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Association of interferon- γ and interferon regulatory factor 1 polymorphisms with asthma in a family-based association study in Taiwan

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Summary

Background Asthma is a multi-factorial disorder caused by complex interactions between genetic and environmental factors. IFN- γ and IFN regulatory factor 1 (IRF-1) affect Th1/Th2 cytokine balance, and influence the differentiation of Th2 cells, which influence the development of asthma.

Objective This study investigated CA repeats polymorphism of the IFN- γ gene and GT repeats polymorphism of the IRF-1 gene, which may predispose individuals to asthma pathogenesis.

Methods In the present study, we used the transmission/disequilibrium test (TDT) to investigate the relationship between asthma and the IFN- γ and IRF-1 polymorphisms by studying 348 subjects composed of 232 parents and 116 asthmatic children.

Results For global TDT test, IFN- γ CA repeats and IRF-1 GT repeat polymorphisms showed a significant association with asthma in children ($P = 0.009$ and 0.017 , respectively). We demonstrated that 13 CA repeats (138 bp) of IFN- γ gene and 11 GT repeats (306 bp) of IRF-1 gene are significantly preferentially transmitted to asthmatic children (T/NT = 89/61, $\chi^2 = 8.43$, $P < 0.005$ and T/NT = 75/49, $\chi^2 = 8.18$, $P < 0.005$, respectively). The offspring will have an increased risk of asthma when their parents transmit IFN- γ 13 CA repeats (OR = 1.83, $P = 0.009$) and IRF1 11 GT repeats (OR = 1.88, $P = 0.007$) to them. But we observed that the IFN- γ and IRF-1 polymorphisms are not associated with IgE concentrations.

Conclusion These findings provide strong evidence of which IFN- γ CA repeat and IRF-1 GT repeat polymorphisms influence the risk of asthma for children in Taiwan.

Keywords asthma, IFN- γ , IFN regulatory factor 1, polymorphism

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Introduction

T helper type 1/ type 2 (Th1/Th2) cytokine imbalance, with excess Th2 products in concert with decreased Th1 products, has been shown to be associated with chronic inflammatory disease, such as asthma or atopy. IFN- γ is a major pleiotropic Th1 cytokine, which directly suppresses IgE synthesis and inhibits the development towards Th2 cells [1, 2]. IFN- γ and receptor activate the intracellular signal pathways, which induce expression of IFN regulatory factor-1 (IRF-1) gene [3, 4]. IRF-1 is a transcription factor and originally identified by its ability to induce the transcription of IFN and the IFN-induced genes [5].

Functional studies have shown increased levels of IL-4 and reduced levels of IFN- γ produced by atopic asthmatics compared with non-atopic asthmatics and controls [6, 7].

In a knockout mouse study, deficiency of IRF-1 gene leads to strongly impaired Th1 response and enhanced response of Th2-related cytokines, with an increase in the antigen-specific IgE response [8]. There have been several studies that have provided strong evidence of linkage of asthma and atopy to IFN- γ (12q24.1) and IRF-1 (5q31.1) [9–11]. The IFN- γ gene was identified on chromosome 12q24.1 [12] and consists of four exons and three introns [13]. The IRF-1 gene was mapped on chromosome 5q31.1 [14], which is approximately 300 kb telomeric to IL4 [9]. Several studies suggested a significant association for a CA repeat polymorphism of IFN- γ to asthma and allergic rhinitis [9, 15, 16], particularly in patients with an onset age of 3 years or younger [15]. However, some studies failed to detect any linkage and association between asthma and IFN- γ intron one CA repeat [17]. The IRF-1

gene has previously been reported to be linked to the total serum IgE [9]. A case-control study has shown a borderline allelic association between IRF-1 gene and bronchial hyper-reactivity [11], but not in asthmatic children [15].

The two potential functional polymorphisms including CA tandem repeat in the first intron of IFN- γ gene and the GT tandem repeat within intron 7 of IRF-1 gene have been tested for possible effect on asthma and related phenotypes. This genetic basis study on asthmatic families was conducted to address inherited pre-disposition for IFN- γ and IRF-1 genes in Taiwan, and to eliminate the possibility of a false-positive association from hidden population stratification.

