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ARTICLE

Effect of filaggrin loss-of-function mutations on atopic dermatitis in young age: a longitudinal birth cohort study

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease, and skin barrier defects are often observed in patients with AD. So far, few association studies between *FLG* loss-of-function mutations and onset of AD in longitudinal studies of early childhood have been reported. In the present study, we aimed to investigate the effect of *FLG* loss-of-function mutations on the development of AD in a longitudinal birth cohort study. The status of AD diagnosis at each age until 6 years was collected from the Tokyo Children's Health, Illness, and Development (T-CHILD) study. We analyzed eight loss-of-function mutations in *FLG* in 712 participants. *FLG* loss-of-function mutations were significantly associated with AD onset in infancy (≤2 years) ($P < 0.001$, OR 3.54, 95% CI 1.88–6.65), but not with AD onset in childhood (≥3 years) ($P = 0.981$, OR 0.99, 95% CI 0.29–3.36), and none of the children in the present cohort who developed AD at 5 years of age or later carried *FLG* loss-of-function mutations. Our data support the notion that the effect of *FLG* loss-of-function mutations is prominent during a very early stage of life.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by eczema with itching and dysfunction of the skin barrier [1, 2]. Recently, the prevalence of AD has increased, causing considerable morbidity in childhood, estimated to be between 15% and 30% among children (the morbidity is between 2% and 10% among adults) [2]. It is also reported that AD commonly occurs by 1 year of age and that ~85% of patients develop the symptoms by 5 years of age [2, 3]. Development of AD is influenced by genetics and by various environmental factors, which show complex interactions, and the estimate of the genetic components for AD is as high as 84% [2, 4]. AD has been found to be accompanied by ichthyosis vulgaris, which is the most common inherited disorder of skin barrier dysfunction [5]. Loss-of-function mutations in the filaggrin gene (*FLG*) were first identified in patients with ichthyosis vulgaris [5], and subsequently, the genetic association with AD has been extensively reported [6]. *FLG* is an important key protein for functioning of the skin barrier, which prevents invasion of toxic chemicals, bacteria, and allergens and also acts as a moisturizing factor [7]. Skin barrier dysfunction and dry skin are associated with AD; therefore, *FLG* loss-of-function mutations may influence the physical skin barrier, resulting in antigen penetration of the lower layers of the epidermis and activation of the immune response [7].

An epidemiologic study related to *FLG* loss-of-function mutations in AD patients provided evidence of a strong association with early-age onset, but not with late-childhood or adult onset [8], and there may be a possible influence of different ratios of genetic and acquired environment factors. Early-age-onset AD patients may have more predisposing genetic factors,

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while in late-childhood-onset or adult-onset AD patients, the development of the disease may be associated with more environmental exposure. However, few association studies between *FLG* loss-of-function mutations and onset of AD in longitudinal studies of early childhood have been reported [9].

In the present study, we aimed to investigate the effect of *FLG* loss-of-function mutations on the development of AD in a longitudinal birth cohort study.

Materials and methods

Ethical issues

This study was approved by the human genome research ethics committees of the University of Tsukuba (acceptance number 242) and the National Center for Child Health and Development (acceptance number 533) and was conducted according to the Declaration of Helsinki.

Study participants

The Tokyo Children's Health, Illness, and Development (T-CHILD) study (Seiiku Cohort Study) was a single-center, prospective, and hospital-based birth cohort study. The design of this study has been described in detail elsewhere [10]. A total of 1701 pregnant women at less than 16 weeks' gestation were enrolled at the National Center for Child Health and Development hospital between October 2003 and December 2005, and 1550 children were born to the enrolled women. During the follow-up periods at 4–5 years of age, 738 children participated in the genome study (Fig. 1).

Questionnaire

The schedule for sending the questionnaires is shown in Supplementary Fig. 1. The questionnaire, including questions on the status of allergic diseases, was mailed at the beginning of each participant's birthday month every year, and the parents were asked to return the questionnaire within 2 weeks of receipt.

Allergic diseases and symptoms including asthma, rhinitis, and wheezing at 5 years of age were investigated on the basis of the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire, which was completed by the parents [11]. The ISAAC questionnaire was translated into Japanese and validated through the process of back translation with the cooperation of sterling committee members [12, 13]. Details of the questionnaire are described in Supplementary Table 1, and the ISAAC questionnaire is available at the study's website (http://isaac.auckland.ac.nz/phases/phasethree/corequestionnaire_6–7.pdf).

