Brief Report

Targeted Filaggrin Gene (*FLG*) Sequencing: A Pilot Study among Indian Children with Atopic Dermatitis

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Abstract

Background: Filaggrin deficiency causes early-onset atopic dermatitis (AD), extrinsic AD, persistent and severe disease, palmoplantar hyper linearity, keratosis pilaris, and increased risk of hand eczema. There is a paucity of data on the prevalence and types of variation in the filaggrin gene (*FLG*) in the Indian population. **Aim and Objectives:** To study the prevalence and characteristics of filaggrin mutations in Indian children affected with AD and to attempt a genotype-phenotype correlation. **Materials and Methods:** A pilot study was done among Indian children with AD aged 4-16 years, attending the Pediatric Dermatology outpatient department between February and September 2022 (7 months). Long-range polymerase chain reaction target enrichment and next-generation sequencing were used to sequence the complete *FLG* gene from peripheral blood samples. The identified variants were analyzed and categorized. **Results:** Among the 30 recruited children with AD, 28 genetic variants in exon 3 of *FLG* were found in 19 (63%) patients. These variants were classified as pathogenic (6, 21.4%), likely pathogenic (3, 10.7%), benign (16, 57.1%), and variant of uncertain significance (3, 10.7%). Among the 9 significant variants, 4 (45%) were novel. Although the patients with filaggrin variants had a higher prevalence of positive family history of atopy, other allergic diseases in the child, higher IgE levels, and a higher percentage of severe AD, the difference was not statistically significant. **Limitation:** Small sample size. **Conclusion:** Significant *FLG* null variants were identified in 23% (among which 45% were novel) of Indian children with AD. The spectrum of identified variants did not reflect the known *FLG* hotspots from other ethnicities, indicating the need for larger studies to determine the relevant hotspots in the Indian population.

Keywords: *Atopic dermatitis, atopic eczema, filaggrin polymorphisms, FLG, Indian children, next-generation sequencing*

Introduction

Atopic dermatitis (AD) is a chronic, relapsing, and remitting pruritic dermatosis in children and adults with a prevalence of 15-20% in children and 1-3% in adults.[1] The pathogenesis of AD is complex, characterized by inflammation, immune dysregulation, and skin barrier dysfunction, and recently, deficiency of filaggrin encoded by *FLG* has been found to play a significant role. Filaggrin deficiency tends to be associated with early-onset, extrinsic, persistent, and severe disease, keratosis pilaris and increases the sensitivity and severity of food allergies and vulnerability to infections. Variations in *FLG* predispose to palmar hyperlinearity with more than 60% manifesting as criss-cross hyperlinearity of the thenar eminence.[2] The *FLG* gene is complex and cumbersome to sequence, due to the variable number of tandem repeats in exon 3. The prevalence of *FLG* polymorphisms in the normal population is 3% in Asia.[3] International studies have identified hotspots such as R501X, 2282del4, S3247X, and R2447X.[1] Most studies, including the few available Indian studies, have looked at these hotspots using restriction fragment length polymorphism, despite the lack of validation of these hotspots in the Indian population. Except for a few studies on hand eczema and asthma and a few recent studies on atopic eczema, there is a scarcity of data from India: both hotspot based and whole gene sequencing based.[1] In this context, we undertook this project to study the variations in the entire *FLG* by next-generation sequencing among Indian children with AD.

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Materials and Methods

This was a pilot study done in children aged 4-16 years, fulfilling the United Kingdom (UK) diagnostic and American Academy of Dermatology (AAD) criteria of AD, attending the Pediatric Dermatology outpatient department between February 2022 and September 2022 (7 months) in a tertiary care center. The study aimed to determine the prevalence and types of *FLG* variants in Indian children affected with atopic eczema. Informed consent was taken from parents of all children <16 years, and assent was taken from children >7 years. Basic demographic details, age of onset, clinical features, disease course, triggers, treatment details, history of atopy in self and family, history of dry skin in the family members, and details of additional contact dermatitis if any (e.g., nickel) were recorded in a proforma. A detailed cutaneous examination was done including examination for filaggrin markers (hyperlinear palms, keratosis pilaris, ichthyosis vulgaris), and disease severity was graded using the scoring systems SCORAD (the Scoring of Atopic Dermatitis) and EASI (Eczema and Severity Index), and quality of life was assessed using Child Dermatology Life Quality Index (CDLQI). Serum Immunoglobulin E (IgE) levels were measured at baseline in all affected individuals.