Methods and materials

Study population

Small nuclear families consisting of both parents and their asthmatic children were recruited from the Department of Pediatrics in two medical centres: the Memorial Hospital of Kaohsiung Medical University and Kaohsiung Veterans General Hospital in southern Taiwan. The subjects were 116 small nuclear families including 116 affected children and 232 parents. Asthma was defined by an interview of all subjects with our paediatricians according to the GINA guideline [18]. The International Study of Asthma and Allergic Disease in Children Chinese version questionnaire was used to assess respiratory symptoms and atopic diseases for all subjects who participated in this study [19]. The protocol was approved by the IRB of Kaohsiung Medical University. Informed written consent was obtained from the subjects participating in the study or from the parents of asthmatic children who were below 18 years.

Clinical and phenotype measurements

Questionnaires for children were completed by the parents. We measured serum total IgE levels in all participants using the Microparticle Enzyme Immunoassay (MEIA; IMX System Abbot, Tokyo, Japan). The definition of high responsiveness of total IgE was greater than 100 IU/mL. Specific IgE was detected on asthmatic patients by the MAST system (Multiple Allergens Simultaneous Test, Immunosystems, Allentown, PA, USA) or the Cap system (Pharmacia, Uppsala, Sweden). Positive responses for house dust (HD) and mites were defined as a level larger than grade 2 for MAST tests, or when titre was higher than 0.35 kUA/L for CAP tests of specific IgE against HD and *Dermatophagoides pteronyssinus* (Der p) or *Dermatophagoides farinae* (Der f). The Phadiatop test was analysed by the immunoassay system Pharmacia UniCAP (Pharmacia), an *in vitro* test detecting specific IgE production in response to a mixture of common inhalant allergens and expressed as a positive or negative response with respect to a reference sample.

Clinical and laboratory data were also collected from medical charts, which included total IgE levels, specific IgE and lung function.

Genotyping

Genomic DNA was extracted using a commercial DNA purification kit from peripheral blood (Amersham Biosciences, Piscataway, NY, USA) for all individuals. PCR was used to amplify the region containing a CA repeat polymorphism within the first intron of the IFN- γ gene. The forward primer was 5'-GCTGTCATAATAATATTCAGAC-3', and that reverse primer was 5'-CGAGCTTTAAAGA-TAGTTCC-3' [15]. The 5' end of the forward primer was fluorescence labelled with 6-carboxyfluorescein dye. We amplified in a volume of 15 μ L containing 50 ng of genomic DNA, 1 μ L of each primer (5 μ M), 9.0 μ L premix. The PCR conditions in this study were 95°C for 12 min, followed by 10 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s, and 20 cycles of 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s. The length of PCR products was from 136 to 148 bp, containing 12–18 CA repeats (seven types alleles) of IFN- γ . We also genotyped the GT repeat polymorphism of intron 7 of the IRF-1 gene. The forward primer was 5'-GGTTTGAGAGGC TGAGTCAC-3' and the reverse primer was 5'-TCACTGAG AAACGGTCACTTC-3' [15]. The PCR condition was similar to the IFN- γ gene. The length of the PCR products was from 306 to 322 bp, which contained six types of GT repeat alleles of IRF. Molecular typing at IFN- γ CA and IRF-1 GT microsatellite polymorphisms were performed by PCR, followed by use of a sequencer. The fragments were separated by electrophoresis (ABI Prism 310 Genetic Analyzer; Applied Biosystems, Perkin-Elmer, Foster City, CA, USA), and size analysis was performed with GeneScan and Genotyper software, and GeneScan 400 Rox as the size standard (Applied Biosystems). Fifty-two (15%) subjects of our samples were randomly selected to verify genotype by re-genotyping. All the re-genotyping results were consistent with the first experiment.

Statistical analysis

Family-based associations with asthma were evaluated by the transmission disequilibrium test (TDT) [20]. TDT can test whether an allele of a marker closely linked to genes that contribute to disease susceptibility are transmitted more frequently from heterozygous parents to the affected children. We have applied PedCheck [21] and MERLIN [22] software to check for any inconsistent Mendelian inheritance, non-paternity or other genotyping errors. Any inconsistency was zeroed out to avoid bias. We excluded two individuals from analysis because of genotyping errors found in the samples. Under the null hypothesis (that there is no linkage), each allele is transmitted with equal frequency to affected children. The TDTPHASED

Table 1. Descriptive characteristics of asthma and allergies in asthmatic families

