

Filaggrin null mutations in childhood atopic dermatitis among the Chinese

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Summary

Atopic dermatitis (AD) is associated with loss or reduced expression of filaggrin (*FLG*). We evaluated five *FLG* null mutations, namely R501X, 2282del4, R2447X, S2554X, and S2889X, in 174 Chinese children with AD and 191 matched controls. 2282del4, R2447X, S2554X and S2889X mutations were not found in these patients. Heterozygous carriage of R501X was only found in four male patients, and associated with long-term disease severity. *FLG* mutations prevalent in Caucasian and other Asian populations are rarely found in our series.

Introduction

Atopic dermatitis (AD) is characterized by skin dryness and chronic inflammation. It is suggested that skin barrier dysfunction is the major predisposing factor (Smith *et al.*, 2006). Disruption of the skin barrier allows penetration of allergens into skin and sensitization of antigen-presenting cells (Candi *et al.*, 2005). AD was first found to have strong association with the epidermal differentiation complex (EDC) on chromosome 1q21. EDC is a dense cluster of genes encoding a number of essential structural proteins involved in epidermal terminal differentiation including filaggrin (*FLG*). *FLG* is one of the most abundantly expressed proteins in outer epidermis which plays key role in facilitating epidermal differentiation and maintenance of barrier function. Immunohistological and ultrastructural observations indicated a complete absence or marked reduction of *FLG* in keratohyalin granules of the epidermis in AD patients (Smith *et al.*, 2006; Nomura *et al.*, 2007). *FLG* degradation products, amino acids and hygroscopic derivatives, contribute to moisture retention in the cornified layers of epidermis (Rawlings & Harding, 2004). As a result, loss or reduction of *FLG* expression correlates with dry skin and impaired barrier function.

Recently, *FLG* null mutations have been found to be an important predisposing factor for atopic march (Marenholz

et al., 2006; Palmer *et al.*, 2006), and they were strongly associated with early-onset and persistent AD (Barker *et al.*, 2007). *FLG* mutations also correlated with asthma severity (Palmer *et al.*, 2007). Because *FLG* null mutations are associated with severe and persistent AD, the identification of these mutations may serve as more accurate prognostic indicator for AD phenotypes. *FLG* mutations have so far been identified in European, American, Austrian and Japanese ancestry. They are present in up to 10% of the European population and 48% of European AD cohorts (Barker *et al.*, 2007; Sandilands *et al.*, 2007). Strong associations between AD and *FLG* mutations have also been replicated consistently in independent studies, making *FLG* the most promising genetic target for AD.

Five mutations R510X, 2282del4, R2447X, S2554X and S2889X were chosen in this study for the following reasons: R501X and 2282del4 were carried by half of the Caucasian patients with moderate-to-severe AD, with a combined allele frequency of 0.330 and odds ratio (OR) 13.4 (Palmer *et al.*, 2006). S2554X had the highest OR among the comprehensive analysis of *FLG* mutation (Sandilands *et al.*, 2007). We also added two mutations S2554X and S2889X that were found only in Japanese (Nomura *et al.*, 2008), in whom genotype frequencies of a number of allergy candidate genes are close to those in southern Chinese (Leung *et al.*, 2003, 2004, 2009). The purpose of this study was to evaluate whether these five *FLG* null mutations reported in Caucasians and Japanese might also predispose Chinese children to AD and atopy, to investigate their associations with disease severity, quality of life, serum IgE levels, eosinophil counts, skin and epidermal sensitization in AD, and to develop a novel non-invasive diagnostic test through genotype-phenotype correlations through skin hydration (SH) and transepidermal water loss (TEWL) measurements.

Patients and methods

Patients with AD < 18 years of age were recruited from the paediatric dermatology clinic of a university teaching hospital. AD was diagnosed according to criteria proposed by Hanifin and Rajka (1980). Severity and symptomatology including pruritus and sleep loss (in the preceding 3 days) of AD patients were evaluated using SCORAD index (CREAD, 1993; Kunz *et al.*, 1997). Subjects were excluded if information regarding pruritus or sleep loss could not be recalled by patients and caregivers, or if obvious parasomnias were evident from history.