Five ml of venous blood collected in an EDTA vacutainer from the affected children was used to isolate genomic DNA. Genetic screening for variants in the entire *FLG* gene was carried out in the Molecular Endocrinology Laboratory by long-range polymerase chain reaction (PCR)-based target enrichment followed by sequencing using the previously established protocols from the laboratory (Chapla *et al*., 2015).[4] The coding and splice site regions of *FLG* were amplified in three amplicons—two with long-range PCRs (6394bp and 7447bp) and one with a conventional uniplex PCR -381bp using TaKaRa LA PCR TM Kit (ver. 2.1). Targeted Sequencing was performed on IonTorrent PGM using Ion PGM™ 400 Sequencing Kit (Ion Torrent, Life Technologies), utilizing 318 chips (10 to 12 samples). The variant search engine Varsome and web-based insilico prediction tool MutationTaster were used to predict the pathogenicity of the identified variants.

The workflow is depicted in Figure 1.

Statistics

Descriptive statistics for quantitative values were expressed as mean (± standard deviation) in accordance with the data distribution. Frequencies and percentages were used to describe categorical variables. Chi-square/ Fischer's exact test was used to compare categorical data.

Results

During the 7-month study period, 30 children fulfilling the inclusion criteria were recruited. The mean age of the study population was 10.4 ± 2.9 years with a slight male predominance (56.6%). The age of onset of the disease was <2 years in 58% of the children, and the site of onset was the face in 13 (43%) children, followed by antecubital fossa in 11 (36%). While personal history of atopy was present in 26 children (86.6%), family history was present in 17 (56.6%) of the

