of age and control subjects, alleles 1 and 4 revealed higher frequencies, and allele 6 showed a lower frequency with marginal significance (allele 1: 33.1% vs 26.6%, P = .075; allele 4: 1.1% vs 0%, P = .081; and allele 6: 13.5% vs 20.2%, P = .082). This polymorphism showed no association with patient subgroups classified by severity of asthma and serum IgE concentration (data not shown).

TABLE I. Allele frequencies of IFNG gene CA repeat polymorphism in patients with atopic asthma and control subjects

					Atopic asthma subgroups			
IFNG polymorphism	CA repeat No.	Control subjects, n = 218 (436 alleles) (%)	Atopic asthma, n = 158 (316 alleles) (%)	*P* value	Onset <3 y, n = 87 (174 alleles) (%)	*P* value	Presence of family history, n = 92 (184 alleles) (%)	*P* value
Allele 1	12 repeat	40 (9.2)	39 (12.3)	NS	21 (12.1)	NS	21 (11.4)	NS
Allele 2	13 repeat	251 (57.6)	175 (55.4)	NS	92 (52.9)	NS	100 (54.3)	NS
Allele 3	14 repeat	5 (1.1)	9 (2.9)	NS	6 (3.4)	NS	4 (2.2)	NS
Allele 4	15 repeat	116 (26.6)	78 (24.7)	NS	46 (26.4)	NS	46 (25.0)	NS
Allele 5	16 repeat	21 (4.8)	4 (1.3)	.0069	1 (0.6)	.0076	3 (1.6)	NS
Allele 6	17 repeat	0 (0.0)	2 (0.6)	NS	1 (0.6)	NS	2 (1.0)	NS
Allele 7	18 repeat	3 (0.7)	9 (2.8)	.034	7 (4.0)	.0074	8 (4.3)	.0037
Whole distribution				.0018*		.0004*		.0038*

*Whole distributions of alleles between the patient subgroups and control subjects were evaluated by using the 2-sided Fisher exact test for a 2×7 table.
NS, Not significant.

TABLE II. Allele frequencies of the *IRF1* gene GT repeat polymorphism in patients with atopic asthma and control subjects

					Atopic asthma subgroups			
IRF1 polymorphism	GT repeat No.	Control subjects, n = 218 (436 alleles) (%)	Atopic asthma, n = 158 (316 alleles) (%)	*P* value	Onset <3 y, n = 87 (174 alleles) (%)	*P* value	Presence of family history, n = 92 (184 alleles) (%)	*P* value
Allele 1	11 repeat	116 (26.6)	102 (32.3)	.10	59 (33.1)	.075	50 (27.2)	.92
Allele 2	12 repeat	174 (39.9)	110 (34.8)	.17	65 (36.5)	.58	67 (36.4)	.47
Allele 3	13 repeat	6 (1.4)	2 (0.6)	.47	0 (0.0)	.19	1 (0.5)	.68
Allele 4	14 repeat	0 (0.0)	2 (0.6)	.17	2 (1.1)	.081	2 (1.1)	.088
Allele 5	15 repeat	22 (5.0)	8 (2.5)	.091	4 (2.2)	.18	6 (3.3)	.40
Allele 6	16 repeat	88 (20.2)	59 (18.7)	.64	24 (13.5)	.082	40 (21.7)	.67
Allele 7	17 repeat	30 (6.9)	32 (10.1)	.14	19 (10.7)	.10	18 (9.8)	.25
Allele 8	19 repeat	0 (0.0)	1 (0.3)	.42	1 (0.6)	.29	0 (0.0)	
Whole distribution				.044*		.0058*		.27*

*Whole distributions of alleles between the patient subgroups and control subjects were evaluated by using the 2-sided Fisher exact test for the 2×8 table.

