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ORIGINAL ARTICLE

Influence of MILR1 promoter polymorphism on expression levels and the phenotype of atopy

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The recently identified cell surface immunoreceptor MILR1 (mast cell immunoglobulin-like receptor 1; synonyms, Allergin-1) has been shown to suppress immunoglobulin E (IgE)-mediated, mast cell-dependent responses in both mice and humans. We performed a mutation search of *MILR1* together with a genetic association study to determine whether polymorphisms in *MILR1* are associated with atopy in human. Mutation screening of *MILR1* was performed using DNA from 146 unrelated Japanese. Genotyping of the identified polymorphisms was done with 1505 individuals from the general Japanese adult population. Atopy, as defined by positive responses for specific IgEs against at least one of the 26 common allergens, was evaluated using MAST-26. Five polymorphisms (rs6504230, c.−170_−166delAGGAA, rs8071835, rs143526766 and rs12936887) and two rare missense variants (Val273Ala and Leu311Val) were identified by mutation screening. The C allele of rs6504230 had protective effects against atopy ($P = 0.002$). A luciferase reporter assay using the promoter region of *MILR1* revealed that the C allele of rs6504230 was associated with increased expression of MILR1, which was in accordance with the results of expression quantitative trait loci analysis using human leukocytes. Our data indicates that the rs6504230 polymorphism affects MILR1 expression levels in humans, leading to a susceptibility to producing specific IgE antibodies against common allergens.
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INTRODUCTION

Atopic diseases such as asthma, atopic dermatitis and allergic rhinitis are some of the most common diseases in the world, and their prevalence is still increasing in some countries.[1] Atopy is defined as a genetic predisposition toward the development of immediate hypersensitivity reactions against common environmental antigens such as house-dust mites and pollen, with the production of specific immunoglobulins E (IgEs) against these allergens; atopy is considered the strongest predisposing factor for allergic diseases.[2] A study of twins from the general Australian population revealed that the association for atopy was greater in monozygous pairs (odds ratio 14.6; 95% confidence interval, 7.1–30.1) than dizygous pairs (odds ratio 2.5; 1.4–44.5).[3] Other studies have shown that heritability for atopic traits is between 0.6 and 0.7, and monozygous twins have always a higher concordance of the traits under study than dizygotic twins.[4]

Meta-analysis of genome-wide linkage studies of asthma and related traits revealed that 3p25.3–q24 and 17q12–q24 reached genome-wide significance for skin prick test positivity, one of the atopic phenotypes in European families.[5] In candidate gene approaches, interlukin-4 (IL4), IL4 receptor, IL13, the beta-chain of the high-affinity receptor for IgE (FcεRI) and the major histocompatibility complex have been shown to be associated with atopy and its related phenotypes.[6,7] A recent meta-analysis of genome-wide association studies (GWAS) on allergic sensitization identified 10 loci that reached the genome-wide significance level, including single-nucleotide polymorphisms (SNPs) in *TLR6*, *C11orf30*, *STAT6*, *SLC25A46*, *HLA-DQB1*, *IL1RL1*, *LPP*, *MYC*, *IL2* and *HLA-B*.[8] In addition, GWAS of self-reported pollen, dust mite, and cat allergy revealed genome-wide significant association at *TLR1*, *TLR6*, *TLR10*, *HLA-C*, *MICA*, *PTGER4*, *PLCL1*, *LPP*, *NFATC2*, *ADAD1*, *FOXA1* and *TTC6*.[9]

We recently identified MILR1 (synonyms, Allergin-1), mast cell immunoglobulin-like receptor, that contains an immunoreceptor tyrosine-based inhibitory motif-like domain[10] and showed that coligation of MILR1 and FcεRI suppressed IgE-mediated degranulation of bone marrow-derived cultured mast cells and that mice deficient in MILR1 developed enhanced passive systemic and cutaneous anaphylaxis. Because *MILR1* is located on chromosome 17q23.3 in humans, one of the linkage region for atopy defined by skin prick test,[5,11] we hypothesized that variants in *MILR1* may be associated with atopy. In the present study, we screened for mutations in the 5′-flanking region and the coding region of *MILR1* and conducted an association study for atopy in the general Japanese population. We found that a promoter polymorphism, rs6504234,

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affects the level of MILR1 expression and that rs6504234 in *MILR1* is associated with atopy.

