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Functional Polymorphisms in the Promoter Region of Macrophage Migration Inhibitory Factor and Atopy

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Macrophage migration inhibitory factor (MIF) is a pleiotropic lymphocyte and macrophage cytokine; it is likely to play an important role in innate immunity. Genome-wide search for atopy susceptibility genes recently identified human chromosome 22q11, where the gene encoding MIF resides, as a region of interest for atopic traits. Both the $-173\text{G}/\text{C}$ and -794 [CATT]₅₋₈ repeat polymorphisms in the MIF promoter region are associated with altered levels of MIF gene transcription *in vitro*. We, therefore, hypothesized that these potentially functional polymorphisms may influence susceptibility to atopy and asthma. A case-control analysis examined the genetic influence of these promoter polymorphisms on the development of atopy and asthma in a Japanese population ($n = 584$). Evidence for significant association between the $-173\text{G}/\text{C}$ and -794 [CATT]₅₋₈ repeat polymorphisms and atopy was found; odds ratio for homozygotes of -173C allele was 3.67 (compared with homozygotes of -173G allele, 95% confidence interval = 1.43–9.46, $p < 0.01$), and odds ratio for noncarriers of the -794 [5-CATT] allele was 3.51 (compared with 5-CATT repeat homozygotes, 95% confidence interval = 1.82–6.78, $p < 0.0005$). No associations with asthma were detected. These results indicate that promoter polymorphisms in the MIF promoter region are risk factors for atopy and implicate MIF in the pathogenesis of atopy in a Japanese population.

Keywords: candidate gene; case-control analysis; specific IgE

Macrophage migration inhibitory factor (MIF) was initially described as an immune activity isolated from the supernatants of T lymphocytes (1) and has been implicated in macrophage activation and in antigen-driven T cell responses (2, 3). A recent investigation indicated that MIF regulates innate immune responses by macrophages through modulation of expression of toll-like receptor 4 (4); toll-like receptor 4 is the principal receptor for bacterial endotoxin recognition. There is evidence that endotoxin exposure during early life is protective against development of atopy and asthma (5–7); it is hypothesized that bacterial signals, such as endotoxin, play a functional role in maturation of T helper cell type (Th) 1-type immune responses, suppressing the Th2 response (8).

We previously demonstrated expression of MIF protein in serum and induced sputum of patients with asthma (9). Bronchoalveolar lavage fluid obtained from patients with asthma having atopy also contains significantly elevated levels of MIF, compared with volunteers not having atopy (10). Furthermore,

increased levels of MIF protein are associated with atopic dermatitis (11).

A recent linkage study has found that human chromosome 22q11, where the gene encoding MIF is located, shows evidence of linkage with atopy-related phenotypes (12). Polymorphisms with potential functional relevance have also been identified in the MIF promoter; a single nucleotide polymorphism at position -173 (G to C) (13) and a tetranucleotide CATT repeat beginning at nucleotide position -794 (14) have been found to be associated with altered levels of MIF gene transcription *in vitro*. Further evidence of the functional importance of these variants includes findings of significant association with several immune-mediated inflammatory diseases including juvenile idiopathic arthritis (13), sarcoidosis (15), and rheumatoid arthritis (14). Given the role of MIF in innate immune responses against microbial pathogens and regulation of inflammatory responses, we hypothesized that common allelic variations in these potentially functional polymorphisms may be involved in the genetic-environmental interaction underlying the pathophysiology of atopy and asthma. In a case-control association study using 584 unrelated Japanese subjects, we investigated whether the above two polymorphisms in the MIF promoter region contribute to the risk of development of atopy and asthma.

METHODS

Complete details are provided in the online supplement.

Study Population

We recruited 584 unrelated Japanese subjects (Table 1). Total serum IgE levels (IU/ml) and specific IgE responses to 10 common inhaled allergens were determined. An increase in specific IgE antibody levels (IgE ImmunoCAP [Pharmacia & Upjohn Diagnostics] radioallergosorbent test ≥ 0.35 UA/ml, or multiple radioallergosorbent test ≥ 1.0 lumicount) was considered a positive response (16). We defined atopy as a positive response to at least 1 of the 10 allergens as described previously (17).