Analysis of *IFNGR1* and *IFNGR2* gene polymorphisms in patients with atopic asthma

Because patients with systemic lupus erythematosus, a disorder in T_H1/T_H2 balance, showed a positive association with a polymorphism of the *IFNGR1* gene but not of the *IFNGR2* gene,[22] we analyzed polymorphisms of *IFNGR1* and *IFNGR2* genes. As shown in Table III, there were no significant differences in the genotype and allele frequencies of *IFNGR1* Val14Met or *IFNGR2* Glu64Arg polymorphisms between patients and control subjects. These polymorphisms also showed no association with patient subgroups classified by severity of asthma, serum IgE concentration, age at onset, and positive family history (data not shown).

Various combinations of the 4 polymorphisms and atopic asthma

To evaluate the synergistic effects of the polymorphisms of the 4 genes, we performed association studies between various combinations of the 4 polymorphisms and atopic asthma. Representative results were as follows: negative *IFNG* allele 5/positive *IRF1* allele 1, control subjects n = 89 (46.3%) versus patients n = 84 (54.5%), $P = .38$; negative *IFNG* allele 5/negative *IRF1* allele 5: control subjects n = 178 (92.7%) versus patients n = 145 (94.2%), $P = .92$; negative *IFNG* allele 5/positive *IFNGR1* Met 14: control subjects n = 4 (3.4%) versus patients n = 8 (6.8%), $P = .25$; negative *IFNG* allele 5/negative *IFNGR2* Gln 64: control subjects n = 29 (24.6%) versus patients n = 26 (22.0%), $P = .64$. Similarly, other combinations did not demonstrate any synergistic effects.

DISCUSSION

Linkage and association studies identified a number of putative atopy genes. Among them, T_H1/T_H2 cytokine and receptor genes, including *IL4* promoter, *IL4R*, and *IL13* genes have been shown to be associated with atopy or serum IgE levels.[24-26]

In the present study we have shown a significant correlation between the CA repeat polymorphism of the *IFNG* gene and atopic asthma. The significance was more prominent in patients with younger age at onset or with a family history of atopic asthma. However, there were no significant associations between this polymorphism and patient subgroups classified by severity of

TABLE III. Distributions of *IFNGR1* and *IFNGR2* genotypes in patients with atopic asthma and control subjects

	Control subjects, n = 118 (%)	Patients, n = 118 (%)	*P* value
IFNGR1			
Genotype frequency			
Val 14/Val 14	113 (95.8)	110 (93.2)	
Met 14/Val 14	5 (4.2)	8 (6.8)	.39*
Met 14/Met 14	0 (0)	0 (0)	
Allele frequency			
Val	231 (97.9)	228 (96.6)	
Met	5 (2.1)	8 (3.4)	.40
IFNGR2			
Genotype frequency			
Arg 64/Arg 64	31 (26.3)	28 (23.7)	
Arg 64/Gln 64	53 (44.9)	65 (55.1)	.65*
Gln 64/Gln 64	34 (28.8)	25 (21.2)	
Allele frequency			
Arg	115 (48.7)	121 (51.3)	
Gln	121 (51.3)	115 (48.7)	.58
Total	118	118	

*Analysis performed among the 3 genotypes.

asthma or serum IgE concentration. Possible explanations for the negative results might include that severity of asthma depends, at least in part, on the strategies for management of asthma[27]; that atopic asthma in infancy is not necessarily associated with high levels of serum IgE; and that environmental factors and managements alter IgE levels.[28] The association between this polymorphism of the *IFNG* gene and T_H1-dominant disorders, such as type 1 diabetes[20] and Graves' disease[21] or lung allograft fibrosis,[29] was previously reported. The frequency of allele 5 was significantly increased in patients with T_H1-dominant disorders, whereas it was decreased in atopic asthma, a T_H2-dominant disease, in our study, suggesting that the *IFNG* gene with allele 5 is associated with an increased production of IFN-γ. A recent report has shown that transcription of the *IFNG* gene is regulated by several regions in the promoter and within the intronic sequences.[30] Pravica et al[31] studied the correlation between the CA repeat polymorphism and in vitro IFNγ production. They showed that allele 2 (which corresponds to allele 1, 12 CA repeats, in our study) of the *IFNG* gene was associated with higher IFN-γ production. However, IFN-γ production in other individual alleles has not been studied independently either in vivo or in vitro. In addition, the allele distribution (5 alleles) in white subjects was different from that (7 alleles) in Japanese subjects. Thus it is possible that the polymorphism affects the regulation of *IFNG* gene expression, although whether it has a direct effect on IFN-γ production remains to be determined. Besides the CA repeat microsatellite in the first intron, 5 SNPs in the first and third introns and 3′ UTR have been recently reported in the human *IFNG* gene.[32] Because none of the polymorphisms locate within exons or the promoter region of the *IFNG* gene,[33] it was suggested that CA repeat polymorphism does not associate with functional ones in the coding or promoter region of the *IFNG* gene but affects the expression of the *IFNG* gene by itself. To obtain a definitive conclusion, it would be necessary to perform functional and association studies of these polymorphisms, as well as to evaluate the linkage disequilibrium between the CA repeat polymorphism and other SNPs in the *IFNG* gene.