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Table 1. Restriction enzymes and polymerase chain reaction primers and conditions for *FLG* genotyping

Mutation	Restriction enzyme	Primer	Annealing condition
R501X ^a	<i>NlaIII</i>	5'-GCT GAT AAT GTG ATT CTG TCT G-3'	57 °C, 1 min
		5'-ACC TGA GTG TCC AGA CCT ATT-3'	58 °C, 45 s
2282del4	<i>DraIII</i>	5'-CAC GGA AAG GCT GGG CTG A-3'	57 °C, 45 s
		5'-ACC TGA GTG TCC AGA CCT ATT-3'	
S2447X + S2554X ^a	/	5'-AAT AGG TCT GGA CAC TCA GGT-3'	60 °C, 1 min
		5'-GGG AGG ACT CAG ACT GTT T-3'	
R2447X	<i>BssSI</i>	5'-CCA CAC GTG GCC GGT CAG CA-3'	64 °C, 1 min
		5'-GTC CTG ACC CTC TTG GGA CGT-3'	
S2554X	<i>Hpy188I</i>	5'-GCA GAC AAA CTC GTA ACG AT-3'	58 °C, 1 min
		5'-TGT CTG GAG CCA TCT CTT AG-3'	
S2889X	<i>BamHI</i>	5'-GAT GGA CGG GGC CCA GCA CTA-3'	64 °C, 30 s
		5'-CTT GGT GGC TCT GCT GAT GGG A-3'	

^a Nest PCR is needed for a more specific R501X, S2554X and R2447X products. Two pairs of specific primers are needed to first make a longer length PCR product and then followed by a shorter one. Two sets of primers are needed for R501X alone, while S2554X and R2447X share the same first pair of primers. The first PCR product size for R501X is 1.5 kbp while the final product is 312 bp.

Thereafter, SH and TEWL were measured using previously standardized method (Hon *et al.*, 2008). The patients were allowed to rest in an air-conditioned waiting area prior to entering the consultation room. After acclimatization in the consultation room with the patient sitting comfortably in a chair for 20–30 min, SH (arbitrary unit (a.u.)) and TEWL (in $\text{g m}^{-2} \text{h}^{-1}$) were then measured in duplicates with the Mobile Skin Center® MSC 100 equipped with a corneometer CM 825 and a Tewameter TM 210 probe (Courage & Khazaka electronic GmbH, Cologne, Germany), following manufacturer instructions at 2-cm distal to the right antecubital flexure. The study was site-specific, and we did not assess individual lesions under the fixed limits of ambient temperature and humidity of this investigation. Oozing and infected areas were avoided by moving the probe slightly sideways (Shahidullah *et al.*, 1969; Hon *et al.*, 2008).

AD severity was also assessed with the Nottingham Eczema Severity Score (NESS) (Emerson *et al.*, 2000; Hon *et al.*, 2003) and quality of life with the validated Chinese version of Children's Dermatology Life Quality Index (CDLQI) (Chuh, 2003; Hon *et al.*, 2006).

Venous blood was collected from the AD patients, centrifuged at 4 °C and 2041 g for 10 min, and then stored at –70 °C until analysis. Non-allergic controls matched for ethnicity, age and sex and without current drug treatment were studied for comparison (Leung *et al.*, 2009). All restriction enzymes and polymerase chain reaction (PCR) primer sequences are listed in supplementary Table 1. The mutations were first screened by restriction fragment length polymorphism (RFLP). Genotyping results were confirmed in one-third of the samples and positive cases by direct sequencing using ABI-3130 Genetic Analyzer and BigDye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Informed written consent was obtained from parents, and the Clinical Research Ethics Committee of the University approved the study.

Data were expressed as proportion or mean and standard deviation (SD) unless otherwise stated. Chi-square or Fisher exact test was used to compare proportions, and Student's *t*-test was used to analyse numerical variables. All comparisons were made two-tailed, and *P*-values less than 0.05 were considered to be statistically significant.