All subjects gave written informed consent for enrollment in the study and all associated procedures. The Ethics Committee of the School of Medicine, Hokkaido University, approved the study.

Identification of Polymorphisms

For each individual, we typed the $-173\text{G}/\text{C}$ promoter polymorphisms using the assay that combines kinetic (real-time quantitative) polymerase chain reactions with allele-specific amplification (18) in which primers were designed (Primer Express software; PE Applied Biosystems, Foster City, CA) to specifically amplify either the -173G or -173C allele in separate polymerase chain reactions. The polymerase chain reaction products were detected using the ABI 7700 Sequence Detection System with the dsDNA-specific fluorescent dye SYBR Green I (PE Applied Biosystems). For typing of CATT tetranucleotide repeat polymorphism beginning at -794 , DNA was amplified by polymerase chain reaction using a carboxyfluorescein-labeled reverse primer. The polymerase chain reaction products were separated by electrophoresis through a performance-optimized polymer-4 gel using an ABI 310 (PE Applied Biosystems). For each individual, allele sizes were calculated using the Genescan Analysis computer program (PE Applied Biosystems).

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TABLE 1. GENOTYPE FREQUENCIES OF *MIF* PROMOTER POLYMORPHISMS IN 584 JAPANESE SUBJECTS

Characteristics	Subjects without Atopy (n = 235)		Subjects with Atopy (n = 349)		p Values*
	Subjects without Asthma (n = 155)	Subjects with Asthma (n = 80)	Subjects without Asthma (n = 152)	Subjects with Asthma (n = 197)	
Age, yr, median (range)	43.0 (18–72)	57.0 (27–81)	27.5 (19–69)	37.0 (16–78)	< 0.0001
Sex, female/male	64/91	54/26	47/105	95/102	< 0.0001
Smoking, n, never/ex/current	103/2/50	52/17/11	114/7/31	108/43/46	< 0.0001
Total serum IgE, log IU/ml (SD)	1.52 (0.044)	1.92 (0.061)	2.15 (0.044)	2.61 (0.039)	< 0.0001
Genotype, n					
–173G/C					
GG	91	49	94	106	
GC	59	28	47	70	
CC	5	3	11	21	
–794 CATT repeat					
5, 5	25	20	24	29	
5, 6	45	31	51	64	
5, 7	26	9	16	25	
5, 8	1	0	0	1	
6, 6	32	10	35	34	
6, 7	22	8	19	32	
6, 8	1	1	0	0	
7, 7	3	1	7	12	

* One-way analysis of variance or χ^2 test was used when appropriate.

Statistical Analysis

The χ^2 test was used to compare qualitative risk factors (sex, smoking status) among the four groups (healthy control subjects without atopy, healthy control subjects with atopy, subjects with asthma not having atopy, and subjects with asthma having atopy). One-way analysis of variance was used to compare quantitative risk factors (age, serum IgE levels). We used the Hardy–Weinberg equilibrium program (19) to compare observed numbers of genotypes with the numbers of genotypes expected under Hardy–Weinberg equilibrium. An estimated haplotype program was used to test for linkage disequilibrium between the two polymorphisms (19).

The association of the *MIF* promoter polymorphisms was measured by odds ratio with 95% confidence intervals, as estimates of relative risk for development of atopy and asthma. The –794 [CATT]_{5–8} genotypes were combined into three categories: 5, 5 genotype; 5, X genotypes; and X, X genotypes (allele X represents any allele other than five repeats of CATT). Odds ratios were adjusted for potentially confounding factors using a multivariate logistic regression model.

We estimated haplotype frequencies for –173G/C and –794 [GATT]_{5–8} repeat polymorphisms using the estimated haplotype program and tested the statistical association between the *MIF* promoter haplotypes and atopy using the program Haplo.Score that provided a global test for association, as well as haplotype-specific tests (20).

