levels of IgE (Weidinger et al. 2006; Weidinger et al. 2007), and allergic sensitization (Weidinger et al. 2006), and Marenholz et al. (Marenholz et al. 2006) reported that these mutations predispose carriers to asthma, allergic rhinitis, and allergic sensitization only in the presence of AD. Other *FLG* null mutations have also been found to be associated with AD in a Caucasian population (Sandilands et al. 2007). In Japanese population, the 3321delA and S2554X mutations were associated with ichthyosis vulgaris and AD (Nomura et al. 2007). Most of the previous studies related to *FLG* mutations were conducted with European populations, and studies of *FLG* variants other than the null mutations in relation with AD have not been conducted.

In the present study, we examined tag single nucleotide polymorphisms (SNPs) and null mutations in *FLG* for possible associations with AD and atopic phenotypes in a Japanese population.

Materials and methods

Subjects

Probands in the AD families were patients with AD who visited the Dermatology Department of the University Hospital of Tsukuba (Japan) and dermatology departments of several hospitals in Ibaraki, Japan. AD was diagnosed in subjects

according to the criteria of Hanifin and Rajka (Hanifin and Rajka 1980). All patients had pruritus, typical appearance of AD, and a tendency toward chronic or chronically relapsing dermatitis. A full verbal and written explanation of the study was given to all family members interviewed, and 105 families (381 members) gave informed consent and participated in this study. The mean age of the probands and their siblings was 13.3 years (range, 0.9 to 42 years). For a case-control study, 376 independent AD patients (ages 16-64 years, mean 29.7 years) were recruited. Control subjects for the case-control study were 923 healthy adults (ages 19-78 years, mean 46.2 years) with no history of any allergic disease. A full verbal and written explanation of the study was given to patients and all family members interviewed, and all provided informed consent. This study was approved by the Committee of Ethics of the University of Tsukuba. The subjects for case-control study were classified according to AD alone, elevated total serum IgE level (>1000 IU/L), and early onset (< 2 years of age). Among 376 patients with AD, the number of patients with AD alone (i.e., AD patients without another atopic disease such as asthma and rhinitis) was 75 (20%). The number with an elevated total serum IgE was 212 (56%), and the number with early onset was 112 (30%).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes or oral brushed cells using standard protocol. R501X and 2282del4 were genotyped by restriction enzyme digestion of polymerase chain reaction (PCR) products amplified from DNAs of 96 unrelated Japanese AD patients. The R501X and 2282del4 variants were PCR amplified with the following primer sequences, 5'-CTGGAGGAAGACAAGGATCG -3' and 5'-TTGTCTGCTTGCACTTCTGG -3' for the R501X and 5'-ATCAGGCACTCGTCACACAC-3' and 5'-AGTGCCTGGAGTTGTCTCGT-3' for 2282del4. PCR products were digested with *Nla*III for R501X and *Dra*III for 2282del4 at 37°C for 16 h. Digested PCR fragments were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining and ultraviolet transillumination. Expected product sizes for R501 were 213 base pairs (bp) and 32 bp and for X501 allele were 177 bp, 36 bp, and 32 bp. Expected product sizes for the wild-type allele of 2282del4 were 458 bp, and for the deletion allele were 240 bp and 214 bp. 3321delA was genotyped with sizing of a fluorescently labeled PCR fragment on an Applied Biosystems 3100 DNA Sequencer (Foster City, CA) as described previously (Nomura et al. 2007). Genotype information for the *FLG* region in Asian populations (Japanese and Chinese) was downloaded from the HapMap database

(http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B36/), and tag SNPs were selected with Tagger software (de Bakker et al. 2005) implemented in Haploview software (Barrett et al. 2005) with an r^2 threshold of 0.8 and allele frequencies of 0.05. Tag SNPs (rs11582620, rs11586114, rs1933064, rs2065958, rs3814299, rs12730241) were genotyped with TaqMan Assay-on-Demand™ SNP Typing Systems (Applied Biosystems). S2554X was genotyped on a TaqMan Assay-by-Design system for SNP genotyping (Applied Biosystems), with the following primer sequences: forward, 5'-CGGCTCCAGGCACTCA-3', reverse, 5'-ATCCCCAGTTCCTGCTTGTC-3' reporter 1 (VIC), 5'-CCCCTCTGATTGTC-3' and reporter 2 (FAM), 5'-CCCCTCTCATTGTC-3'. The genotyping accuracy was confirmed based on the direct sequences of samples obtained from carriers and noncarriers of the S2554X null mutation.

