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NOVEL FILAGGRIN MUTATION BUT NO OTHER LOSS-OF-FUNCTION VARIANTS FOUND IN ETHIOPIAN ATOPIC-DERMATITIS PATIENTS

Running head: Novel filaggrin mutation but lack of known mutations in Ethiopian AD and IV patients

M.C.G. Winge¹, K.D. Bilcha³, A. Liedén², D. Shibeshi³, A. Sandilands⁴, C.-F. Wahlgren¹, W.H.I. McLean⁴, M. Nordenskjöld², M. Bradley^{1, 2}

- ^{1.} Dermatology Unit, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden
- ^{2.} Department of Molecular Medicine & Surgery and Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden
- ^{3.} Department of Dermatovenereology, Faculty of Medicine, Addis Ababa University, Addis Ababa, Ethiopia
- ^{4.} Epithelial Genetics Group, Human Genetics Unit, Division of Pathology and Neuroscience, University of Dundee, Ninewells Hospital and Medical School, Dundee, DD1 5EH, Scotland

Correspondence:

Mårten Winge, marten.winge@ki.se

Dermatology Unit, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital Solna, SE-17176 Stockholm Sweden

Phone +46851776538

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Conflict of interest disclosures:

W.H.I.M. has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

Bulleted statements:**What is already known about this topic?**

- Filaggrin (*FLG*) loss-of-function variants are considered the causative genetic factor for ichthyosis vulgaris (IV) and a strong risk factor for developing atopic dermatitis (AD) in European and Asian populations.

What does this study add?

- This study suggests that *FLG* loss-of-function-variants are less common in Ethiopian AD and IV patients compared to European and Asian patients.

ABSTRACT

Background: Filaggrin is a key protein involved in maintaining skin barrier function and hydration. Mutations in the filaggrin gene (*FLG*) cause ichthyosis vulgaris (IV) and are a major predisposing factor for atopic dermatitis (AD) in individuals of European and Asian descent. It has been proposed that *FLG* mutations are population-specific and a difference in the spectra of mutations between different ancestral groups has been described. However, it is unknown whether *FLG* mutations in the African population are a causative genetic factor for IV and predispose to AD, or whether other mechanisms are more prominent.

Objective: The present aim was to investigate the role of *FLG* mutations as predisposing factors for IV or AD among individuals from Ethiopia.

Clinical material and methods: A case series of Ethiopian AD (n=103) and IV patients (n=7) together with controls (n=103; subjects without past or present history of AD, dry skin or atopic manifestations) were collected at the outpatient dermatology clinics at ALERT Dermatology Hospital, Tikur Anbessa Hospital and Gondar University Hospital, Ethiopia. AD was diagnosed by a dermatologist using the UK Working Party's diagnostic criteria. The IV diagnosis was based on clinical examination and genetic testing of the steroid sulfatase gene to exclude X-linked recessive ichthyosis. Patients were studied with direct sequencing (n=40) and/or allelic discrimination (n=110). Immunohistochemical (IHC) analysis was performed for filaggrin expression in the skin of patients (n=7) and controls (n=2).

Results and conclusions: The Ethiopian patients and controls were genotyped for the four previously-described common European *FLG* null mutations (R501X, 2282del4, S3247X, R2447X) and no carriers were found. In one patient with AD a novel heterozygous two base pair deletion, 632del2, leading to a premature stop

was revealed by direct sequencing. No additional carrier of this deletion or other mutations was found. In addition, no difference in filaggrin expression was detected in AD or IV skin compared to healthy control skin. Our result indicates that *FLG* loss-of-function-variants are less common in IV and AD patients in the Ethiopian population suggesting that other factors may be of importance in the pathogenesis in this ethnic group.

INTRODUCTION

Atopic dermatitis, (AD; Online Mendelian Inheritance in Man #603165), is an inflammatory skin disorder that affects up to 20 % of children and 3-5 % of adults in the western world (1, 2). The prevalence of AD in the Ethiopian population is reportedly as low as 1-2 % for both children and adults in Southern Ethiopia (3), and up to 19 % among 13-14-year-old children in the capital Addis Ababa (4), but has not yet been extensively studied. AD is diagnosed on clinical features, frequently using the U.K. Working Party's criteria: pruritus, typical distribution, early onset, dry skin and a personal or family history of atopic disease (5). The etiology is a complex interaction between environmental factors and genetic predisposition, indicated by high family incidence and pair wise concordance rates of 0.77 in monozygotic twins and 0.23 in dizygotic twins (6).