Luciferase Reporter Gene Assay

We constructed three plasmids (corresponding to the three most prevalent haplotypes: G/5-CATT, G/6-CATT, and C/7-CATT). A549 cells (1×10^5) were then transfected with 0.1 μ g of one of the three constructs and 0.1 μ g of pRL-TK vector, an internal control for transfection efficiency. After 24 hours, we measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Tokyo Japan).

RESULTS

Characteristics of the 235 subjects without atopy (155 subjects without asthma, 80 subjects with asthma) and 349 subjects with atopy (152 subjects without asthma, 197 subjects with asthma) are presented in Table 1. The mean age was highest for subjects with asthma not having atopy, and females predominated in this group. Subjects with atopy (both healthy control subjects and subjects with asthma) had higher total serum IgE levels than

subjects without atopy (unpaired *t*-test, $p < 0.001$). In addition, subjects with asthma (both subjects with atopy and subjects without atopy) had higher total serum IgE levels than healthy control subjects (unpaired *t*-test, $p < 0.001$). Alleles of the two promoter polymorphisms were in Hardy–Weinberg equilibrium in subjects without atopy.

We found that both the –173G/C and –794[CATT]_{5–8} repeat promoter polymorphisms were significantly associated with atopy (Table 2); odds ratio for CC homozygotes of the –173G/C polymorphism was 3.67 (compared with GG homozygotes, 95% confidence interval = 1.43–9.46, $p < 0.01$), and odds ratio for noncarriers of the 5-CATT allele of the –794 [CATT]_{5–8} repeat polymorphism was 3.51 (compared with 5-CATT homozygotes, 95% confidence interval = 1.82–6.78, $p < 0.0005$). In contrast, there were no significant differences in genotype distribution of these promoter polymorphisms between healthy control subjects and subjects with asthma (Table 2). Because we initially studied two markers (–173G/C and –794 CATT polymorphisms) in two phenotypes (atopy and asthma), we multiplied our significance levels by 4 (two markers \times two phenotypes), although these statistical tests were not independent due to the linkage disequilibrium between the two polymorphisms. Using this correction, the association between the two promoter polymorphisms and atopy was significant at $p = 0.05$.

The –173G/C and –794 [CATT]_{5–8} promoter polymorphisms were in significant linkage disequilibrium, with the –173C allele strongly associated with the 7-CATT repeat allele ($p < 0.000001$). The three most frequent haplotypes common to both groups with and without atopy were G/5-CATT, G/6-CATT, and C/7-CATT. These three haplotypes constituted 89.9% of haplotypes in the group with atopy and 90.1% of haplotypes in the group without atopy (Table 3). The haplotype composed of these two promoter polymorphisms was significantly associated with atopy, with a *p* value of 0.009 from 10,000 simulations of a global score test, as implemented in Haplo.Score (20). The haplotypes most strongly associated with atopy, as judged by the haplotype-specific scores, were G/5-CATT ($p < 0.0001$) and C/7-CATT ($p = 0.0036$), on the basis of 10,000 simulations; the G/5-CATT haplotype was

TABLE 2. IMPACT OF THE -173G/C AND -794 [CATT]₅₋₈ POLYMORPHISMS ON ATOPY AND ASTHMA

	-173 G/C	OR (95% CI)	-794 [CATT] ₅₋₈ Repeat	OR (95% CI)
Adjustments	-173G		5-CATT	
Atopy				
None	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/-	0.94 (0.66-1.34)	+/-	1.19 (0.75-1.90)
	-/-	2.80 (1.25-6.26)*	-/-	1.51 (0.93-2.46)
Age, sex, smoking, total IgE levels, and disease status (with asthma or healthy)	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/-	1.09 (0.69-1.73)	+/-	2.22 (1.20-4.11)*
	-/-	3.67 (1.43-9.46)†	-/-	3.51 (1.82-6.78)‡
Asthma				
None	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/-	1.10 (0.55-1.56)	+/-	0.94 (0.51-1.33)
	-/-	1.79 (0.92-3.49)	-/-	0.82 (0.51-1.33)
Age, sex, smoking, atopic status	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/-	1.07 (0.73-1.58)	+/-	0.84 (0.50-1.40)
	-/-	1.31 (0.62-2.80)	-/-	0.59 (0.35-1.0)

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

Adjustment for matching factors and potential confounding factors was performed by unconditional logistic-regression analysis. The analysis for atopy was adjusted for age, sex, smoking status (never, ex, or current), log-transformed total serum IgE levels, and disease status (subjects with asthma or healthy control subjects).

