

Clinical characteristics of Korean patients with filaggrin-related atopic dermatitis

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Summary

Background. Filaggrin is a key protein involved in skin barrier function. Mutations in the gene encoding filaggrin (*FLG*) have been identified as the cause of ichthyosis vulgaris and have been shown to be major predisposing factors for atopic dermatitis (AD).

Aim. To investigate the clinical characteristics of patients with AD with *FLG* mutations and determine the differences between patients with AD with and without *FLG* mutations.

Methods. We identified *FLG* mutations in patients with AD by complete sequencing and SNaPshot methods, and then analysed the data on clinical characteristics from questionnaire responses.

Results. We found that earlier age of AD onset ($P < 0.05$), tendency to respiratory atopy ($P = 0.03$), more severe clinical characteristics of AD (higher Eczema Area and Severity Index, $P = 0.02$) and decrease in skin hydration ($P = 0.04$) were associated with *FLG*-related AD.

Conclusion. Our data demonstrate that *FLG* mutations are indicators of a poor prognosis in AD, and are predisposing factors that exist in early infancy and persist into adulthood.

Introduction

Mutations in the filaggrin gene (*FLG*) have been reported as important predisposing factors for atopic dermatitis (AD) and atopic asthma, indicating that these mutations predispose to atopic march, to early onset of AD that persists into adulthood and to more severe asthma.^{1,2} Interestingly, distinctive differences in *FLG* mutations have been found between ethnic groups. Specifically, the original *FLG* mutations p.R501X and c.2282del4 found in European populations were not found in an East Asian population.³

There is accumulating evidence from epidemiological studies of AD that disease onset occurs within the

first 6 months of life in 45% of children and within the first 12 months in 60%, and that 85% are affected before their fifth birthday.⁴ In children with AD onset before 2 years of age, 20% will have persistent AD by 7 years of age. Onset after adolescence has been recorded in 16.8% of adults with AD.⁵

We hypothesized that skin barrier abnormalities are the primary defects leading to AD symptoms in *FLG*-related AD, and as a result, *FLG*-related AD should have special characteristics compared with non *FLG*-related AD. We therefore investigated the clinical characteristics of patients with AD with the two *FLG* mutations, p.K4022X and 3321delA, and evaluated skin barrier function and clinical severity in patients with AD with and without the two *FLG* mutations.

Methods

The study was approved by the institutional research ethics committee of Chung-Ang University Hospital, and all participants provided informed written consent.

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Study population

We recruited 1110 Korean patients with AD, of whom 68 harboured the *FLG* mutations, p.K4022X and 3321delA (*FLG*-related AD), while the other 1042 had no apparent *FLG* mutation (non-*FLG*-related AD). The demographics and clinical parameters of the study cohort are summarized in Table 1.

Filaggrin genotyping

Genotyping for the p.K4022X and 3321delA mutations was performed using PCR. We tried to follow the reported 'overlapping long-range PCR' method for confirming *FLG* polymorphisms and mutations, but found that modifications were necessary under most reaction conditions. We established the reaction conditions for PCR and sequencing of most of the DNA fragments through trial and error.

Exons 3 (*FLG* repeats 1–3) and exon 3 (7–10) were particularly problematic. We obtained a single-band product of the correct size by PCR for both, but sequencing did not progress smoothly. For this reason, we performed sequencing analysis by dividing exon 3 (1–3) into two overlapping fragments. In the case of exon 3 (7–10), PCR with previously reported⁶ forward and reverse primers did not work well.⁶ Using newly designed primers, we produced the desired PCR product and used it to conduct sequencing analysis. Additional repeats after repeat 8 and 10 (repeats 8.2 and 10.2, respectively) obtained PCR products for exon 3 (7–8), (7–10), (9–10) and (10).

Briefly, genomic DNA from patients with atopy was extracted from peripheral blood samples (G-DEXTM Genomic DNA Extraction Kit; iNtRON Biotechnology, Sungnam, South Korea). PCR amplifications were performed in 40 µL reaction volumes containing 200 ng

genomic DNA, 2.5 mmol/L MgCl₂, 200 µmol/L of each dNTP, 10 pmol/L of each primer, 2 µL 5X BD (Solgent Co. Ltd. Daejeon, Korea) and 2 U *Taq* DNA polymerase (TaKaRa Bio Inc. Otsu, Japan) using the a PCR system (GeneAmp 2700; Applied Biosystems, Princeton, NJ, USA). Genomic DNA was amplified using the primers listed in Table 2. The amplified PCR products were separated by electrophoresis in 0.8% agarose gel.