The epidermal differentiation complex (EDC) has been identified as a susceptibility locus for both AD and psoriasis and is located on human chromosome 1q21 (7). The EDC contains a cluster of genes that encode proteins of importance for the terminal differentiation of the epidermis, the outermost skin layer, and other epithelia. Located within the stratum corneum, the cornified envelope (CE) is an insoluble protein matrix vital for skin-barrier function and integrity. It replaces the plasma membrane of the granular cells during cornification. EDC proteins share significant sequence similarities, and phylogenetic association suggests that these proteins are derived from a common ancestor, evolving to meet tissue-specific demands such as developmental epidermal differentiation (8). The >400 kiloDalton (kDa) polyprotein profilaggrin is normally expressed in the keratohyalin granules in the granular layer of the epidermis. This polyprotein precursor is cleaved into 10-12 filaggrin monomers of 37 kDa that aggregate the keratinocyte cytoskeleton, prior to being cross linked and contributing to the formation of the CE. Subsequently, in the stratum corneum, filaggrin is enzymatically degraded into metabolites including urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA). These metabolites add to a pool of amino acids, ions and other metabolites and act as natural moisturizing factors (NMF). NMF play a central role in maintaining hydration of the SC and affect enzyme activity, pH and antimicrobial defence (9).

In 2006, loss-of-function variants in the *FLG* gene were identified in patients of Scottish, Irish and European-American descent, as the causative genetic factor in the most common form of ichthyosis, ichthyosis vulgaris (IV; Online Mendelian Inheritance in Man #146700) (10). AD patients may have decreased expression of filaggrin in the skin (11). When tested, the *FLG* variants were strongly associated with AD in an Irish AD cohort (12). At present, more than 40 loss-of-function mutations have been reported in European and Asian populations, and filaggrin deficiency is considered a strong risk factor for developing AD (13). *FLG* mutations have not yet been reported in native African individuals, indicating that the global contribution of *FLG* mutations to the development of AD and IV still remains to be further elucidated.

MATERIAL AND METHODS

Subject collection

Patients (n=110) and controls (n=103; subjects without past or present history of AD, dry skin or atopic manifestations) were identified at the dermatology outpatient clinics at ALERT Dermatology Hospital, Tikur Anbessa Hospital and Gondar University Hospital, in collaboration with College of Medicine and Health Sciences, University of Gondar and Faculty of Medicine, Addis Ababa University, Ethiopia. All patients were investigated by the same dermatologist (K.D.B) who also performed a clinical examination and recorded medical history using a standardized questionnaire. AD (n=103) was diagnosed according to the UK Working Party's diagnostic criteria while disease severity for AD was assessed using the scoring atopic dermatitis index (SCORAD). Cases were categorized into mild (<15 points), moderate (15-40 points) or severe (>40 points) AD according to objective SCORAD criteria (14). Other atopic manifestations such as allergic asthma and allergic rhinoconjunctivitis were assessed through the questionnaire. Blood for genetic testing was sampled from all patients and controls. Serum was taken from AD patients (n=53) when possible.

The diagnosis of IV (n=7) was based on clinical findings by the same dermatologist (KDB). X-linked recessive ichthyosis (XLRI; Online Mendelian Inheritance in Man#308100) was excluded with genetic testing.

Punch biopsies were taken from moderate (n=3) and severe AD patients (n=3), IV patients (n=1) and healthy controls (n=2) for immunohistochemistry (IHC) analysis. All selected patients had palmar hyperlinearity.

All the study participants gave written or oral informed consent, which complies with Declaration of Helsinki principles. This study was approved by the Ethics Review Board of the University of Gondar, and the regional ethical committee, Karolinska Institutet, Sweden.