Statistical analysis

Transmission disequilibrium test (TDT) and pedigree disequilibrium test (PDT) were performed with the unphased program (http//www.mrc-bsu.cam.ac.uk/personal/frank/software.unphased/). Linkage disequilibrium (LD) between SNPs, as expressed by D', was calculated with

Haploview software (Barrett et al. 2005). The significance of differences in the allele frequencies between case and control groups in case-control comparisons was determined by χ^2 test. To combine the family and case-control data, control alleles in AD families were constructed as non-transmitted parental allele, and case alleles as transmitted parental alleles as described by Kirov et al. (Kirov et al. 1999).

Results

The X501 and 2282del4 alleles were not identified in 96 independent Japanese patients with AD. The allele frequencies for all SNPs in the parents in AD families and in controls did not deviate from Hardy-Weinberg equilibrium predictions ($P > 0.05$).

TDT revealed that the minor alleles of rs2065958 and rs12730241 were overtransmitted to AD-affected offspring ($P < 0.05$, Table 1). However, these results were not replicated in the AD case-control study (Table 2). In the AD case-control study, we genotyped two non-synonymous SNPs, rs2065958 and rs3814299, because rs12730241 is in nearly complete linkage disequilibrium with rs2065958 ($r^2 = 0.95$). The null allele of S2554X tended to be overtransmitted to AD-affected offspring, though the P value did not reach statistical significance. In the case-control comparison, the null allele of S2554X was associated statistically significantly with AD (Table 2). S2554X was also associated with high IgE levels and the phenotype of patients with AD alone. Five percent of patients with the phenotype of patients with AD alone carried the S2554X null mutation, whereas only 1% of healthy control subjects had the null mutation ($P = 2.4 \times 10^{-5}$). Three percent of AD patients and 4% of those with the phenotype of patients with AD alone carried the 3321delA allele, whereas 1% of healthy control subjects had the null mutation. However, this difference

was not statistically significant ($P > 0.05$). Association was observed between 3321delA and the high-IgE phenotype ($P = 0.0036$). Combined null mutation carriers (subjects carrying either X2554 or 3321delA alleles) were observed more in patients with the AD and high-IgE phenotypes than in control subjects. The most significant association was observed for the phenotype of patients with AD alone (7 of 67 patients, carrier frequency 9.5 %, $P = 4.2\times10^{-5}$). Subjects with compound heterozygous null mutations were not observed in our family or case-control samples.

To combine the TDT and case-control data, the proband of each family was selected, and an artificially constructed case population consisting of parental alleles transmitted to the affected child and a control population of nontransmitted alleles in the AD family trios were determined (Kirov et al. 1999), and these "cases" and "controls" in the family trios were combined with the genotype data (Table 3). The combined *P* value was significant for the S2554X polymorphism and null mutations of *FLG* ($P = 0.0001$), whereas rs2065958 and rs12730241were not associated with development of AD.

Discussion

In the present study, we found that the null allele of S2554X was associated with development of AD, confirming previous studies showing that *FLG* null mutations are associated with AD (Nomura et al. 2007). In the present study, 1% of healthy subjects without any allergic diseases carried *FLG* null mutations, whereas no control subjects carried the null mutations in a previous study (Nomura et al. 2007). Allele frequency of the *FLG* null mutations in the AD patients were similar to those reported previously (Nomura et al. 2007). The null alleles of R501X and 2282del4 were not detected in 96 Japanese AD subjects.