Sequencing

DNA sequencing was performed using a DNA sequencer (3100/3700; Applied Biosystems) after purification with QIAquick PCR purification kits (Qiagen, Valencia, CA, USA). We performed SNaPshot sequencing for 1003 patients, but full gene sequencing only for 107 patients, because of the cost.

Characteristics and disease severity

Based on genotype, patients with AD were divided into *FLG*-related and non-*FLG*-related groups. The *FLG*-related AD group was then further divided into subgroups (homozygous or heterozygous for the p.K4022X or 3321delA *FLG* alleles). Disease characteristics such as onset, presence of respiratory atopic symptoms and family history were based on responses to questionnaires.

Using a corneometer (MPA 5; Courage and Khazaka Electronic Co., Germany), transepidermal water loss (TEWL), which reflects the amount of water escaping through the SC, and skin hydration were measured on the subjects' forearms.

Atopy severity was measured by a dermatologist using the Eczema Area and Severity Index (EASI), which is often used in clinical trials,⁷ but excluding two subjective symptoms (itch and sleep loss).

Laboratory tests

Peripheral blood eosinophil count (normal range 40–400/µL), eosinophil cationic protein (ECP) (2–16 µg/L) and total serum IgE (1–100 kU/L) were measured. Extrinsic AD was defined as AD with concomitant sensitization and/or IgE level > 100 kU/L.

Statistical analysis

All statistical analyses were performed using SPSS for Windows (v19; IBM SPSS, Armonk, NY, USA). The χ^2 test and independent two-sample *t*-test were used to compare continuous variables. Data with *P* < 0.05 were considered statistically significant. To test for

Table 1 Demographic characteristics of patients with atopic dermatitis divided according to mutation.

	Mutation	
	c3321delA	p.K4022X
Patients, n/N (%)	18/1110 (1.62)	50/1110 (4.50)
Age at examination, years*	10 (1–34)	14 (1–38)
Mean age of AD onset, n (%)		
Birth to 2 years	11 (61)	26 (52)
< 10 years	6 (33)	13 (26)
> 10 years	1 (6)	11 (22)
IgE, kU/L*	872 (8.15–5000)	497 (3.73–5000)
EASI*	8.3 (0.2–30)	7.1 (0.2–30)

EASI, Eczema Area and Severity Index. *Mean (range).

Table 2 PCR primers used for *FLG* mutation analysis.

<i>FLG</i>	Direction	Sequences 5'→3'	Annealing temperature, °C	Product size, bp
Exon 1	Forward	CGTGAGGAAGCTGGGAAGTA	60	381
	Reverse	TTATGCCCTCATTTTCCTTCT		
Exon 2	Forward	CTACTAAGTCCAGCTGTAAGTG	60	431
	Reverse	GCTCTATCTTTGGTCTTGTCAG		
Exon 3 repeats 1–3 (1)	Forward	GCTGATAATGTGATTCTGTCTG	60.1	1710
	Reverse	GACCCCGATGATTGTTCTCTGT		
1–3 (2)	Forward	CACGGAAAGGCTGGGCTGA	67.2	2642
	Reverse	GACCCCGATGATTGTTCTCTGT		
3–5	Forward	GCAAGCAGACAACTCGTAAG	65	1916
	Reverse	ACATCAGACCTTTCCTGGGAC		
4–7	Forward	GACAACATTCATCTGTAGTCG	59.9	2609
	Reverse	CTGGCTAAACTGGATCCCCA		
7–8	Forward	CCACACGTGGCCGGTCAGCA	65	1224/2196
	Reverse	CTACCGAATGCTCGTGGTGGT		
7–10	Forward	CCCAGGACAAGCAGGAACT	61.8	3331/4303
	Reverse	GCTTCATGGTGATGCGACCA		
9–10	Forward	CCCAGGACAAGCAGGAACT	65	1367/2342
	Reverse	GCTTCATGGTGATGCGACCA		
10	Forward	GCCCATGGGCGGACCAGGA	65	1753/2728
	Reverse	CTGCACTACCATAGCTGCC		
End	Forward	CTAGTACCGCTAAGGAACATGG	60	781
	Reverse	TGGTCTCTTCGATATTCTGA		

associations with binary traits, logistic regression models were used, with age, sex and genotype as independent variables. In addition, IgE level was evaluated using a linear regression model on log-transformed IgE values with the same covariates.