Genetic Testing

Genomic DNA was isolated from peripheral blood using QIAamp® DNA mini kit. The *STS* gene was analyzed using multiplex ligation-dependent probe amplification (MLPA; n=7) and direct sequencing (n=7). The *FLG* gene was analyzed using direct sequencing (n=40) and/or allelic discrimination (n=103). The Polyphen (15) and the Alamut mutation interpretation software (Interactive Biosoftware, Rouen, France) were used to predict pathogenicity for sequence alterations compared to current reference sequence.

MLPA

MLPA analysis was run for the *STS* gene using the P160 A2 kit from MRC-Holland®, as previously described (16) with minor modifications. Typically 100 ng genomic DNA was amplified and analyzed on the ABI® 3130xl genetic analyzer.

Direct sequencing

In addition to MLPA, the *STS* gene was fully sequenced using primers and PCR conditions previously described (17). The *FLG* gene was sequenced using a series of overlapping PCR fragments (10). The novel variant 623del2 was not amenable to genotyping by allelic discrimination and was therefore screened for by direct sequencing of the amplified overlapping PCR fragment covering this region. In brief, 50 ng DNA was amplified with 1.25 µl 10 mM dNTPmix (2.5mM of each), 2.5 µl 10xRxn buffer - MgCl₂, 2 µl 50 mM MgCl₂, 2.5 µl PCR Enhancer, 0.3 µl Invitrogen® PlatinumTaq DNA Polymerase, 10.45 µl H₂O and 2.5 µl each of forward and reverse primer. Sequencing was analyzed using an ABI® 3730 DNA Analyzer instrument.

Allelic discrimination

FLG genotyping was performed in patients and controls for the four most prevalent European *FLG* mutations R501X, 2282del4, S3247X and R2447X by allelic discrimination. Genomic DNA was PCR-amplified in 384-well plates. Each well contained 5 ng genomic DNA, 2.5 µl TaqMan Universal PCR Master Mix, 0.125 µl specific Taqman assay solution and 2.375 µl H₂O. Allelic discrimination was carried out on the ABI PRISM® 7900HT Sequence Detection System and the SDS 2.2.1 sequence detection system program (Applied Biosystems®). Primers and PCR conditions for tested *FLG* mutations were as described previously (18).

Immunohistochemistry

Punch biopsies were taken from lesional skin in IV and AD patients and from normal skin in healthy controls. All biopsies were formalin-fixed and paraffin-embedded. Five-µm sections were cut on glass slides, dewaxed, rehydrated and demasked with TE-buffer. Vector Laboratories standard ABC-Elite protocol for paraffin sections (Vector Laboratories Inc. Burlingame, CA, USA) was used with minor modifications. Briefly, paraffin sections were blocked with 0.3 % H₂O₂, normal horse serum (dilution 1/ 10). Sections were incubated overnight at 4°C with a mouse monoclonal anti-human filaggrin (dilution 1/200; SPM181, Abcam, Cambridge, UK) followed by incubation with a diluted biotinylated anti-mouse secondary antibody (dilution 1/200, Vector Laboratories Inc. Burlingame, CA, USA). The sections were washed and incubated with preformed avidin-biotin-enzyme complex (ABC-ELITE reagent, Vector Laboratories Inc. Burlingame, CA, USA.) and developed with 3-amino-9-ethylcarbazole (AEC) substrate. Counterstaining was made with Mayer's haematoxylin. Irrelevant mouse IgG2a was used as control and no unspecific staining was found with the control antibody.

IgE

Total IgE and allergen-specific serum IgE-levels were measured against *Dermatophagoides pteronyssinus* (*Der p*), hen's egg, and mold mix (Mx2) including *Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Candida albicans*, *Alternaria alternate* and *Helminthosporium halodes* using the ImmunoCAP method (Phadia AB, Uppsala, Sweden).

Statistical analysis

Differences in total serum-IgE and allergen-specific serum IgE-levels were compared between mild to moderate and severe AD by Mann-Whitney U test. P-values <0.05 were defined as significant.

