Title
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 −173G/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 −173G/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 < 0.0005). 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.

**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 luimic count) 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 −173G/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 (CATT)5–8, DNA was amplified by polymerase chain reaction using a carboxyfluorescein-labeled reverse primer. 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).

**Key Words:** candidate gene; case–control analysis; specific IgE

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Subjects without Asthma (n = 233)</th>
<th>Subjects with Asthma (n = 349)</th>
<th>p Values</th>
<th>Subjects without Asthma (n = 155)</th>
<th>Subjects with Asthma (n = 197)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr, median (range)</td>
<td>43.0 (18–72)</td>
<td>57.0 (27–81)</td>
<td>&lt; 0.0001</td>
<td>27.5 (19–69)</td>
<td>37.0 (16–78)</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>64/91</td>
<td>54/26</td>
<td>&lt; 0.0001</td>
<td>47/105</td>
<td>95/102</td>
</tr>
<tr>
<td>Smoking, n, never/ex/current</td>
<td>103/2/50</td>
<td>52/17/11</td>
<td>&lt; 0.0001</td>
<td>114/7/31</td>
<td>108/43/46</td>
</tr>
<tr>
<td>Total serum IgE, log IU/ml (SD)</td>
<td>1.52 (0.044)</td>
<td>1.92 (0.061)</td>
<td>&lt; 0.0001</td>
<td>2.15 (0.044)</td>
<td>2.61 (0.039)</td>
</tr>
</tbody>
</table>

* One-way analysis of variance or χ² test was used when appropriate.

**Statistical Analysis**

The χ² test was used to compare qualitative risk factors (sex, smoking status) among the four groups (healthy control subjects without atopy, healthy control subjects with asthma, 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 [CAT]₇–₈ repeat polymorphism was significantly associated with atopy, with a p value of 0.0036, on the basis of 10,000 simulations of a global score test, as implemented in Haplo.Score (20). The haplotypes most strongly associated with the 7-CATT repeat allele (p = 0.000001). 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]₇–₈), repeat promoter polymorphisms in two phenotypes (atopy and asthma), we multiplied our significance levels by 4 (two markers × 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.

**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]₇–₈ 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]₇–₈ 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).
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 haplotype 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 2. Impact of the −173G/C and −794 [CATT]_{3-8} Polymorphisms on Atopy and Asthma

<table>
<thead>
<tr>
<th>Adjustments</th>
<th>−173 G/C</th>
<th>OR (95% CI)</th>
<th>−794 [CATT]_{3-8} Repeat</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+/+</td>
<td>1.0 (Reference)</td>
<td>+/+</td>
<td>1.0 (Reference)</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>0.94 (0.66–1.34)</td>
<td>+/-</td>
<td>1.19 (0.75–1.90)</td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>2.80 (1.25–6.26)</td>
<td>-/-</td>
<td>1.51 (0.93–2.46)</td>
</tr>
<tr>
<td>Age, sex, smoking, total IgE levels, and disease status (with asthma or healthy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td></td>
<td>1.0 (Reference)</td>
<td>+/+</td>
<td>1.0 (Reference)</td>
</tr>
<tr>
<td>+/-</td>
<td></td>
<td>1.09 (0.69–1.73)</td>
<td>+/-</td>
<td>2.22 (1.20–4.11)</td>
</tr>
<tr>
<td>-/-</td>
<td></td>
<td>3.67 (1.43–9.46)</td>
<td>-/-</td>
<td>3.51 (1.82–6.78)</td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+/+</td>
<td>1.0 (Reference)</td>
<td>+/+</td>
<td>1.0 (Reference)</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>1.10 (0.55–1.56)</td>
<td>+/+</td>
<td>0.94 (0.51–1.33)</td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>1.79 (0.92–3.49)</td>
<td>-/-</td>
<td>0.82 (0.51–1.33)</td>
</tr>
<tr>
<td>Age, sex, smoking, atopic status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td></td>
<td>1.0 (Reference)</td>
<td>+/+</td>
<td>1.0 (Reference)</td>
</tr>
<tr>
<td>+/-</td>
<td></td>
<td>1.07 (0.73–1.58)</td>
<td>+/+</td>
<td>0.84 (0.50–1.40)</td>
</tr>
<tr>
<td>-/-</td>
<td></td>
<td>1.31 (0.62–2.80)</td>
<td>-/-</td>
<td>0.39 (0.35–1.0)</td>
</tr>
</tbody>
</table>

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.

### Table 3. Estimated Haplotype Frequencies of −173G/C and −794 CATT Repeat Polymorphisms

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

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 (χ² = 260.95 [3 degrees of freedom (df)], p < 0.000001) and without atopy (χ² = 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 = 172, 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.
activation and antibody production specific immune responses is additional evidence that the}

...atory 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 1 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 1 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 1 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 pathophysiological mechanisms by which these polymorphisms affect development of atopy.

**Conflict of Interest Statement:** N.H. has no declared conflict of interest; E.Y. has no declared conflict of interest; D.T. has no declared conflict of interest; J.N. has no declared conflict of interest; M.N. has no declared conflict of interest.

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