The analysis for asthma was adjusted for age, sex, smoking status (never, ex, or current), and atopic status.

* p < 0.05.

† p < 0.01.

‡ p < 0.0005.

associated with a lower risk of atopy and the C/7-CATT haplotype was associated with an increased risk of atopy (Table 3).

Transfection of the clone containing the C/7-CATT haplotype into A549 cells resulted in significantly reduced luciferase activity, relative to cells containing the other two common haplotypes (Figure 1).

DISCUSSION

In the present case-control study using a Japanese population, we found significant association between atopy and two promoter polymorphisms of the MIF gene. The G/5-CATT haplo-

type was associated with reduced risk for development of atopy, and the C/7-CATT haplotype was associated with increased risk for development of atopy. In previous *in vitro* functional studies, levels of MIF expression significantly differed among -173G/C genotypes in a cell type-specific manner. Promoter sequence analysis indicates that the -173C allele creates a potential activator protein 4 transcription factor-binding site (13). With the CATT tetranucleotide polymorphism, the 5-CATT allele was shown to be associated with lower basal and stimulated MIF promoter activity *in vitro* than the 6-, 7-, or 8-repeat alleles (14). Using the A549 epithelial cell line, we characterized *in vitro*

TABLE 3. ESTIMATED HAPLOTYPE FREQUENCIES OF -173G/C AND -794 CATT REPEAT POLYMORPHISMS

Haplotype	Atopy (%) (n = 349)	Nonatopy (%) (n = 235)	Haplotype-specific Score	p Values (Empirical)
G/5-CATT	32.14	38.83	-3.54	< 0.0001
G/6-CATT	40.54	37.63	1.54	0.13
G/7-CATT	1.38	1.63	0.009	0.99
G/8-CATT	0.00	0.00	-	-
C/5-CATT	5.54	4.15	-0.57	0.57
C/6-CATT	3.01	3.22	0.1	0.92
C/7-CATT	17.24	13.90	2.89	0.0036
C/8-CATT	0.14	0.64	-0.74	0.47
Total	100.00	100.00		

There is significant evidence for linkage disequilibrium between the -173G/C and the -794 tetranucleotide repeat polymorphisms both in groups with atopy ($\chi^2 = 260.95$ [3 degrees of freedom (df)], $p < 0.000001$) and without atopy ($\chi^2 = 132.11$ [3 df], $p < 0.000001$).

Haplotype frequencies were estimated using the Estimating Haplotype-Frequencies program, as described elsewhere (19). Frequencies of haplotypes composed of the MIF promoter polymorphisms differed significantly between subjects with atopy and subjects without atopy, with a p value of 0.009 from 10,000 simulations of global score tests (global-stat = 17.2, df = 6), as implemented in Haplo.Score (20). The analysis was adjusted for age, sex, smoking status (never, ex, or current), disease status (subjects with asthma or healthy control subjects), and log-transformed total serum IgE levels.

Note that a global score does not give effect estimates, whereas negative haplotype-specific scores are associated with a protective effect and positive haplotype-specific scores are associated with an increased risk.

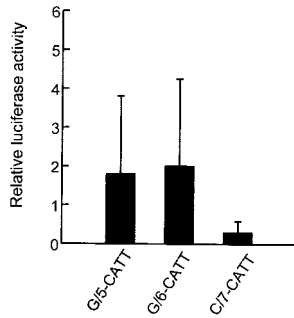


Figure 1. Transcriptional regulatory activity affected by *MIF* promoter haplotype. *MIF* promoter activity was determined by dual luciferase assays, with results expressed as relative luciferase activity (luciferase activity divided by renilla activity). The C/7-CATT haplotype had lower promoter activity than the G/5-CATT or G/6-CATT haplotype ($p = 0.0027$ for comparison among three haplotypes by Friedman test).

function of the three most common haplotypes. The C/7-CATT haplotype had lower promoter activity than the G/5-CATT and G/6-CATT haplotypes, suggesting functional importance of the *MIF* promoter haplotype in determining levels of *MIF* gene transcription. There is, however, no clear and simple discernable relationship between these polymorphisms and the differences observed in transcription levels of the three haplotype constructs, illustrating the complex nature of transcriptional regulation of the *MIF* gene. Thus, the physiologic relevance of the functional consequences of these promoter polymorphisms remains uncertain, and we cannot exclude the possibility that they act as markers of another important genetic abnormality without themselves being functionally relevant.