MATERIALS AND METHODS

Subjects for genetic analysis

A cross-sectional analysis of 1505 Japanese men and women aged 22–81 years was carried out for subjects who visited the Tsukuba Medical Center for an annual medical check-up between June 2008 and May 2009.[12] All the participants were asked about their respiratory health, medical history, lifestyle and exposure to environmental irritants (for example, cigarette smoke, allergens and air pollution). Total serum IgE levels were measured by fluorescence-enzyme immunoassay (SRL, Tokyo, Japan). Specific serum IgE antibody was measured with the multiple allergen simultaneous test (MAST)-26 chemiluminescent assay systems (Hitachi Chemical Company, Tokyo, Japan). Of the 1505 subjects, 1464 subjects completed both the questionnaires and the MAST-26 analysis. Genomic DNA was extracted from peripheral blood samples from all participants by an automated DNA extraction system (QuickGene-610L; Fujifilm, Tokyo, Japan). The characteristics of the study population are shown in Table 1. Positive sensitization (atopy) was defined as the demonstration of specific IgE levels greater than or equal to a lumicount 2.76 (class 2) for any allergens included in MAST-26, and those subjects whose lumicount was <2.76 for all 26 allergens included in MAST-26 (Supplementary Table 1) and who had no history of any allergic diseases served as the controls for the study. MAST-26 has been reported to produce results that correlate well with those produced by capsulated hydrophilic carrier polymer radioallergosorbent test (CAP RAST) and by the intradermal skin test.[13]

Written informed consent was obtained from each participant in accordance with institutional requirements and the principles of the Declaration of Helsinki. This study was approved by the Ethical Committee of the University of Tsukuba (No 103-5).

Mutation screening and genotyping

All exons and exon–intron junctions as well as the 5′-flanking region (348 bp) and 3′-flanking region (84 bp) of *MILR1* were amplified by PCR with the genomic DNA obtained from 146 unrelated Japanese subjects. Direct sequencing was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies, Carlsbad, CA, USA) on an ABI 3130 autosequencer (Life Technologies) using primers described in Supplementary Table 2. Primers were designed using the human genome reference sequence (NM_001085423, hg18/GRCh36). The genotype data for *MILR1* in the general Japanese population were obtained from the HapMap website (http://www.hapmap.org/), and tag SNPs were selected using the Tagger software implemented in the Haploview software (version 3, release 27), with an r^2 threshold of 0.8 and allele frequencies of 0.1.[14,15] Three SNPs, rs6504230, rs8071835 and rs12936887, were selected as tag SNPs.

Genotyping of subjects was done with the TaqMan SNP Genotyping Assay (Life Technologies) using primer pairs described in Supplementary Table 3. The reactions were performed in a 384-well format using a total reaction volume of 5 µl with 5 ng of genomic DNA. The plates were then placed on a thermal cycler (GeneAmp 9700 PCR systems, Life Technologies) and heated to 95 °C for 10 min, followed by 60 cycles of 92 °C for 15 s and 60 °C for 1 min. The end point fluorescence readings were performed using an ABI Prism 7900 HT System (Life Technologies). Genomic regions around c.−170_−166 delAGGAA polymorphisms were amplified with primers 5′-FAM-CACCAA ACTCCTTTTGGCATA-3′ and 5′-GGTTTGGATGAGGTTTGCAT-3′. PCR product (wild type; 242 bp, c.−170_−166delAGGAA; 237 bp) were electrophoresed on an ABI 3130 autosequencer (Life Technologies), and c.−170_−166delAGGAA genotypes were determined using GeneMapper v4.1 (Life Technologies). Genomic regions around the rs143526766 were amplified with primers 5′-CGTGGCCTTTTATATTCTCTCTTG-3′ and 5′-ACAGTGCTGAC CAGTGGAAA-3′. PCR products were subjected to direct sequencing using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Life Technologies) on an ABI PRISM 3130 Genetic Analyzer (Life Technologies).