In the present study, a questionnaire regarding doctor-diagnosed AD was available from 6 months to 6 years of age, but the ISAAC questionnaire regarding AD (with questions such as "Has your child had this itchy rash at any time in the past 12 months?") was available only from 4 to 6 years of age, not from 6 months to 3 years of age. Therefore, we used the questionnaire regarding doctor-diagnosed AD for the analysis. AD diagnosis was defined at 6 months and 1 year of age as a positive response to the following question: "Has your child been diagnosed by a doctor as having atopic dermatitis," and at 1.5 years old, as a positive response to the following question: "Since becoming 1 year old, has your child been diagnosed by a doctor as having atopic dermatitis?" AD diagnosis from 2 to 6 years of age was defined as a positive response to the following question in each age group:

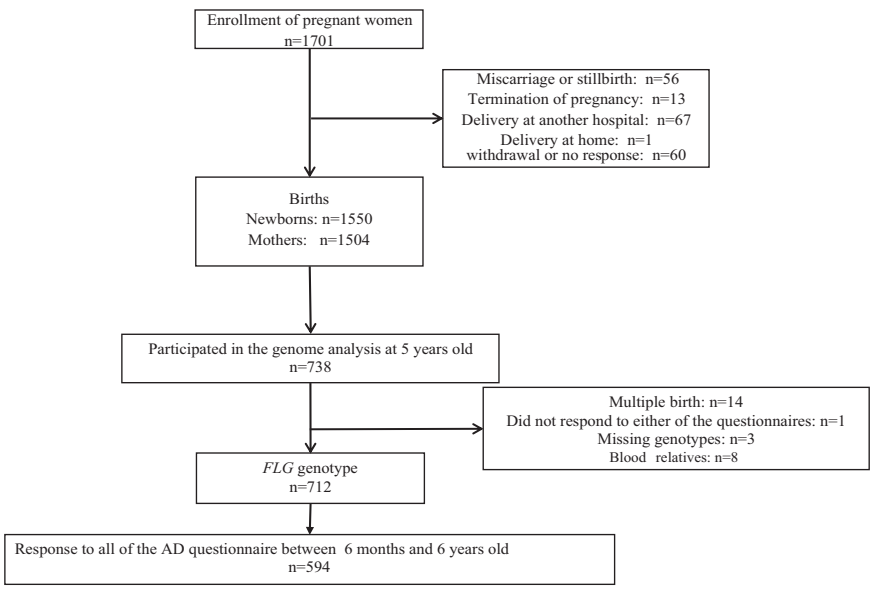

Fig. 1 Flowchart of selection of participants

"Does your child currently have a diagnosis of atopic dermatitis from a doctor?"

Among the participants who answered all the questions regarding doctor-diagnosed AD from 6 months to 6 years of age, we defined AD onset in infancy (≤2 years) as a positive response to the question described above regarding any time from 6 months to 2 years of age. We defined AD onset in childhood (≥3 years) as a positive response to the question regarding any time from 3 to 6 years of age, but not to the question regarding any time from 6 months to 2 years of age.