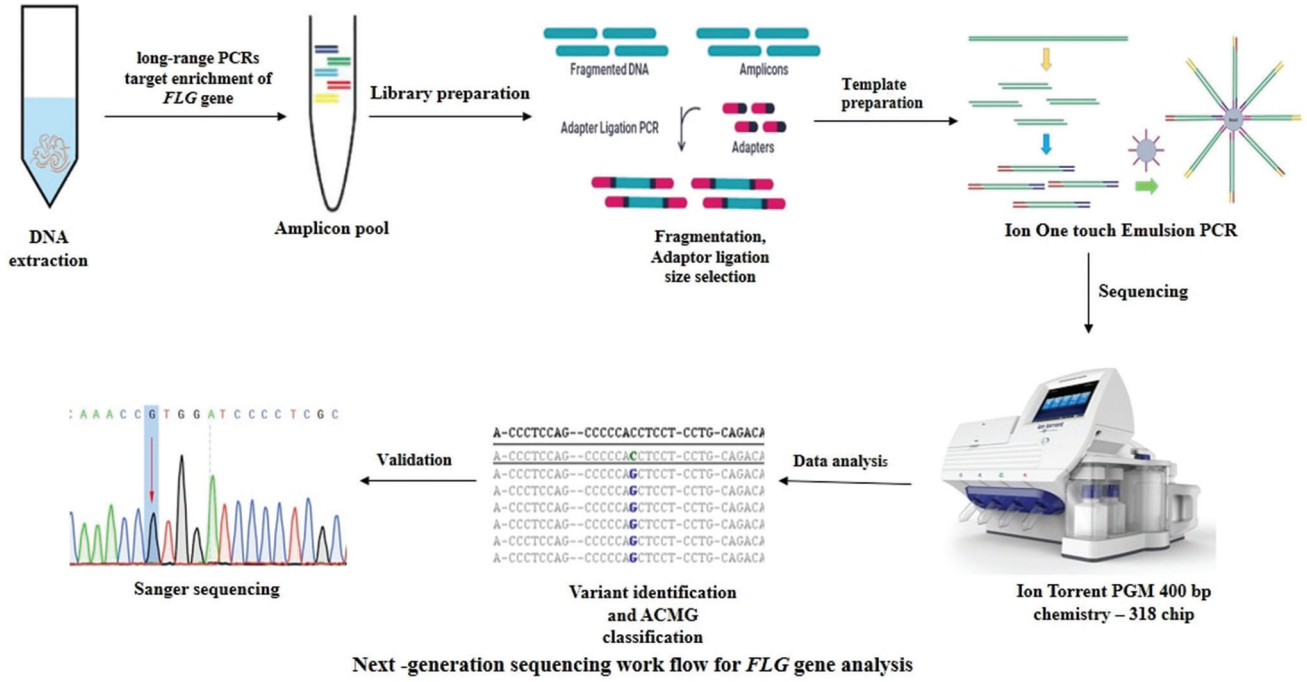

Figure 1: Depicts the workflow of genetic testing performed in-house for our patients with atopic dermatitis

children. Affected children were predominantly born of non-consanguineous marriage (90%) and the entire cohort had a history of dry skin. Seasonal variation was found in 23 (76.6%) (winter-18, summer-3, extremes of both-2) of the patients. There was a history of food allergy (milk products, chocolates) in 3 children (10%). High IgE levels (range: 500-2500 U/ml) (mean IgE: 987 ± 592 U/ml) were found in 26 children (86.6%), and all children had hyperlinear palms (100%). All patients had classical AD features with few having scalp scaling (9 patients-30%) and prurigo lesions (3 children-10%) in addition. Based on SCORAD and EASI severity scoring, mild, moderate, and severe disease was seen in 17 (56.6%), 8 (26.6%), and 5 (16.6%) children, respectively, and the mean CDLQI was 12.47 ± 6.6.

Genotyping with genotype-phenotype correlation

Among the 30 patients studied, 28 genetic variants in *FLG* on exon 3 were found in 19 (63%) patients. These variants were classified as pathogenic (6, 21.4%), likely pathogenic (3, 10.7%) [Table 1], benign (16, 57.1%), and VUS (a variant of uncertain significance) (3, 10.7%). None of the VUS variants were found to be damaging on insilico prediction analysis. The classification of variants was determined according to the ACMG (American College of Medical Genetics) criteria at the point of submission; however, it is subject to change as new evidence emerges over time. Although patients with *FLG* variants had a higher prevalence of positive family history of atopy, other allergic diseases, high IgE levels, and higher disease severity, these differences were not statistically significant [Table 2].

Discussion

Variations in *FLG* have been considered as a significant predisposing factor for the development of atopic AD in European, Asian, and other ethnicities worldwide to differing degrees. As depicted in Table 3, various studies have looked at *FLG* variants and their relationship with AD.[5-15] The incidence of *FLG* null mutations reported in the literature ranges from 20 to 30%, akin to our data (23%). Those with *FLG* null mutations are known to have 2 to 4.78 times more risk of AD than those without.[7] Most commonly, studies have looked at hotspots-like R501X, 2282del4, S3247X, and R2447X, commonly reported in the Western population.[6] Our study did not identify the hotspots as mentioned in the Western literature except for 1 variant (R2447X), reiterating that variations in *FLG* are influenced by ethnicity. In an Indian study, the reported prevalence of *FLG* null variants was 34.7%,[7] and 16 (80%) out of the 20 different loss of function variants observed in this study were novel, and those with these variations had early-onset and persistent disease. We found 9 pathogenic/likely pathogenic variants in our study population of which one is a well-known polymorphism (c. 2282del4) and one has been reported previously (c.7031C>G).[7] Three other variants (c.7339C>T, c.6109C>T, and c.3191G>A) found in our study have been reported as novel