Results

Of the 1110 patients with AD, 71.7% had extrinsic AD, 40% had asthma and 44.6% had early disease onset (≤ 2 years of age). Mean total IgE of patients with AD was 841.35 KU/L (data not shown).

Frequency of *FLG* null alleles in patients with atopic dermatitis and controls

There were 18 patients with AD who were heterozygous for the 3321delA mutation, while 49 patients were heterozygous and 1 was homozygous for the p.K4022X *FLG* mutation (Table 3).

FLG mutation strongly predisposes to early onset of atopic dermatitis

FLG null alleles were associated with early onset of AD. Mean age of onset was 9 years in patients with *FLG* mutations and 14 years in patients without *FLG* mutations (data not shown). A higher risk of

developing AD by 2 years of age was seen in patients with than in those without *FLG* mutations (54.41% vs. 44.05%, $P = 0.03$) (Table 4).

FLG mutation strongly predisposes to early onset of wheezing and asthma

Based on the results from our questionnaires, we found that a diagnosis of asthma or 'wheezing with whistling' in the prior 12 months had been given to 55.89% of patients with *FLG* gene mutation but only 38.9% of those without *FLG* mutation. Given this significant difference ($P = 0.03$), the symptoms of asthma and a change to atopic march appear to be associated with *FLG* mutation (Table 4).

FLG mutation and family history

There was a stronger association with family history in the *FLG*-related AD group than the non-*FLG*-related

Table 3 Frequency of *FLG* null alleles in patients with atopic dermatitis and controls.

Genotype	3321delA	p.K4022X
Aa	18/1110	49/1110
aa	0/1110	1/1110
AA	1082/1110	1050/1110

	AA (wild type)	Aa + aa (heterozygote + homozygote)	P
Total population			
Early onset (≤ 2 years) (%)	459/1042 (44.05)	37/68 (54.41)	< 0.05
Asthma symptom (%)	406/1042 (38.96)	38/68 (55.89)	0.03
Family history (%)	312/1042 (29.94)	25/68 (36.76)	0.10
EASI	5.01	7.41	0.02
Skin hydration	11.76	8.8	0.04
TEWL	14.2	16.4	0.70
IgE level, KU/L	846.6	1096.1	0.94
Extrinsic AD only (IgE > 100)			
n/N (%)	799/1100 (72.64)	48/68 (70.59)	< 0.05
IgE level, KU/L	898.7	1183.3	< 0.05
ECP level, $\mu\text{g/L}$	27.68	43.35	0.04

EASI, Eczema Area and Severity Index; ECP, eosinophil cationic protein.

AD group, but this was not significant ($P = 0.10$) (Table 4). However, we found a significantly ($P = 0.03$) high familial tendency in the *FLG*-related AD group for the extrinsic subtype but not for the intrinsic subtype of AD (data not shown).

Significant correlation observed between Eczema Area and Severity Index and *FLG*-related atopic dermatitis

There was a significant difference ($P < 0.05$) in AD severity between *FLG*-related and non-*FLG*-related AD as indicated by total EASI (7.41 vs. 5.01), and the significance increased as the severity of EASI increased (Table 4).

Significant decrease in skin hydration in *FLG*-related atopic dermatitis

There were significant differences in skin hydration between two groups, with mean skin hydration values on the subjects' forearms being lower for *FLG*-related AD than for non-*FLG*-related AD ($P = 0.04$) (Table 4).

Transepidermal water loss (TEWL) values in each group are also summarized in Table 4. There was no significant difference between the *FLG*-related and non-*FLG*-related groups ($P = 0.70$).

Association between *FLG* mutations and laboratory results

Total eosinophil, ECP and serum IgE levels were analysed. There were no significant differences in total eosinophil count or serum IgE level between the two groups, but there was a significant difference ($P = 0.04$) in mean value of ECP between the groups.

Table 4 Summary of clinical characteristics, disease manifestations and laboratory findings for patients with atopic dermatitis, classified by type.

We divided the samples into subgroups of extrinsic and intrinsic AD, and analysed the serum IgE level for each group. Our results demonstrated that there was no significant difference related to the presence of a *FLG* mutation in the intrinsic AD group, but the IgE level was significantly higher in the *FLG*-related extrinsic AD group (Table 4).