Table 1 Characteristics of the study population

Characteristic	
Sex (male: female)	676:788
Mean age ± s.d.	50.5 ± 9.6
Atopy	872/1464 (60%)
Total IgE, geometiric mean (IU ml^{-1})	64.4 (range <5 to 32 000)
Current/former smoker	557/1464 (38%)
Current/former asthma	95/1464 (6.5%)

Abbreviation: IgE, immunoglobulin E.

In silico analysis

GeneVar (http://www.sanger.ac.uk/resources/software/genevar/) was used to access HapMap expression quantitative trait loci (eQTL) data, and the association between SNP genotype and gene expression was estimated using the Spearman's rank correlation coefficient.[16,17]

Construction of luciferase reporter plasmids

Real-time (RT)–PCR was performed to examine *MILR1* expression using cDNA derived from the K562 human leukemia cell lines and human embryonic kidney 293T cell line. The primers used for RT–PCR are given in Supplementary Table 2. Genomic fragments of the 5′-flanking region of *MILR1* were amplified with the primers shown in Supplementary Table 4 to construct plasmids encompassing −1472 to +28 bp, −759 to +28 bp, −318 to +28 bp and −117 to +28 bp of the human *MILR1* promoter relative to the translation initiation site. The PCR product was digested with *Xho*I and *Hin*dIII and ligated into a pGL4.10 vector (Promega, Madison, WI, USA) containing the firefly luciferase gene as a reporter and then transformed into *Escherichia coli* DH5α (TOYOBO, Osaka, Japan). Plasmid DNA was extracted using the QIAGEN EndoFree Plasmid Maxi Kit (QIAGEN, Hilden, Germany). In order to generate vectors containing region rs6504230:T>C (c.−171T>C), a plasmid construct containing −318 to +28 bp of the human *MILR1* (wild-type rs6504230:T) was subjected to site-directed mutagenesis with the PrimeSTAR mutagenesis kit (Takara, Tokyo, Japan) using primers 5′-TCTTCTTACAGGAACTCACAGAGGAAGACAGAT-3′ and 5′-GTTCCTGTAA GAAGAGAGATATTTGGTAGATTG-3′ to introduce rs6504230:C. All reactions were performed according to the manufacturer's instructions, and the accuracy of the plasmid constructs was verified by direct sequencing.

Transient transfections and luciferase assays

We seeded 1×10^5 K562 cells per well in 24-well plates and transfected them with pGL4.10 containing the *MILR1* promoter. The pRL-TK plasmid (Promega) was cotransfected as a normalizing control. After 24 h of incubation, cells were collected, and luciferase activity were determined using 5 µl of lysate sample with the Dual-Luciferase Assay System (Promega) and a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Statistical methods

Deviation from the Hardy–Weinberg equilibrium was examined using the χ^2 test. Logistic regression analysis was performed under a log-additive model adjusted for sex, age, smoking status and asthma by using the SNPassoc package from R software.[18] *P*-values were corrected by Bonferroni corrections for allelic association study, and $P<0.01$ (0.05/5) were considered statistically significant. Power calculation was performed with the Genetic Power Calculator.[19]

RESULTS

Mutation search and case–control analysis

We screened for *MILR1* mutations in DNA obtained from 146 unrelated Japanese and found five polymorphisms (rs6504230, rs8071835, rs12936887, rs143526766 and c.−170_−166delAGGAA) and two rare missense variants (Val273Ala for 1 in 146 subjects and Leu311Val for 1 in 146 subjects; Figure 1 and Supplementary Table 5). c.−170_−166delAGGAA was in complete linkage

disequilibrium with rs6504230:C ($D' = 1.0$). None of the polymorphisms deviated from the expected values based on the Hardy–Weinberg equilibrium ($P > 0.05$). The characteristic differences between atopic and non-atopic subjects (controls) are shown in Table 2. Atopic subjects were significantly younger than non-atopic subjects, and male subjects were more susceptible to atopy than female subjects. To control for confounders, logistic regression analysis was performed, adjusting for sex, age, smoking status and asthma. Our analysis showed that rs6504230 was significantly associated with atopy, suggesting that the C allele of rs6504230 had a protective effect against allergic sensitization (Table 3).