Table 1: List of pathogenic/likely pathogenic variants identified in our cohort and their phenotype (9 variants in 7 patients)

Nucleotide change	*FLG* Pathogenic/ likely pathogenic variants	Previously reported in literature	Age of onset of disease	Personal history of atopy	Family history of atopy	Ig E Levels (U/ml) (Cut off: 0-378 U/ml)	Clinical phenotype	SCORAD baseline severity score 0-25- Mild 26-50-Moderate >51- Severe
c.7031C>G	p. Ser2344Ter	Yes	2 years	Yes – asthmatic	Yes	675-elevated	Hyperlinear palms	Mild (15)
c.3448C>T	p. Arg1150Ter	No	4 months	Yes – asthmatic	Yes	elevated->1000	Hyperlinear palms, ichthyosis, periorbital pigmentation, scalp scaling	Severe (62)
c.2476C>T	p. Arg286Ter	No	2 years	Yes-allergic rhinitis	Yes	500-elevated	Hyperlinear palms, keratosis pilaris, periorbital pigmentation, perifollicular accentuation	Mild (10)
c.7339C>T	p. Arg2447Ter	Yes	4 years	Yes-asthmatic	Yes	700-elevated	Hyperlinear palms	Mild (12)
c.3418C>T c.6109C>T	p. Arg1140Ter p. Arg2037Ter	No Yes	5 months	Yes-asthmatic	Yes	855-elevated	Hyperlinear palms, periorbital pigmentation, scalp scaling	Severe (58)
c.2282_2285 delCAGT	p. Ser761CysfsTer36	Yes	9 years	Yes-asthmatic	Yes	550-elevated	Hyperlinear palms, scalp scaling	Mild (20)
c.3191G>A c.3358delC	p. Trp1064Ter p. Gln1120ArgfsTer2	Yes No	3 years	Yes-asthmatic	Yes	595-elevated	Hyperlinear palms, ichthyosis, periorbital pigmentation	Moderate (30)

Table 2: Clinical characteristics of Indian children with and without variation in *FLG*

Characteristics	Entire cohort *n* (%)	AD with FLG variations *n* (%)	AD without FLG variations *n* (%)	#*P*
Number of patients	30 (100%)	7 (23%)	23 (77%)	-
Age±SD, years	10.4±2.9	11±3.46	10.3±2.03	-
Sex				
Male	17 (56.6%)	2 (6%)	15 (50%)	0.084
Female	13 (43.3%)	5 (17%)	8 (27%)	
Elevated Ig E levels	26 (86.6%)	7 (100%)	19 (83%)	0.548
Hyperlinear palms*	30 (100%)	7 (100%)	23 (100%)	-
Age of onset				
<2 years	18 (60%)	4 (57%)	14 (61%)	1.000
3-10 years	12 (40%)	3 (43%)	9 (39%)	
11-16 years	0 (0%)	0 (0%)	0 (0%)	
Family history of atopy	17 (56.6%)	5 (71%)	12 (52%)	0.427
Allergic disease association	26 (86.6%)	7 (100%)	19 (83%)	0.548
SCORAD				
Mild	17 (56.6%)	4 (57%)	13 (57%)	0.522
Moderate	8 (26.6%)	1 (14%)	7 (30%)	
Severe	5 (16.6%)	2 (29%)	3 (13%)	
Xerosis	17 (57%)	5 (71%)	12 (52%)	0.427
Pityriasis alba	26 (87%)	7 (100%)	19 (83%)	0.548
Ichthyosis	4 (13%)	2 (29%)	2 (8.7%)	0.225
Keratosis pilaris	2 (6%)	1 (14%)	1 (4%)	0.418