Discussion

FLG mutations have been found to be an important predisposing factor for AD.⁷ Attention has focused on impaired epidermal barrier function, which is a hallmark feature of AD.⁸ It has also been demonstrated that two functional *FLG* mutations are strong risk factors for AD and asthma in the context of AD in European populations.⁹ However, distinct differences have been found in *FLG* mutations between ethnic groups. Furthermore, there are more differences than similarities in *FLG* mutations between ethnic groups. The 3321delA mutation is the most prevalent mutation in five East Asian populations (Korean, Japanese, Taiwanese, Chinese and Singaporean Chinese). Meng *et al.* reported that 3321delA is significantly associated with various AD clinical phenotypes, including skin xerosis, ichthyosis vulgaris, palmar hyperlinearity, keratosis pilaris, white dermatographism, food intolerance and disease severity.¹⁰ Another Singaporean study showed that a combined null *FLG* genotype of 17 mutations detected in cases and controls was strongly associated with increased AD severity.¹¹ However, this association was inconsistent with other studies in the Chinese Han population.¹²

In the current study, we analysed whether the mutations 3321delA and p.K4022X correlated with

clinical characteristics in Korean patients with AD. These two mutations were chosen because they have been observed in many patients with AD, including East Asian patients. The 3321delA mutation was found to be common among East Asians, and the p.K4022X mutation was previously reported in Japanese patients.¹³

The association of *FLG* mutations with asthma is complex. Filaggrin is not expressed in the respiratory epithelium.¹⁴ The assumption is that AD is a causal risk factor for asthma and systemic allergen sensitization in the context of *FLG* mutations.¹⁵ The mechanisms of this relationship are as yet unclear, but increased allergen and pathogen penetration through the SC, followed by stimulation of keratinocyte-derived thymic stromal lymphopoietin in inflamed epidermis may lead to distal effects in the lung.¹⁶ Another study showed that a defect in a single gene may predispose to different allergic phenotypes of the atopic march, and that expression of the subsequent disease stages requires the presence of eczema.¹⁷ These findings highlight the role of the epidermal barrier in the pathogenesis of allergic disease.

In our results, we saw a significant difference in EASI between our two AD groups, with the *FLG*-related AD group having a more severe clinical manifestation than the non-*FLG*-related AD group, and this difference being more prominent in patients with moderate to severe AD by EASI.⁷ Skin hydration was statistically significantly lower in the *FLG*-related AD group, whereas there was no significant difference in TEWL between the two groups. This is an unexpected finding, and might indicate underdetection of *FLG* null mutations, and therefore inability to detect more subtle differences in TEWL. Low skin hydration in the *FLG*-related AD group might be related indirectly to barrier defects but could primarily be related to a deficiency in water-binding filaggrin breakdown products. TEWL was reported to be generally increased in patients with AD, although controversy remains as to whether the defective barrier function in patients with AD is a primary cause of AD or a secondary consequence of dermatitis.¹⁸

Our laboratory tests revealed that there was a significant difference in ECP in the extrinsic AD subgroup while, consistent with previous findings, no association was seen with the intrinsic subtypes of AD.¹ Therefore, it seems conceivable that the key event in AD is a genetically determined primary epidermal barrier disruption that causes reduced resistance to environmental agents such as allergens, irritants and microbes. ECP may serve as a suitable laboratory parameter for

monitoring disease activity in AD.¹⁹ Although we did not analyse the correlation between clinical severity score and serum ECP, the serum ECP level was significantly higher in the *FLG*-related AD group.

Conclusion

We identified correlations between clinical manifestations and questionnaire results, earlier onset of AD, tendency to respiratory atopy, clinical characteristics of more severe AD and decrease in skin hydration in *FLG*-related AD compared with non-*FLG*-related AD.

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What's already known about this topic?

- Filaggrin is essential for triggering a cascade of biochemical and cellular events that lead to the formation of the stratum corneum.
- *FLG* mutations have been reported as predisposing factors for AD and atopic asthma.

What does this study add?

- *FLG* mutations are indicators of a poor prognosis in AD and are predisposing factors that exist in early infancy and persist into adulthood.
- *FLG*-related AD showed earlier onset, more severe type, tendency to asthma, and decrease in skin hydration, compared to non-*FLG*-related.

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