Publically available eQTL data showed that the C allele of rs6504230 was associated with increased expression levels of *MILR1* ($P < 0.0001$; Supplementary Figure 1).

Figure 1 Polymorphisms and variants identified in *MILR1*. Closed boxes represent exons, and arrows indicate the positions of the variants.

Table 2 Characteristics of atopic and non-atopic subjects

	Atopy	*Non-atopy (control)*	P-*value*
Sex (male:female)	425:427	251:342	<0.001
Mean age ± s.d.	48.6 ± 9.4	53.26 ± 9.3	<0.001
Smoking status (non:current or past)	217:340	340:532	0.36
Asthma (no:yes)	795:77	573:19	<0.001

Transcriptional activities in the 5′-flanking region of *MILR1*

We first selected cell lines expressing MILR1 using RT-PCR. The PCR product sizes of *MILR1* isoforms[20] (Allergin-1L and Allergin-1S) were 1104 and 820 bp, respectively. *MILR1* was expressed in K562 cells but not in 293T cells (Supplementary Figure 2). To examine transcriptional activity in the 5′-flanking region of the *MILR1* gene, we constructed plasmids that contained sequences from −1472 bp, −759 bp, −318 bp and −117 bp to +28 bp, relative to the translation initiation site (NM_001085423). The expression of *MILR1* in K562 cells was confirmed by reverse transcription-PCR (data not shown). These constructs were then transiently transfected into K562 cells. Deletion analysis revealed that the region driving maximal reporter gene expression in K562 cells was located −318 bp proximal to the translation initiation site (Figure 2). To determine whether rs6504230:T>C (c.−171T>C) affected the MILR1 expression levels, we generated luciferase reporter gene constructs that contained

Table 3 Case–control study for Allergin-1 polymorphisms

Polymorphism	*Population*	*Genotype count*			*MAF*	P-*values*[a]	*Odds ratio (95% CI)*[a]
		T/T	T/C	C/C			
rs6504230	Atopy	376	396	94	0.34	0.002	0.77 (0.66–0.91)
	Controls	206	290	82	0.39		
		−/−	del/−	del/del			
c.-170_-166delAGGAA	Atopy	852	17	0	0.01	0.9	1.05 (0.48–2.3)
	Controls	567	11	0	0.01		
		GG	GT	TT			
rs8071835	Atopy	779	85	2	0.051	0.55	0.9 (0.64–1.27)
	Controls	479	56	3	0.058		
		GG	GT	TT			
rs12936887	Atopy	466	339	62	0.27	0.26	1.11 (0.93–1.32)
	Controls	327	212	40	0.25		
		GG	GA	AA			
rs143526766	Atopy	826	46	0	0.026	0.95	1.02 (0.62–1.67)
	Controls	563	29	0	0.024		

[a]*P*-values were calculated by logistic regression analysis under a log-additive model adjusted by sex, age, smoking status and asthma.