#*P* value calculated using Chi-square test/Fisher's exact test. *Palmar hyperlinearity could not be correlated as they were present in all patients

variants in the recent Indian study.[7] However, four variants (45%) (c.3448C>T, c.2476C>T, c.3418C>T, and c.3358delC) are novel and not reported to the best of our knowledge. This highlights the fact that the traditional hotspots identified in the Caucasian population do not stand true in all races including ours. Forty-five percent of the mutations (4/9) were novel, emphasizing the lack of data regarding the *FLG* variants in our population and reflecting the lack of studies analyzing the entire coding region of the gene.[1] Previous studies have found an association of *FLG* variants with earlier age of onset, high IgE levels, xerosis, ichthyosis vulgaris, palmar hyperlinearity, keratosis pilaris, white dermographism, and severe disease.[13,15-17] In our study, though the patients with *FLG* variants had a higher prevalence of positive family history of atopy, other allergic diseases, high IgE levels, and a higher percentage of severe AD, these differences were not statistically significant, probably due to the smaller sample size studied. Interestingly, frank ichthyosis was not identified in 71% of AD patients with *FLG* mutations, which indicates the poor value of this clinical feature in picking up *FLG* variants. We did not find any specific and statistically significant phenotypic markers of *FLG* variations in AD as was reported by Park *et al*.,[18] possibly due to the multifactorial nature of the disease. The major limitation of our study was the low sample size. Despite this limitation, the study expands the spectrum of null variants in *FLG*, especially highlighting the novel variants. Further studies in a larger number of patients are required to get a complete understanding of the various null variants in *FLG*, the various roles it plays in AD, and to explore newer preventive and therapeutic strategies in AD. We will also be able to identify *FLG* hotspots in the Indian population from studies performed on larger sample sizes and use it for further clinical and research settings.

Limitation

A small sample size limits our study, and we intend to conduct further studies with larger numbers to achieve a better genotype-phenotype correlation.

Conclusion

Every population is likely to have a unique set of *FLG* variations. Population differences highlighted by the diverse *FLG* variations make it unique and complex to perform a worldwide screening. It is, therefore, imperative to establish global population genetic maps to identify novel variants restricted to a particular population. We found a high prevalence of *FLG* variants (23%) in this Indian cohort. However, a study with a large sample size is required to reaffirm this finding. This study also reports 45% novel variants, dismissing the role of hotspot testing in Indian patients until further large studies are done in our population to establish the relevant hotspots and a better genotype-phenotype correlation.