Results

One hundred and seventy-four children with AD (92 boys and 82 girls) and 191 age- and sex-matched controls were recruited. The mean (SD) SCORAD and CDLQI in AD patients were 48.6 (19.7) and 7.5 (5.2), respectively. Nineteen (11.3%) patient had mild AD, 74 (44.0%) moderate AD and 75 (44.6%) severe AD according to objective SCORAD scores ($n = 168$) (CREAD, 1993).

Four (2.3%) of our 174 AD children were heterozygous carriers of wild-type R501X, whereas none of the controls carried this mutation ($P = 0.051$). Neither AD nor controls carried the other four *FLG* mutations. There was no association between the R501X mutation and asthma or allergic rhinitis. SH and TEWL did not differ between patients with *FLG* wild type and R501X (Table 2, Fig. 1). On the other hand, the R501X mutation was associated with long-term AD severity (NESS; $P = 0.028$) but not with SCORing Atopic Dermatitis (SCOARD) ($P = 0.132$), objective SCOARD ($P = 0.252$), CDLQI ($P = 0.613$), age of onset for AD ($P = 0.947$), total IgE ($P = 0.953$) or eosinophil count ($P = 0.636$) (Table 3). Table 4 compares published minor allele frequencies of our *FLG* mutations in different ethnic populations.

Discussion

Loss or reduction of *FLG* expression correlates with excessively dry skin and impaired barrier function. As far

Table 2. Dermatology factors comparison between R501X and wild-type atopic dermatitis patients ($n = 107$)

FLG	SH, a.u.			TEWL, $\text{g m}^{-2} \text{h}^{-1}$		
	Antecubital flexure	2-cm below	Mid-forearm	Antecubital flexure	2-cm below	Mid-forearm
R501X	21.3 $\pm$ 13.1	19.9 $\pm$ 4.8	25.5 $\pm$ 7.3	10.6 $\pm$ 3.2	7.5 $\pm$ 0.6	6.0 $\pm$ 1.3
WT	27.1 $\pm$ 12.7	25.1 $\pm$ 10.5	24.4 $\pm$ 9.6	13.2 $\pm$ 4.6	9.8 $\pm$ 4.1	8.3 $\pm$ 3.6
<i>P</i> -value	0.432	0.396	0.851	0.330	0.344	0.262

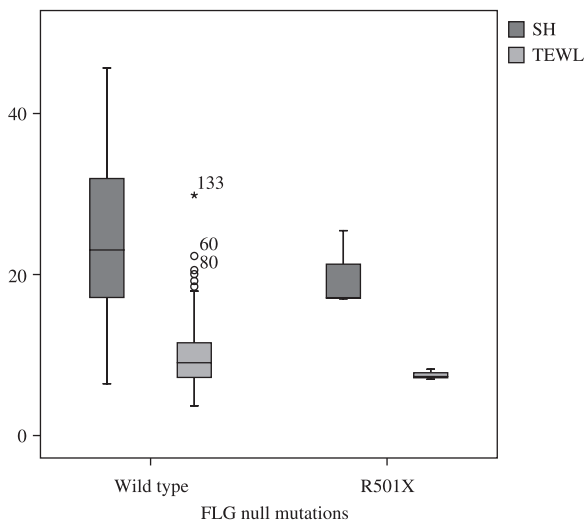
a.u., arbitrary unit; SH, skin hydration; TEWL, transepidermal water loss.

Table 3. Comparison of clinical factors between AD patients with R501X and wild type genotype

FLG	SCORAD ($n = 180$)	Objective SCOARD ($n = 176$)	CDLQI ($n = 145$)	NESS ($n = 166$)	Eczema onset age ($n = 134$)
R501X	33.9 $\pm$ 19.6	26.9 $\pm$ 19.6	6.3 $\pm$ 3.3	7.0 $\pm$ 2.6	2.3 $\pm$ 2.5
WT	48.9 $\pm$ 19.6	37.2 $\pm$ 17.6	7.6 $\pm$ 5.2	10.9 $\pm$ 3.0	2.1 $\pm$ 2.4
<i>P</i> -value	0.132	0.252	0.613	0.028*	0.947

* $P < 0.05$.