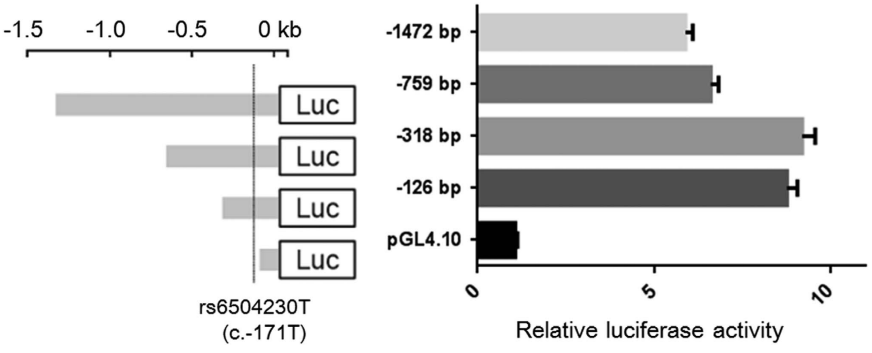

Figure 2 Luciferase activity of K562 cells transfected with plasmids. Each construct was transfected into K562 cells as described in Material and methods. Luciferase activity is relative to Renilla activity, and data represent the average ± s.d. obtained from five samples. The numbers represent positions relative to the translation initiation site as +1.

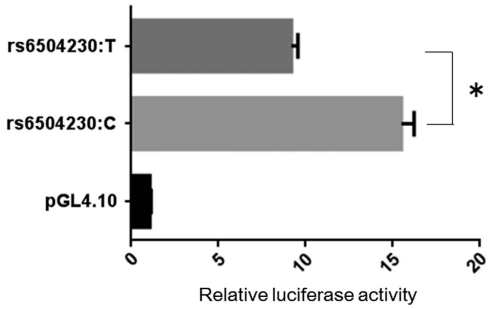

Figure 3 Effect of the three polymorphisms on *MILR1* promoter activity. Luciferase activity is relative to Renilla activity, and data represent the average ± s.d. obtained from five samples. The numbers represent positions relative to the translation initiation site as +1. *$P<0.0001$.

sequences from −318 bp to +28 bp and differed from wild type only at rs6504230:T>C. The construct containing rs6504230:C had higher transcriptional activity than that containing rs6504230:T ($P=1.2\times10^{-7}$; Figure 3).

DISCUSSION

In this study, we identified four polymorphisms and two rare missense variants in *MILR1*. Of them, rs6504230, which is located in the promoter region, was associated with atopy and expression levels of *MILR1*. The C allele of rs6504230 was associated with increased expression levels of *MILR1*, suggesting that increased expression of MILR1 has a protective role against the development of atopy. In the present study, the statistical powers of the case–control analysis for rs6504230, c.-170_-166delAGGAA, rs8071835, rs12936887 and rs143526766 were 0.99, 0.25, 0.78, 0.99 and 0.58, respectively, at the α level of 0.05 and the genotype relative risk of the risk allele of 1.3 for homozygote and 1.2 for heterozygote.[19] Thus, our sample size was relatively small to sufficiently detect the variants with minor allele frequencies <0.05. Therefore, it is probable that the association of atopy with c.-170_-166delAGGAA, rs8071835 or rs143526766 could not be detected because of the small sample size.

MILR1 was first identified in 2011[10] and was reported to mediate an inhibitory signal for FcεRI-mediated degranulation. Mice lacking MILR1 showed more severe anaphylaxis than wild-type mice in the passive cutaneous anaphylaxis model. In humans, MILR1 inhibits IgE-mediated activation of human primary mast cells in bronchoalveolar lavage fluid.[20] Our data showing that increased expression of MILR1 has a protective role against the development of atopy is in agreement with the functional role for MILR1 in suppressing FcεRI-mediated activation.

Recently conducted GWASs identified several loci for allergic sensitization,[8,9] but none of the loci was located in chromosome 17q. Allergic sensitization is believed to be influenced by exposure to allergens and the genetic background. The majority of the study populations described in the two GWAS were Caucasian, and the inconsistent results of these studies can be attributed to the differences in the genetic backgrounds and the types of allergens the subjects were exposed to. Wood *et al.*[21] performed a genome-wide SNP analysis to identify eQTLs using whole blood from 712 individuals, and the results showed that rs6504230 was significantly associated with expression levels of MILR1 (synonyms, C17orf60; $P=3.78\times10^{-17}$). Together with the results from our experiment using the luciferase reporter assay and other publically available eQTL data (Supplementary Figure), these observations show that rs6504230 affects MILR1 expression levels in humans, leading to a susceptibility to producing specific IgEs against common allergens.

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