Genotyping for *FLG* loss-of-function mutations

DNA was extracted from saliva using Oragene DNA (Genotek, Ottawa, Canada) or blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. So far, 10 FLG loss-of-function mutations, including p.Arg501Ter, c.441–442del, c.3321del, p.Ser1695Ter, p.Gln1701Ter, p.Gln1790Ter, p.Ser2554Ter, p.Ser2889Ter, p.Ser3296Ter, and p.Lys4022Ter, have been investigated in the Japanese population [14]. Among these, we genotyped eight mutations (c.3321del, p.Ser1695Ter, p.Gln1701Ter, p.Gln1790Ter, p.Ser2554Ter, p.Ser2889Ter, p.Ser3296Ter, and p.Lys4022Ter) because p.Arg501Ter and c.441–442del were not detected in the general Japanese population [14]. We determined c.3321del by size, using a fluorescently-labeled polymerase chain reaction (PCR). PCR products were mixed with GeneScan™ 500 ROX™ dye Size Standard (Thermo Fisher Scientific, Waltham, MA, USA) and Hi-Di Formamide, after which capillary electrophoresis was performed using an Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific). p.Ser2554Ter, p.Ser2889Ter, p.Ser3296Ter, and p.Lys4022Ter were determined using a TaqMan Assay-by-Design system for single-nucleotide polymorphism genotyping (Thermo Fisher Scientific). To genotype p.Ser1695Ter, p.Gln1701Ter, and p.Gln1790Ter, PCR was performed to amplify the genomic region including p.Ser1695Ter and p.Gln1701Ter (using the primer pair 5′-GTC AGC AGA CAG CTC CAC AG-3′ and 5′-GTG TGT CTG ACT CTT CTG AG-3′; expecting a product size of 134 bp) and to amplify the genomic region including p.Gln1790Ter (using the primer pair 5′-CAC AGG GCC CAG CAC TG-3′ and 5′-ACC GAT TGC TCA TAG TGG GAT C-3′; expecting a product size of 156 bp). The PCR product was then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI PRISM 3130 Genetic Analyzer (Thermo Fisher Scientific). The primer pair used to amplify the region including p.Ser2554Ter was 5′-CCA CAC GTG GCC GGT CAG CA-3′ and the reverse primer 5′-CTA CCG AAT GCT CGT GGT GGT-3′ [15]; then, the primer 5′-TCT CAT CAC AGC CAC ACC AC-3′ was used as the sequence primer. A predesigned copy number TaqMan assay was performed to determine the copy number targeting the genomic region around chr1: 152303147 on build GRCh38 (assay ID, Hs00156031_cn, Thermo Fisher Scientific) with an RNase P internal reference TaqMan assay (Thermo Fisher Scientific) co-amplified in a duplex PCR reaction using the ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The copy number assay was performed in quadruplicate.

Statistical analysis

Association between status of allergic respiratory diseases and *FLG* genotype at 5 years of age was analyzed using logistic regression analysis with an additive genotype model adjusted for sex and was performed using R version 3.1.1 (https://www.r-project.org/). Among the participants who answered all the questions regarding doctor-diagnosed AD from 6 months to 6 years of age, association between a positive AD diagnosis and the *FLG* genotype was also analyzed using logistic regression analysis with an additive genotype model adjusted for sex. Disease-free survival curves of AD development were drawn using the Kaplan–Meier method by means of GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). The Bonferroni correction was applied for correcting the multiple testing, and the thresholds were set at $\alpha = 0.05/3$ (1.7×10^{-2}) for the three analyses: infancy, childhood, and all studied ages. All associations based on the models were presented with odds ratios (ORs), 95% confidence intervals (CIs), and *P* values.

Results

A flowchart describing the strategy for selecting the study participants is shown in Fig. 1. Among the 738 participants in the present genome study, we randomly selected 1 of the children with multiple birth or blood relatives for inclusion in the present analysis. We also excluded 1 participant who did not respond to either questionnaire, resulting in 715 children. The successful genotyping rate of *FLG* loss-of-function mutations was 99.9%, and we used the genotypic data of the 712 children without missing genotypes for further analysis.

The outcomes in terms of allergic respiratory diseases at 5 years of age were determined on the basis of the ISAAC questionnaire. Because we wanted to evaluate the AD status at each age, we selected the questionnaire regarding AD diagnosis by a doctor at 6 months and at 1, 1.5, 2, 3, 4, 5, and 6 years of age (Supplementary Table 1).

The numbers of responders to the questions regarding allergic diseases at 5 years of age and those regarding AD

Table 1 Distribution of *FLG* loss-of-function mutations in the T-CHILD genome study

	c.3321.del	p.Ser1695Ter	p.Gln1701Ter	p.Gln1790Ter	p.Ser2554Ter	p.Ser2889Ter	p.Ser3296Ter	p.Lys4022Ter	Combined
Genotypes[a]									
AA	702	712	711	712	704	690	709	686	642
Aa	10	0	1	0	7	22	3	26	69
aa	0	0	0	0	1	0	0	0	1