FLG is thought to be one of the most important factors in skin barrier function. In children, dry skin is often the earliest sign of AD. Impairment of epidermal barrier function is a clinical hallmark of AD. Microarray analysis revealed decreased expression of *FLG* mRNA in active atopic skin (Sugiura et al. 2005). These findings suggest that dysfunction of *FLG* is an important factor in the development of AD. In the present study, the most significant effect of *FLG* null mutations was observed in the phenotype of patients with AD alone. AD patients often suffer from other atopic diseases such as asthma and allergic rhinitis, and patients with multiple atopic diseases exhibit increased levels of IgE against allergens. AD patients suffering from other

atopic diseases are more likely to exhibit allergic skin inflammation, which leads to the development of AD. In contrast, since FLG plays an important role in the skin barrier function, the skin may be fragile in carriers of the *FLG* null allele regardless of the atopic status of these individuals, and this may result in the development of AD. Therefore, subjects with the phenotype of AD alone are more likely to carry the *FLG* null allele than those with the phenotype of AD along with other atopic diseases.

The study by Palmer et al. (2006)was the first to show that *FLG* null mutations are associated with AD in Caucasian populations. A number of studies have been conducted to replicate the original findings, and some have confirmed and others refuted the association of *FLG* with AD (Marenholz et al. 2006; Ruether et al. 2006; Weidinger et al. 2006; Barker et al. 2007; Morar et al. 2007; Stemmler et al. 2007; Weidinger et al. 2007). To examine the association of common *FLG* variants with development of AD, we performed tag SNP analysis of Japanese AD families and case-control subjects. Two SNPs, including one nonsynonymous mutation, were associated with AD by PDT analysis, but this finding was not confirmed in the case-control subjects. The statistical power of the case-control study for these SNPs was more than 80% at the alpha level of 0.05 if the relative risk for AD in those persons carrying a putative risk allele is 1.5 compared with that in persons without the

allele. Therefore, our number of case-control samples is sufficient to detect alleles confirming moderate risk, but may not be sufficient to detect alleles with weak risk.

The results of our family-based association study of S2554X did not reach statistical significance, however the null allele of S2554X tended to be overtransmitted to affected offspring in our Japanese AD families. In the case-control comparison, X2554 was significantly associated with development of AD, and the combined *P* value for the family and case-control data was significant. Because of the low allele frequencies of the null alleles in *FLG*, failure to find an association in the family samples is due to low statistical power. The other null allele, 3321delA, was not associated with AD. The allele frequency of the 3321delA is very low, 2.7% in AD patients and 1.3 % in control. The statistical power in the pedigree samples was less than 0.1 at the alpha level of 0.05 if the relative risk for AD in those persons carrying a putative risk allele is 2.0 compared with that in persons without the allele. In the case-control study, five hundred and sixty-seven cases will be required to achieve statistical power of 0.8 at the alpha level of 0.05 if the relative risk for AD in those persons carrying a putative risk allele is 2.0 compared with that in persons without the allele. Therefore, our sample size is not enough to assess the genotypic relative risk less than 2. However, combined analysis of the *FLG* 3321delA and S2554X null mutations

showed significant association with AD. R501X and 2282del4 were the first null mutations reported to be associated with AD in a European population (Irvine 2007), and a subsequent study identified three additional null alleles of *FLG* (R2447X, S3247X, 3702delG) associated with development of AD (Sandilands et al. 2007). These three null mutations were not found in an Asian population (Sandilands et al. 2007), whereas the3321delA and S2554X null alleles were not found in a European population (Sandilands et al. 2007). Our present tag SNP analysis included common missense mutations (D3105Y and L3970S), but the results were not consistent across the family and case-control data. In the present study, *FLG* null mutations were also associated with high IgE levels. Allergens can penetrate through the skin, leading to allergic sensitization in susceptible individuals. Dysfunction of the skin barrier may accelerate allergen penetration, and therefore, loss of *FLG* function can contribute to allergic sensitization and the high-IgE phenotype.

In conclusion, *FLG* null alleles, not common variants, are associated with development of AD and high IgE levels in the Japanese, confirming the importance of null mutations in *FLG* for disease onset and allergic sensitization in AD patients.