Recognition of endotoxin and Gram-negative bacteria by the host requires cooperative interplay between the endotoxin-binding protein (lipopolysaccharide-binding protein) (21), CD14 (22), and toll-like receptor 4 (23). Microbial toxins are powerful inducers of MIF release by immune cells, and, by upregulating basal expression of toll-like receptor 4 in the macrophage, MIF promotes recognition of endotoxin-containing particles and Gram-negative bacteria by the innate immune system (4). Several epidemiologic studies suggest that lack of exposure to endotoxin in early childhood is a risk factor for development of atopic phenotypes (5–7). Furthermore, genetic variants in the genes encoding endotoxin-signaling molecules such as CD14 (24) and CARD15 (25) have been described and found to be associated with levels of total serum IgE or allergy, supporting the hypothesis that exposure to endotoxin modulates IgE regulation by activating innate immune systems. We speculate that individuals carrying the C/7-CATT haplotype have lower expression of MIF in response to inhaled endotoxin at the respiratory mucosa, lower expression of toll-like receptor 4, and, consequently, lower endotoxin-inducible expression of interleukin-12 and interleukin-18, resulting in enhanced Th2 differentiation.

MIF is involved in antigen-specific immune responses; neutralizing anti-MIF antibodies inhibited T cell proliferation and interleukin-2 production *in vitro*, suppressing antigen-driven T cell activation and antibody production *in vivo* (26). MIF has recently been shown to be coded for by the same gene as glycosylation-inhibiting factor (27); glycosylation-inhibiting factor has been described as an immunosuppressive cytokine in a series of studies of regulation of antigen-specific IgE responses (28). Glycosylation-inhibiting factor is involved in antigen presentation involving B and T cell receptors and regulates generation of Th effectors from naive CD4 T cells (29), consequently regulating the balance of Th1/Th2-type immune responses. The importance of regulatory roles of MIF/glycosylation-inhibiting factor in antigen-specific immune responses is additional evidence that the *MIF* gene is a promising candidate for atopy or antigen-specific IgE responsiveness.

It is important to note that the significant association between atopy and the two promoter polymorphisms could be due to

type I error or population stratification (30). However, as we evaluated only two loci in an entirely Japanese population and as the control group was in Hardy–Weinberg equilibrium for the two polymorphisms, the usual problems associated with population stratification may be of limited importance in the present study. As for type I error, none of the reported p values were adjusted for multiple comparisons, because not all of the statistical tests were independent, due to the linkage disequilibrium between the two polymorphisms and the dependence between genotype and haplotype. In addition, given strong prior evidence for *MIF* as a candidate gene for atopy and evidence for functionality of *MIF* promoter polymorphisms, the present results appear to significantly support the hypothesis that individuals carrying certain genotypes of the present *MIF* promoter polymorphisms are at increased risk of developing atopy under certain additional environmental and genetic conditions. Nevertheless, we acknowledge that type I error and population stratification may have influenced the present findings and that these findings are preliminary and do not by themselves conclusively confirm an etiologic relationship. Additional evidence is needed from studies of other groups of individuals with and without atopy, especially in cohorts well characterized in terms of levels of endotoxin exposure in infancy.

In conclusion, MIF is an excellent positional and biologically plausible candidate gene for atopy and may be involved in the endotoxin-signaling pathway, contributing to the development of atopy. However, given the great diversity of functions performed by MIF, further functional studies of genetic variation in the *MIF* promoter region are needed to clarify the pathophysiologic mechanisms by which these polymorphisms affect development of atopy.

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