[a]AA, homozygous wild-type for filaggrin (*FLG*) loss-of-function mutation; Aa, heterozygous for *FLG* loss-of-function mutation; aa, homozygous for *FLG* loss-of-function mutation

diagnosis at each age are described in Supplementary Table 2. Table 1 shows the distribution of *FLG* loss-of-function mutations in the T-CHILD genome study. The allele frequencies for *FLG* loss-of-function mutations in this study were 0.70%, 0.07%, 0.63%, 1.54%, 0.21%, and 1.83% for c.3321del, p.Gln1701Ter, p.Ser2554Ter, p.Ser2889Ter, p.Ser3296Ter, and p.Lys4022Ter, respectively; p.Ser1695Ter and p.Gln1790Ter were not detected (0%). Whereas 69 children (9.7%) were heterozygous for *FLG* loss-of-function mutations, 1 child (0.14%) was homozygous for the mutations. There was one homozygote for p.Ser2554Ter, and the direct sequence results of the homozygote are shown in Supplementary Fig. 2. We also determined the status of the copy number using the probe near the p.Ser2554Ter variant in the p.Ser2554Ter mutant-type homozygote, four heterozygotes, and two wild-type homozygotes. All the participants analyzed were determined as carrying two copies (Supplementary Fig. 3).

Table 2 shows the results of the association analysis between the *FLG* genotype and allergic respiratory diseases in the participants at 5 years of age. *FLG* loss-of-function mutations were not associated with allergic respiratory diseases at 5 years of age ($P > 0.05$). Among the 712 participants, the parents of 594 participants answered all the questions regarding doctor-diagnosed AD at each age. Table 3 shows the effect of *FLG* loss-of-function mutations on AD onset in infancy and childhood. Although associations were strong for AD at all ages (i.e., AD developed at any time from 6 months to 6 years; $P = 0.002$, OR 2.58, 95% CI 1.43–4.66), the association was notably stronger with higher ORs for infancy-onset (≤2 years) AD ($P < 0.001$, OR 3.54, 95% CI 1.88–6.65), but not for childhood-onset (≥3 years) AD ($P = 0.981$, OR 0.99, 95% CI 0.29–3.36). Also, none of the children who carried *FLG* loss-of-function mutations developed AD at the age of 5 years or later.

A Kaplan–Meier plot of the disease-free survival curves of AD development showed that a significant difference existed depending on the carrier status of *FLG* loss-of-function mutations ($P < 0.001$; Fig. 2).

Discussion

The findings of this longitudinal birth cohort study demonstrated that the association between *FLG* loss-of-function mutations and AD and the effect of the *FLG* loss-of-function mutations were prominent during a very early stage of life.

Several studies have shown *FLG* loss-of-function mutations and development of AD at an early age. Rupnik et al. reported that *FLG* loss-of-function mutations were associated with AD until 8 years of age, but not in children aged

Table 2 Effect of *FLG* loss-of-function mutations for allergic diseases at 5 years of age

Allergic disease	Disease status (positive/negative (%positive))		OR (95% CI)[a]	*P* values
	FLG AA	*FLG* Aa + aa		
Ever asthma	98/530 (15.6%)	15/54 (21.7%)	1.47 (0.81–2.67)	0.21
Current asthma	65/563 (10.4%)	10/59 (14.5%)	1.43 (0.71–2.89)	0.32
Ever rhinitis	255/373 (40.6%)	28/41 (40.6%)	0.98 (0.60–1.61)	0.93
Current rhinitis	238/389 (38.0%)	26/43 (37.7%)	0.97 (0.59–1.60)	0.90
Ever wheeze	193/434 (30.8%)	22/47 (31.9%)	1.05 (0.62–1.78)	0.87
Current wheeze	111/508 (17.9%)	15/53 (22.1%)	1.28 (0.70–2.33)	0.42

[a]Odds ratio (95% CI)

Table 3 Effect of *FLG* loss-of-function mutations on onset of AD in infancy and childhood

Age	Disease status (positive/negative (% positive))		OR (95% CI)[a]	*P* values
	FLG AA	*FLG* Aa + aa		
Onset at infancy (≤2 years)	57/479 (10.6%)	17/41 (29.3%)	3.54 (1.88–6.65)	<0.001[b]
[c]Onset at childhood (≥3 years)	36/443 (7.5%)	3/38 (7.3%)	0.99 (0.29–3.36)	0.981
All age (6 months–6 years)	93/443 (17.4%)	20/38 (34.5%)	2.58 (1.43–4.66)	0.002[b]

[a]Odds ratio (95% CI)

[b]$P < 1.7 \times 10^{-2}$

[c]Odds ratio and *P* values for onset in childhood were calculated in participants with the AA genotype ($n = 479$) and in those with Aa + aa genotypes ($n = 41$) in which infant-onset AD was excluded