	Asthmatic offspring	Parents
<i>N</i>	116	232
Sex (M/F)	77/39	116/116
Age $\pm$ SD	9.4 $\pm$ 3.6	39.2 $\pm$ 6.3
Asthma		
Asthma diagnosed by physicians (%)	100	14.2
The age at first time diagnosis $\pm$ SD	5.3 $\pm$ 2.8	16.7 $\pm$ 13.6
Nocturnal waking with wheezing (%)	37.8	3.6
Wheezing and shortness of breath after exercise (%)	31.5	3.2
≥ 1 asthma attack, in the past 12 months (%)	57.1	7.8
Allergy		
Log IgE $\pm$ SD	2.58 $\pm$ 0.57	1.93 $\pm$ 0.67
IgE ≥ 100 U/mL (%)	86.1	42
Phadiatop test (%)	85.1	–
House dust (%)	37.1	4.8
Der p (or Der f) (%)	78.2	23.4
Cockroach (%)	17.1	3.6
FEV ₁ < 80% predicted (%)	28.4	–

Repeat	bp		
IFN- γ CA allele (%)			
12	136	29 (13.1%)	67 (15.5%)
13	138	101 (45.5%)	165 (38.2%)
14	140	3 (1.4%)	6 (1.4%)
15	142	77 (34.7%)	171 (39.6%)
16	144	6 (2.7%)	9 (2.1%)
17	146	1 (0.5%)	2 (0.1%)
18	148	5 (2.3%)	13 (3.1%)
IRF-1 GT allele (%)			
11	306	78 (36.8%)	140 (33.2%)
12	308	56 (26.4%)	135 (32.1%)
15	314	14 (6.6%)	20 (4.8%)
16	316	22 (10.4%)	44 (10.5%)
17	318	41 (19.3%)	81 (19.3%)
19	322	1 (0.5%)	2 (0.1%)

Der p, *Dermatophagoides pteronyssinus*; Der f, *Dermatophagoides farinae*; IRF-1, IFN regulatory factor-1.

program of the UNPHASED software version 2.403 is a suite of programs for family-based association analysis from unphased genotype data [23]. Bonferroni corrections (multiplying the *P*-values by the number of alleles compared) were used to correct for multiple comparisons.

Results

Table 1 summarizes the phenotype characteristics of the 116 asthmatic families included in the analysis. Self-reported symptoms of asthma and allergies were described for asthmatic children and their parents. Asthmatic probands had higher total IgE levels, and higher positive rates

Table 2. IFN- γ CA repeat and IRF-1 GT repeat polymorphisms are associated with asthma, as determined by a transmission disequilibrium test

Repeat	allele	Transmitted	Non-transmitted	χ^2 -test	<i>P</i> -value
IFN- γ CA repeat					
12	136	26	32	0.73	0.393
13	138	89	61	8.43	0.004**
14	140	3	3	0.00	1.000
15	142	71	91	4.17	0.041*
16	144	5	3	0.52	0.472
17	146	0	1	1.39	0.239
18	148	5	8	0.72	0.396
Global <i>P</i> -value					0.009**
IRF-1 GT repeat					
11	306	75	49	8.18	0.004**
12	308	48	73	7.66	0.006**
15	314	14	7	2.52	0.113
16	316	19	24	0.66	0.417
17	318	31	34	0.17	0.682
19	322	1	1	0.00	1.000
Global <i>P</i> -value					0.017*

Repeat	Allele	Transmitted	Non-transmitted	OR	<i>P</i> -value
IFN- γ CA repeat					
Non-13	Non-138	110	138	1	
13	allele-138	89	61	1.83	0.009*
IRF-1 GT repeat					
Non-11	Non-306	113	139	1	
11	allele-306	75	49	1.88	0.007*

***P* < 0.01.

**P* < 0.05.

IRF-1, IFN regulatory factor-1.

of specific IgE against HD, Der p (or Der f) and cockroaches than those of their parents.

The family-based associations between asthma and repeat polymorphisms of IFN- γ and IRF-1 genes were detected using TDTPHASED program as described in Table 2. Under the hypothesis of no linkage disequilibrium, each allele is transmitted with equal frequency to affected children. A total of seven different alleles for IFN- γ CA and six different alleles for IRF-1 GT were observed in the Taiwanese asthmatic families. Allele 138 (13 CA repeats) of the IFN- γ gene and allele 306 (11 GT repeats) of the IRF-1 gene were most common and at frequencies of 44.7% and 39.9% in transmitted alleles, respectively, and 15 CA repeats and 12 GT repeats were most common and at frequencies of 45.7% and 38.8% in non-transmitted alleles. Our data demonstrated that 13 CA repeats (138 bp) of the IFN- γ gene and 11 GT repeats (305 bp) of the IRF-1 gene were highly significant preferentially transmitted to asthma (T/NT = 89/61, χ^2 = 8.43, *P* < 0.005 and T/NT = 75/49, χ^2 = 8.18, *P* < 0.005, respectively), and 12 GT repeats