Furthermore, a significant association was also observed between whole-allele distribution of the *IRF1* gene and atopic asthma, especially in patients with onset at less than 3 years of age, as shown in Table II. Although several alleles of the *IRF1* gene showed marginal associations with atopic asthma, the *IRF1* gene allele distribution appeared to contribute to its significant association as a whole rather than through a single rare allele with a higher significance because deletion of such an allele did not greatly affect the significance (data not shown). IRF-1 has been found to affect T_H1/T_H2 cytokine balance in IRF-1 knockout experiments. Deficiency of the *IRF1* gene in mice leads to strongly impaired T_H1 responses and enhanced T_H2 responses, with an increase in the antigen-specific IgE response.[12] Hence it is possible that the *IRF1* gene polymorphism might alter T_H1/T_H2 cytokine production and contribute to an inherited predisposition to childhood atopic asthma in the Japanese population. To our knowledge, only one association study showed no significant association between the *IRF1* gene polymorphism and a T_H1-dominant disorder, type 1 diabetes.[34] Further study is needed to determine whether the polymorphism of the *IRF1* gene directly affects gene transcription or is linked to another functional polymorphism.

With respect to *IFNG* receptor genes, our data were consistent with the previous results that a polymorphism in the *IFNGR1* gene was not associated with atopic asthma,[35] partly because of the rare frequencies of the polymorphisms.

Combinations of the genotypes of the *IFNG*, *IRF1*, *IFNGR1*, or *IFNGR2* genes failed to show any synergistic effects on the genetic susceptibility to atopic asthma, at least partly because combinations of the genotypes

decreased the numbers of samples for comparison. In recent articles *IL4* promoter and *IL4R* genes of the *IL4*-mediated pathway have been demonstrated to confer susceptibility to atopic asthma,[24,25] but no synergistic interaction of these genes was identified.[36] It is also important to determine an interaction between *IFNG*- and *IL4*-mediated pathways because Stat 6, activated by IL-4, competitively inhibits the binding of Stat 1 on the IFN-γ activation sequence element of the IRF-1 promoter, thereby reducing the transcription of the *IRF1* gene.[37] When the present data on *IFNG* and related genes were combined with our previous data on *IL4*-mediated pathway genes by using the same population of atopic asthma,[38] the combination of *IRF1* allele 1 or 6 and –590 T/C polymorphism of the *IL4* promoter gene showed a synergistic effect on the association with atopic asthma (positive *IRF1* allele 1/*IL4* TT homozygous, $P = .016$; negative *IRF1* allele 6/*IL4* TT homozygous, $P = .03$; unpublished data), despite the lack of association of these polymorphisms alone with the disease ($P > .05$).

Thus the *IFNG* and *IRF1* genes appear to contribute to an inherited predisposition to childhood atopic asthma in the Japanese population. It is likely that the polymorphisms in the *IFNG* and *IRF1* genes might influence T_H1/T_H2 cytokine production and that *IFNG*- and *IL4*-mediated pathway genes could exert synergistic effects on the susceptibility to atopic asthma in childhood. However, because a difference in genetic background greatly influences allele frequencies of a disease-associated gene, further studies on other polymorphisms of the genes or analyses in different races might provide us more information on the contribution of the *IFNG* pathway.

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