SCOARD, SCORing Atopic Dermatitis; CDLQI, Children's Dermatology Life Quality Index; NESS, Nottingham Eczema Severity Score.

**Figure 1.** Box plot of dermatological conditions measured at 2 cm distal to right antecubital flexure, between R501X carriers and patients with wild-type genotype.

as we are aware, this is the first report to demonstrate the *FLG* null mutations in southern Chinese AD patients. In contrast to prevalent expression in European cohorts, *FLG* mutations were found in a very small proportion of Chinese AD patients.

FLG is located on chromosome 1q21, and consists of three exons. Exon 3 encodes most of the *FLG* repeat constituents that were almost identical at DNA level, making routine PCR and conventional sequencing almost impossible. Recently, PCR protocols were developed to cover all coding regions of *FLG*. To date, 23 loss-of-function nonsense or frame-shift mutations in *FLG* have been identified by these sequencing methodologies. Three of these *FLG* mutations are specific to oriental subjects (Nomura *et al.*, 2007, 2008; Sandilands *et al.*, 2007).

The five mutations studied, R510X, 2282del4, R2447X, S2554X and S2889X, are located in *FLG* repeats 1, 7 and 8. As strong genetic association between AD and *FLG* mutations has been consistently observed, we speculated that these mutations also play similar pathogenic roles for AD in Chinese. Surprisingly, this study found the R501X mutation in only four male patients out of 174 AD children (2.3%) and in none of the controls. Obviously, *FLG* null mutations reported in Caucasians were rare in Chinese patients ($P = 0.051$) and these mutations are likely to be ethnic-specific. We suggest future studies to identify novel *FLG* mutations in different populations by sequencing this important AD candidate gene, with the ultimate aim to establish an international *FLG* mutation database to facilitate subsequent research into its immunogenetic roles for AD.

Several large European cohort studies also suggested that *FLG* null mutations predispose to other atopic disorders, namely asthma and allergic rhinitis. These mutations also predispose to atopic march, early-onset AD that persists into adulthood as well as more severe asthma (Palmer *et al.*, 2006, 2007; Marenholz *et al.*, 2006; Barker *et al.*, 2007). Thus, *FLG* null mutations may be an indicator of severe and persistent disease phenotype. Nevertheless, due to the small number of cases carrying the loss-of-function mutation, this study could not detect any significant association between *FLG* mutations and atopies ($P = 1$).

From the many publications on the importance of *FLG*, it would be clinically interesting and relevant to develop a novel non-invasive diagnostic test for AD through genotype-phenotype correlations. Interestingly, we found that R501X mutation was associated with long-term AD severity as assessed by NESS ($P = 0.028$). Nevertheless, R501X mutation showed poor associations with other clinical factors and allergic markers. The small number of patients with R501X mutation precludes any definite conclusion to be made.

Table 4. Published minor allele frequencies of our *FLG* mutations in different ethnic populations

SNP	rs number	Location	Minor allele	Our MAF	Published MAF in HapMap				Japanese (Nomura <i>et al.</i> , 2007, 2008)
					Han Chinese	Japanese	Caucasian	African	
R510X	3765526	Intron	G	0.498	0.422	0.488	0.492	0.220	ND
2282del4	9526569	Intron	C	0.202	0.211	0.133	0.325	0.450	ND
R2447X	1046295	3'-UTR	G	0.480	0.489	0.420	0.448	0.602	ND
S2554X	3794379	NearGene-3	T	0.318	0.267	0.300	0.125	0.142	0.059
S2889X	3794378	NearGene-3	C	0.206	0.229	0.135	0.370	0.490	0.098

MAF, minor allele frequency; ND, not done; UTR, untranslated region.

In conclusion, the five mutations prevalent in Caucasians and Japanese are rare in southern Chinese children. Future analysis of novel *FLG* variants and other epidermal structural protein mutations will shed light on the understanding of the genetics of AD.

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