Table 3. IFN- γ CA repeat and IRF-1 GT repeat polymorphisms were associated with asthma and related phenotypes, as determined by a transmission disequilibrium test

	Trios	IFN- γ CA repeat			IRF-1 GT repeat		
		Allele	T/NT	<i>P</i> -value	Allele	T/NT	<i>P</i> -value
All asthma	116	13 CA	89/61	0.004**	11 GT	75/49	0.004**
Asthma subgroup							
With wheezing and elevated IgE [†]	72	13 CA	68/49	0.01*	11 GT	56/40	0.02*
With specific IgE against common aeroallergens [‡]	92	13 CA	66/44	0.008**	11 GT	51/37	0.03*
With FEV1 < 80% predicted	31	13 CA	20/15	0.29	11 GT	12/10	0.62

[†]Elevated IgE ≥ 100 U/mL.[‡]Specific serum IgE against common aeroallergens (Phadiatop test).***P* < 0.01.**P* < 0.05.

IRF-1, IFN regulatory factor-1.

Table 4. Allele- and genotype-specific IgE concentrations in asthmatic offspring

Genes	Repeats	Allele	<i>n</i>	IgE mean $\pm$ SD	<i>P</i> -value ^a
IFN- γ	13 CA	138	76	769.6 $\pm$ 1381.8	0.69
	Non-13 CA	Non-138	31	663.0 $\pm$ 767.2	
IRF-1	11 GT	305	62	720.6 $\pm$ 969.6	0.23
	Non-11 GT	Non-305	40	520.4 $\pm$ 452.4	
Genes	Repeats	genotype	<i>n</i>	IgE mean $\pm$ SD	<i>P</i> -value ^b
IFN- γ	13 CA/13 CA	138/138	23	556.8 $\pm$ 483.1	0.57
	13 CA/non-13 CA	138/non-138	53	861.9 $\pm$ 1620.6	
	Non-13 CA/non-13 CA	Non-138/non-138	31	663.0 $\pm$ 767.2	
IRF-1	11 GT/11 GT	305/305	12	403.8 $\pm$ 276.1	0.15
	11 GT/non-11 GT	305/non-305	50	796.7 $\pm$ 1059.6	
	Non-11 GT/non-11 GT	Non-305/non-305	40	520.4 $\pm$ 452.4	

IgE concentrations were logarithmically transformed before statistical testing to meet the assumption of normal distribution; however, untransformed data are shown.

^a*P*-value was tested by *t*-test.^b*P*-value was tested by ANOVA.

IRF-1, IFN regulatory factor-1.

(307 bp) of the IRF-1 gene was transmitted less frequently than other alleles to affected offspring (T/NT = 48/73, $\chi^2 = 7.66$, *P* < 0.01). Our results represent a statistically significant finding on asthma after adjustment for Bonferroni multiple corrections by multiplying *P*-values by 7 for IFN- γ CA repeats and 6 for IRF-1 GT repeats. Offspring have an increased risk of asthma when the parents transmit IFN- γ 13 CA repeats (OR = 1.73, *P* = 0.009) and IRF1 11 GT repeats (OR = 1.82, *P* = 0.007) polymorphisms to their children.

There were 72 asthmatic trios with wheezing and elevated IgE and 92 asthmatic trios with specific serum IgE against common aeroallergens (Phadiatop test). Except for a doctor's diagnosis of asthma, we found that 13 CA repeats (138 bp) of the IFN- γ gene and 11 GT repeats (305 bp) of the IRF-1 gene were also significant preferentially transmitted to stricter definitions of asthma such as

asthma with wheezing and elevated IgE (T/NT = 68/49, $\chi^2 = 8.43$, *P* = 0.01 and T/NT = 56/40, *P* = 0.02, respectively), and asthma with specific serum IgE against common aeroallergens (Phadiatop test) (T/NT = 66/44, *P* = 0.008 and T/NT = 51/37, *P* = 0.03, respectively) (Table 3). To test whether IFN- γ and IRF-1 genes are related to intermediate phenotypes of asthma, quantitative trait analysis were performed in affected offspring for (log-transformed) serum total IgE levels. The IFN- γ 13 CA repeats and IRF-1 11 GT repeats had higher IgE levels than other alleles, but neither reached a statistically significant difference (Table 4).