RESULTS

Clinical presentation

Phenotypes for AD patients, IV patients and controls are presented in tables 1-3. Briefly, 43.6% of the AD patients were females. The mean age for AD patients was 9.4 (median 7.0; range 0.3-34.0) years; for IV patients 21.4 (median 11.0; range 0.7-65.0) years and for controls 10.6 (median 9.0; range 0.7-60.0) years. Age of onset for AD was 2.7 (median 1.0; range 0-17.0) years. Ninety-eight percent of the AD patients were diagnosed as having either moderate or severe AD according to objective SCORAD. The mean SCORAD was 36.9 (range 8.2-66.0). For further details see Tables 1-3.

Results of genetic testing

The *FLG* gene was fully sequenced in seven IV patients and 33 AD patients. One patient with severe AD and keratosis pilaris and palmar hyperlinearity was found carrying a heterozygous 2 base-pair deletion in repeat 1, 623del2, leading to a premature stop downstream of the deletion (Figure 1). Screening for the 623del2 mutation was performed in all AD patients with DNA samples of sufficient quality (n=99), 7 IV patients and 103 controls and no additional carriers of the 623del2 mutation were found. None of the mutations previously described in the European or Asian populations were found in the sequenced AD or IV patients. Genotyping with allelic discrimination for the most common European *FLG* variants was performed in 103 Ethiopian controls and 103 Ethiopian AD patients with no carriers found. None of the sequenced ichthyosis patients carried *STS* mutations.

Filaggrin expression in IV, AD and healthy skin

IHC analysis was used to study the expression of filaggrin in lesional skin of moderate (n=3) and severe (n=3) AD patients, IV (n=1) patients and in the skin of healthy individuals (n=2). No skin biopsy was available from the patient carrying the 623del2 mutation. Both AD and IV skin displayed general hyperplasia in the suprabasal

layers of epidermis compared to the control skin. Filaggrin was expressed in the stratum granulosum in IV, AD and healthy control skin (Figure 2).

Total and allergen-specific serum IgE-levels

Among the AD patients, 62.3 % showed elevated total serum IgE (median 120; IQR1-3 44-540 k U/L). Of the allergen-specific serum IgE, 11.3% of patients had elevated levels against hen's egg (100% aged 0-5 years), 11.3 % against mold mix and 15.8 % against *Dermatophagoides pteronyssinus* (77% aged 16-20 years). Patients with severe AD had significantly higher total IgE ($p=0.012$) and higher allergen-specific serum IgE-levels against *Dermatophagoides pteronyssinus* (30.8%) than patients with mild to moderate AD (20.0 %), although this did not reach statistical significance ($p=0.32$). Age and gender distribution was similar in the mild to moderate and severe AD sub-groups. Allergen-specific serum IgE-levels in mild to moderate and severe AD patients are summarized in Table 4.

DISCUSSION

FLG loss-of-function variants are considered the primary genetic cause of IV and a major predisposing factor for AD (19). In the present study none of the reported European or Asian variants were found in Ethiopian patients with IV and AD. Instead, a heterozygote mutation was found causing a premature stop codon downstream of a 2-base pair deletion. Unfortunately neither the patient nor her relatives have been available for further testing, so the relevance of this mutation for the patient's manifestation of AD could not be investigated further. However, it is well established that truncating mutations leads to loss of profilaggrin and filaggrin expression (20), which applies to this novel mutation.

Previous studies have indicated that epidermal barrier dysfunction may be a key mechanism underlying elevated IgE sensitization in humans (21). Elevated allergen-specific serum IgE-levels against *Dermatophagoides pteronyssinus* and mold mix have previously been associated with atopic manifestations in Ethiopia (22) and in Gambia (23), respectively. Our AD patients demonstrated elevated total serum IgE values, and elevated allergen-specific serum IgE-levels were detected in several age groups. Patients with severe AD showed significantly higher total serum IgE-levels ($p=0.012$) and higher allergen specific serum IgE against *Dermatophagoides*

pteronysinus than the mild to moderate AD patients. The relevance of this finding, or other untested allergens, is however unclear and has to be addressed in further studies. Parasite-infestations may be of importance (24), but were not investigated in our present study.