Table 3: Review of literature on *FLG* variants in eczema reported worldwide

Study & year	Country	Aim of the study	Sample size	Type of genetic testing	Percentage of null mutations/variations/hot spots if any	Genotype-phenotype correlation if any
1. Chauhan *et al*.[5] 2020	India	Prevalence of R501X in allergic children (asthma, allergic rhinitis, and AD)	90 children	PCR-RFLP	5.5% of R501X mutant genotypes (AA) in children with atopic diseases which comprised 3.3% and 2.2% of children with asthma and asthma concomitantly with eczema	
2. Handa *et al*.[6] 2019	India	Prevalence of *FLG* mutations in Hand eczema screened for s2889x, 2282del4, R501x, 22417x	163 patients and 86 controls	PCR-RFLP	Prevalence- 33.7% cases, 3.5% controls s2889x-96.4%, no R501x and 22417x	*FLG* polymorphisms specifically associated with hand eczema subtype and severe disease
3. Rajeshwari *et al*.[1] 2023	India	*FLG* gene mutations in AD	30 patients and 15 controls	Sequence analysis of the third exon of *FLG* -PCR amplification of 11 overlapping fragments amplified by 11 sequence-specific primer pairs.	Only 5 of the detected 22 amino acid changes H2507Q, L2481S, K2444E, E2389Q, and S2366T have been previously reported. 1 patient stop codon- S2366STOP P2238N, R2239W, V2243L detected in 70% of samples, S2231E detected in 67% not reported previously	
4. Srinivas *et al*.[7] 2023	India	*FLG* gene mutations in AD	75 children	NGS of the whole FLG gene	Prevalence- 34.3%, 80% were novel variants	serum Ig E levels were significantly associated with *FLG* null variants.
5. Chawla *et al*.[8] 2023	India	Genotyping of FLG in AD and ichthyosis vulgaris (IV)	180 children (AD- 60, IV-60, healthy controls- 60)	Hot spot sequencing – PCR-RFLP-FLG Screened for 2282del4, R501x	Most common *FLG* mutations were R501X (31.6% and 23.3%) and 2282del4 (18.3% and 13.3%) in AD and IV patients with heterozygous genotype.	R501X mutation is one of the robust genetic associations of AD and IV. 2282del4 polymorphism was marginally less as compared to R501X.
6. Nath *et al*.[9] 2020	India	Prevalence of *FLG loss of function* and missense mutations, the nature and extent of dysbiosis and altered microbial pathways with and without mutations in *FLG*.	88 (34- AD, healthy controls-54)	Sequencing of the coding region of *FLG*. The shotgun metagenomic assessment for the microbiome	Prevalence of *FLG* LoFs lesser in cases and controls (8.6%, 0%) than those reported in Europeans (27%, 2.6%). *Staphylococcus aureus* was present only on AD skin but not on healthy skin on which *Staphylococcus hominis, Cuti bacterium acnes* and *Malassezia globose* were significantly more abundant.	Concluded host DNA profile is significantly associated with microbiome composition in the development of AD.
7. On *et al*.[10] 2016	Korea	*FLG* mutations in Korean AD patients	70 patients	Direct DNA sequencing of previously reported 14 *FLG* hotspots	Four *FLG* null mutations (3321delA, K4022X, S3296X, and S2889X) in eleven patients (15.7%).	FLG mutations were significantly associated with elevated Ig E and palmar hyperlinearity.

Contd...

Table 3: Contd...

Study & year	Country	Aim of the study	Sample size	Type of genetic testing	Percentage of null mutations/variations/hot spots if any	Genotype-phenotype correlation if any
8. Cascella *et al*.[11] 2011	Italy	Full sequencing of *FLG* gene in Italian AD patients	220 patients	Sequence analysis of the third exon of *FLG* using 11 overlapping fragments amplified by 11 sequence-specific primer pairs	R1798X, E3603X, and R3638X were reported as novel variants	
9. Brown *et al*.[12] 2008	United Kingdom	*FLG* null mutations and atopic eczema	811 patients	PCR-RFLP	Prevalence- 18.4% Mutations detected- R501X, 2282del4, R2447X, S3247X Eight (4.2%) of 190 atopic eczema cases carry 2 null mutations	
10. Muller *et al*.[13] 2009	Germany	*FLG* loss of function mutations in atopic eczema and asthma – hot spots R501X, 2282 del 4	496 children	PCR-RFLP		Significant association of the *FLG* null variants R501X- and 2282del4 with AD (combined genotype $P<0.0001$) and asthma (combined genotype $P<0.0001$).
11. Rogers *et al*.[14] 2007	United States	Genotyped 2 loss-of-function *FLG* mutations (R501X and 2282del4) in white children (age 5-12 years) with mild to moderate asthma in the childhood asthma	646 patients	PCR-RFLP	Mutations detected- R501X, 2282del4 1/3 (185/646) of the participating children had AD.	Strong associations were observed between FLG variants and AD and between the mutations and total serum Ig E level
12. Morar *et al*.[15] 2007	United Kingdom	*FLG* loss of function mutations in atopic eczema	990 patients	PCR-RFLP	Mutations screened- R501X, 2282 del 4 *FLG* variations in 26.7% of patients with AD, and 14.4% of children without AD	

AD- Atopic Dermatitis, PCR- Polymerase chain reaction, RFLP- Restriction fragment length polymorphism, IV- Ichthyosis vulgaris , LOFs- Loss of function variants, NGS- Next generation sequencing

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Nil.

Conflicts of interest

There are no conflicts of interest.

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