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Table1. TDT and PDT analysis of the *FLG* polymorphisms in the Japanese AD families

Polymorphism	SNP	Allele	Allele frequency	T[a]	NT[b]	TDT *P* value	PDT *P* value
rs11582620	A/G	A	0.89	41	26	0.066	0.059
rs11586114	A/G	G	0.55	90	78	0.35	0.11
rs1933064	A/G	A	0.85	44	35	0.31	0.24
rs2065958(D3105Y)	A/C	C	0.36	69	92	0.069	0.038
rs3814299(L3970S)	A/G	A	0.63	95	70	0.24	0.38
rs12730241	A/G	G	0.057	21	14	0.051	0.021
3321delA	A/-	del	0.014	4	4	1	1
S2554X	C/G	G	0.021	10	4	0.11	0.16

[a]T: Number of alleles transmitted to affected children

[b]NT: Number of alleles not transmitted to affected children

Table 2. Case-control study for AD in *FLG* polymorphisms

Polymorphism	Population[a]	Genotype count (frequency%)						Genotypic P^b	Odds ratio (95% CI)[c]	Allelic P^b
		C/C		C/A		A/A				
rs2065958	AD	58	(15.6)	156	(41.9)	158	(42.5)	0.94	1.0 (0.8-1.2)	0.84
	only AD	9	(12.3)	25	(34.3)	39	(53.4)	0.14	0.6 (0.4-1.0)	0.068
	IgE>1000IU/L	30	(14.2)	90	(42.9)	90	(42.9)	0.89	1.0 (0.7-1.3)	0.62
	early onset	12	(10.7)	57	(50.9)	43	(38.4)	0.20	1.1 (0.8-1.7)	0.81
	Controls	142	(15.5)	393	(43.0)	380	(41.5)			
		A/A		A/G		G/G				
rs3814299	AD	2	(0.5)	52	(13.8)	322	(85.7)	0.64	1.1 (0.8-1.6)	0.54
	AD alone	0	(0)	12	(16.0)	63	(84.0)	0.46	1.3 (0.7-2.5)	0.57
	IgE>1000IU/L	1	(0.5)	34	(16.1)	176	(83.4)	0.26	1.4 (0.9-2.0)	0.21
	early onset	1	(0.9)	14	(12.5)	97	(86.6)	0.98	1.1 (0.6-1.9)	0.84
	Controls	7	(0.8)	111	(12.0)	804	(87.2)			
		A/A		A/-		-/-				
3321delA	AD	356	(97.3)	10	(2.7)	0	(0)	0.077	2.1 (0.9-4.9)	0.077
	AD alone	71	(95.9)	3	(4.1)	0	(0)	0.064	3.2 (0.9-11.5)	0.064
	IgE>1000IU/L	198	(95.6)	9	(4.4)	0	(0)	0.0038	3.4 (1.4-8.2)	0.0036
	early onset	103	(97.2)	3	(2.8)	0	(0)	0.22	2.2 (0.6-7.9)	0.22
	Controls	902	(98.7)	12	(1.3)	0	(0)			
		C/C		C/G		G/G				
S2554X	AD	365	(97.3)	10	(2.7)	0	(0)	0.0012	5.0 (1.7-14.8)	0.0012
	AD alone	71	(94.7)	4	(5.3)	0	(0)	0.000024	10.3 (2.7-39.4)	0.000024
	IgE>1000IU/L	207	(97.6)	5	(2.3)	0	(0)	0.011	4.4 (1.3-15.5)	0.011
	early onset	110	(98.2)	2	(1.8)	0	(0)	0.13	3.3 (0.6-17.4)	0.13
	Controls	918	(99.5)	5	(0.5)	0	(0)			
combined		wild allele / wild allele		wild / at least one null allele		null / null				
(3321delA and	AD	355	(64.7)	20	(5.3)	0	(0)	0.00073	3.0 (1.5-5.8)	0.00067
S2554X)	AD alone	67	(90.5)	7	(9.5)	0	(0)	0.000047	5.5 (2.2-13.8)	0.000042
	IgE>1000IU/L	198	(93.4)	14	(6.6)	0	(0)	0.00015	3.7 (1.8-7.7)	0.00014
	early onset	101	(95.3)	5	(4.7)	0	(0)	0.054	2.6 (1.0-7.3)	0.056
	Controls	900	(98.1)	17	(1.9)	0	(0)			

[a] AD alone; AD patients without other atopic disease. Early onset; patients whose disease onset was younger than 2 years old.

[b] Genotypic *P* and alleleic *P* values were calculated with χ^2 test in comparison with genotype and allele counts in controls, respectively.