Discussion

We observed significantly higher frequency transmission to asthmatic children for the 13 CA repeat polymorphism

of the IFN- γ gene and 11 GT repeat polymorphism of the IRF-1 gene from a family-based association study. In our study, the allele distribution of IFN- γ (seven alleles from 12 to 18 CA repeats) was similar to that in Japanese subjects [15], but was different from that in Indian populations (from 10 to 18 CA repeats) [16]. A significant association between IFN- γ CA repeat polymorphism and asthma was found in our study ($P=0.004$), and was also documented in younger Japanese patients [15], an Indian population [16] and an Afro-Caribbean population [9].

The polymorphisms in the IFN- γ and IRF-1 genes were likely to influence Th1/Th2 cytokine production. von Hertzen and Haahtela [24] suggested a vaccine that induces a strong Th1 immune response, and a long-living memory immunity early in life might prevent the differentiation of Th1 cells into Th2 cells. Several functional studies suggested that the variable-length CA repeat of IFN- γ gene has been shown to influence IFN- γ production. Pravica et al. [25] demonstrated that the presence of IFN 12 CA repeat has been associated with a high production of IFN- γ in a *vitro* study. The presence of the IFN 12 CA repeat correlated with increased IFN- γ release and has been associated with a number of conditions including transplant rejection [26]. Low levels of expression of IFN- γ were shown by asthmatic patients in a previous study [7]. In contrast to the IFN 12 CA repeat, the IFN 13 CA repeat may reduce the levels of IFN- γ [25]. In the present study, we found that IFN 13 CA repeat length was associated with a high risk of asthma. Therefore, functional studies can support our study results. In addition to asthma, the 13 CA repeat polymorphism of the IFN- γ gene was highly significantly associated with IDDM patients [27] and severe rheumatoid arthritis [28]. Several genetic polymorphism studies found that the IFN- γ 12 CA repeat polymorphism has been associated with several autoimmune and chronic inflammatory conditions, such as lung allograft fibrosis [29], kidney transplant rejection [26], duration of survival in pancreatic cancer [30], aplastic anaemia [31] and cervical carcinogenesis [32].

Our study demonstrated that the 11 GT repeats polymorphism of the IRF1 gene, which is also the most common allele, is significantly associated with asthma. Nakao et al. [15] also indicated that *IRF1* gene GT whole-allele distribution is associated with atopic asthma. IRF-1 is a transcription factor that regulates the IFN system, inhibits cell growth and has tumour-suppressor activity. Previous studies suggested that the GT repeat polymorphism of IRF1 locus at 5q31.1 is associated with asthma [15], but not for multiple sclerosis [33] and IDDM [34]. IRF1 can regulate type I IFN gene expression [5]. These two micro-satellites are in possible non-functional or linkage disequilibrium with other functional single-nucleotide polymorphism (SNPs) close to them, because they are in the intron and hence do not affect the coding of the protein. However, the intronic regions of IFN- γ gene play

a role in the regulation of transcription [35]. Different combinations of genotypes to individuals or races greatly influence the risk of asthma.

The advantages and limitations of our study should be discussed. First, TDT were used in this study with affected child-parents trios, which can be more powerful than linkage and sib-pairs study for locating a gene [36]. Second, TDT is better than a genetic case-control study; TDT controls for the confounding of genetic stratification in a racial admixture population [37]. Third, multiple comparisons for multiple alleles were adjusted by using Bonferroni corrections to reduce the false-positive results. We still observed statistically significant findings. There were some limitations in our study. One was that the other SNPs in IFN- γ and IRF-1 genes were not genotyped in this study. There were no sequence variations reported in the exon and 5' flanking region of the IFN- γ gene after screening 265 subjects. Noguchi E et al. [38] detected three silent variants in the promoter and coding regions of the IRF-1 gene, which are rare alleles and not significantly associated with asthma. Therefore, we did not genotype these SNPs. The other weakness was moderate sample sizes in this study, but we still demonstrated that the IFN- γ gene and the IRF-1 gene are significantly associated with asthma.

In this family-based study of the TNF gene and asthma, we have provided strong evidence that CA repeats within the first intron of the IFN- γ gene and GT repeats within the intron 7 of IRF1 polymorphisms are highly associated with asthmatic children in Taiwan.

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