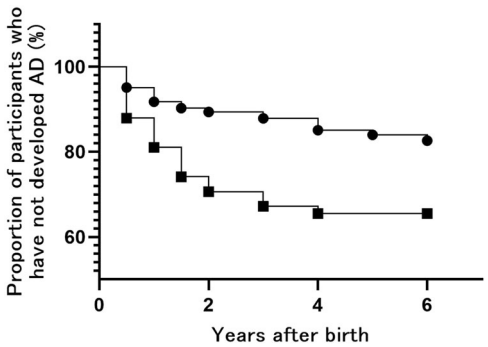

Fig. 2 Proportions of participants who did not develop AD according to the status of *FLG* mutations. Kaplan–Meier plots show the proportions of participants carrying mutations (rectangles) and those not carrying mutations (circles). The Mantel–Cox log-rank test indicated significant differences between the groups ($P < 0.001$)

older than 8 years nor in adulthood (>18 years) [8]. Similar results were also reported showing that the effect of *FLG* loss-of-function mutations on the development of eczema was stronger in early-onset AD [16].

Henderson et al. performed a longitudinal birth cohort study and showed the comprehensive effects of *FLG* mutations for atopic phenotypes, including asthma, sensitizations, and persistence of AD, suggesting the necessity of an early intervention study of AD for *FLG* mutation carriers [9]. Our study has not shown an association between allergic respiratory diseases and *FLG* loss-of-function mutations but has shown the association of AD with *FLG* loss-of-function mutations, which is consistent with the findings of a study in the Japanese population [17]. Because the effect size of allergic respiratory diseases on *FLG* loss-of-function mutations is expected to be lower than that of AD, we cannot exclude the possibility that our sample size is not large enough to detect association between allergic respiratory diseases and *FLG* loss-of-function mutations.

The important function of the skin is to act as a protective barrier against external stimuli, such as toxic chemicals, and thus prevent allergens and pathogens from entering the body. Dysfunction of this permeability barrier causes transcutaneous epidermal water loss (TEWL) and allows unrestricted access of antigens [18]. FLG plays major roles in skin barrier function. Skin barrier function can be measured by TEWL, and Kelleher et al. reported that *FLG* mutation status was not associated with an elevated TEWL at birth, but was associated with an elevated TEWL at 2 and 6 months of age [19]. The use of a moisturizer for skin care is useful to prevent deterioration of AD, and several studies have shown that continual use of moisturizer from the newborn period may reduce the incidence or delay the development of AD [20, 21]. In the present study, *FLG* loss-of-function mutations were significantly associated with infancy-onset (≤2 years) AD, but not with childhood-onset (≥3 years) AD. Skin barrier functions, measured by TEWL and stratum corneum thickness, reach adult values at about 4 years of age [22]; therefore, our data may suggest

that the preventive effect of continual use of moisturizer during infancy on AD would be more beneficial in carriers of *FLG* loss-of-function mutations than in those without the mutations.

Several limitations to the present study should be acknowledged. The population of our study was from a single recruitment center in Tokyo (urban area of Japan); thus, the recruited participants may not be representative of the entire Japanese population. Furthermore, responses to the ISAAC questionnaire for AD/eczema ("Has your child had this itchy rash at any time in the past 12 months?") were not available from 6 months to 3 years of age. Thus, we used the responses of the doctor-diagnosed AD questionnaires from 6 months to 6 years old, but ambiguity may remain without terms to define the period such as "the past 12 months." In addition, the possibility remains that some of the participants may not have been diagnosed owing to their not having seen doctors. Lastly, clinical details such as severity of AD for the *FLG* loss-of-function mutant homozygote were not available in the present study owing to the cohort of children studied being a general population cohort. The participant developed AD during infancy (≤2 years), and at age 5 years, the findings were not positive for allergic respiratory diseases (wheezing, asthma, and rhinitis). Although our direct sequence results and copy number analysis support the participant being homozygous for the *FLG* loss-of-function mutation, because the parents' DNA samples were not available, we cannot completely exclude the possibility of hemiallelic amplification.

In conclusion, the present study supports the notion that the effect of *FLG* loss-of-function mutation is prominent in very early life. A clinical trial to examine the effects of application of emollient for preventing atopic eczema during the first year of life is ongoing, and the trial will also investigate whether *FLG* genotypes can be possible stratifiers of response to emollient intervention [23]. Accumulation of these data will lead to better therapeutic interventions for AD in high-risk infants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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