[c] Odds ratio for the wild type homozygote versus minor allele heterozygote and minor allele homozygote. CI, confidence interval.

Table 3. Combined *P* values of *FLG* polymorphisms in families and case-control study

Polymorphism		Genotype count (frequency%)						Genotypic P^a	Odds ratio (95% CI)[b]	Allelic P^a
		C/C		C/A		A/A				
rs2065958	AD	65	(13.8)	202	(43.0)	203	(43.2)	0.54	0.92 (0.7-1.1)	0.28
	Controls	161	(15.9)	437	(43.0)	417	(41.1)			
		A/A		A/G		G/G				
rs12730241	AD	4	(0.8)	60	(12.6)	412	(86.6)	0.91	1.1 (0.8-1.5)	0.69
	Controls	7	(0.7)	124	(12.1)	892	(87.2)			
		A/A		A/ -		- / -				
3323delA	AD	454	(97.4)	12	(2.6)	0	(0)	0.14	1.8 (0.8-3.8)	0.14
	Controls	999	(98.5)	15	(1.5)	0	(0)			
		C/C		C/G		G/G				
S2554X	AD	459	(96.6)	16	(3.4)	0	(0)	0.000091	5.0 (2.1-12.3)	0.0001
	Controls	1010	(99.3)	7	(0.7)	0	(0)			
combined		wild / wild		wild / null		null / null				
(3321delA and	AD	438	(94.0)	28	(6.0)	0	(0)	0.00015	2.9 (1.6-5.1)	0.00017
S2554X)	Controls	992	(97.8)	22	(2.2)	0	(0)			

[a]Genotypic *P* and alleleic *P* values were calculated with χ^2 test in comparison with genotype and allele counts in controls, respectively.
[b] Odds ratio for the wild type homozygote versus minor allele heterozygote and minor allele homozygote. CI, confidence interval.

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Repeated instillations of *Dermatophagoides farinae* into the airways can induce Th2-dependent airway hyperresponsiveness, eosinophilia and remodeling in mice
Effect of intratracheal treatment of fluticasone propionate

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Abstract

Dermatophagoides farinae are known to be a common environmental allergen causing allergic asthma; however, little is known about their pathophysiological effect via the allergenicities in vivo. Therefore, we first established a mouse model of asthma induced by repeated instillations of *D. farinae*. Second, to investigate whether the asthmatic responses are Th2-dependent, we examined the effect of the deficiency of interleukin-4 (IL-4) receptor α chain gene. Finally, we examined the effect of fluticasone propionate on this model. Mice were instilled with *D. farinae* without additional adjuvants into the trachea 8 times. After the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological examination were carried out. The instillation of the allergen-induced airway hyperresponsiveness, the accumulation of inflammatory cells and increases in the levels of Th2 cytokines and transforming growth factor-β_1 production in the bronchoalveolar lavage fluid dose dependently. The number of goblet cells in the epithelium and the extent of the fibrotic area beneath the basement membrane were also increased in the morphometric study. In contrast, the defect of IL-4/IL-13 signaling through IL-4 receptor α chain completely abrogated all these responses. Furthermore, the simultaneous instillation of fluticasone propionate with the allergen showed significant inhibition or an inhibitory tendency of these changes. These findings demonstrate that the repetitive intratracheal instillations of *D. farinae* can induce airway remodeling through Th2-type inflammation, and that fluticasone propionate inhibits *D. farinae*-induced airway remodeling in mice, and this model would be useful for studying mechanisms involved in the development of allergic asthma.

Keywords: Airway; Asthma; Corticosteroid; *Dermatophagoides farinae*; Eosinophil; Goblet cell; IL-13; Mouse; Subepithelial fibrosis

1. Introduction

Bronchial asthma is one of the most common health problems in the worldwide, especially within industrialized societies, and the prevalence rates have been increasing considerably over the last few decades (Mannino et al., 2002; Robertson et al., 2004; Verlato et al., 2003), for reason that are not yet completely understood. Changes in lifestyle and an increase in indoor allergen exposure caused by higher indoor temperature and humidity have been suggested as potential determinants, and it is reasonable to consider that environmental exposures to allergens are of primary importance for the prevalence and development of asthma, in genetically predisposed individuals, because genes

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