Although this is a first preliminary study, our results suggest that *FLG* mutations are rare in Ethiopian AD and IV patients compared to other described populations. The prevalence of *FLG* mutations is reportedly as high as 17 – 42 % in European populations with moderate-to-severe AD (25), and 20 - 30 % of AD patients in Asian populations (26). It has been proposed that *FLG* mutations are population specific (27) and a difference in the spectra of mutations between different ancestral groups has been described. A theory is that the *FLG* mutations occurred after the major separation of the respective populations (20). In their original study from 2006, Palmer et al included population-based genotyping of 124 individuals from North Africa and 195 from Asia, without finding the two most common European mutations (R501X and 2282del4 (12)). In another study, 188 African American patients with AD and 152 healthy controls were tested and 2282del4 and R501X were found, but at a low prevalence (3.2 % each). These authors explained these findings by the European admixture in the North American population (28). In our present study we wanted to limit the ancestral admixture and focused primarily on one domestic population, where the admixture of other previously-studied populations was expected to be limited. However, extensive genetic diversity is described in the African population, which restricts what generalizations can be made for other African populations than the Ethiopian (29). There is also a risk of underestimating the full spectrum of *FLG* mutations in this population given the sample size of fully sequenced AD and IV patients in the present study.

An interesting question arising is why *FLG* mutations are so prevalent in the European population in general, where the overall prevalence regardless of symptoms has been estimated to 9 % (12). A theory is that it could have been of evolutionary value to carry a mutation causing a more permeable skin barrier. Repeated low-level exposure of pathogens to antigen-presenting cells in the skin might increase the immunity against infections during pandemics, e.g. the bubonic plague that wiped out 30-60 % of the entire European population (30). This sort of balanced selection favoring a heterozygous state has been proposed in other conditions such as the underlying sickle-cell anemia mutations seen in malaria-endemic areas (31). Varying susceptibility to malaria between different ethnic groups has been demonstrated by polymorphisms in several loci affecting immune response pathways (32, 33). This genotypic and phenotypic adaptation may also play a role in other immune-

mediated pathways. If the prerequisites for balanced selection favoring a more permeable skin barrier have not been fulfilled in the African population, this could help explain the low prevalence of *FLG* mutations found in Ethiopian AD and IV patients.

Having *FLG* mutations in a setting such as Africa might even be a disadvantage for a single individual, since filaggrin contains numerous histidine residues which during filaggrin degradation serves as substrates for the formation of UCA. UCA has been proposed to act as a UV-absorbing substance in the stratum corneum (34) and filaggrin deficiency has been associated with lower concentrations of UCA in skin cultures (35) and *in vivo* (36). Skin cultures have shown that irradiation of filaggrin-deficient skin results in increased cyclobutane pyrimidine dimers and caspase 3 activation; both suggesting increased DNA damage and apoptosis (35). Thus, individuals with filaggrin-deficient skin might be subject to increased DNA damage in an environment with high UV radiation.

These theories are so far speculations and our findings of a low incidence of *FLG* mutations in the material analyzed might have other explanations. Secondary down regulation of filaggrin in the absence of mutations has been discussed previously (37). However, our IHC analysis does not indicate any apparent down regulation in lesional skin with the phenotype of AD or IV compared to healthy control skin (Figure 2). The phenotype is of great importance, and it has been suggested that the absence of both palmar hyperlinearity and keratosis pilaris may give a negative prediction value of *FLG* mutations as high as 92 % (38). The novel mutation 623del2 was detected in an AD patient with palmar hyperlinearity and keratosis pilaris. Had our AD material comprised more patients with palmar hyperlinearity and keratosis pilaris, the frequency of *FLG* mutations might have been higher, although in our present study 65.7 % of the sequenced AD and IV patients had at least one of these clinical features.

In summary, we have fully sequenced the *FLG* gene in 40 individuals and genotyped 103 Ethiopian individuals with AD and/or IV for the most prevalent European loss-of-function variants. Neither the European nor the Asian variants were found. In one patient, a novel *FLG* mutation was discovered. No other mutations were found, and no mutations were found in any of the 103 controls. In addition, no difference in filaggrin expression was detected in AD or IV skin compared to healthy control skin. Our result indicates that *FLG* loss-of-function

variants are less common in Ethiopian AD and IV patients and other mechanisms could contribute to the pathogenesis of IV and AD in this ethnic group.

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TABLES 1-4

Table 1	
Phenotype of Ethiopian AD patients (n=103)	%
Female sex	43.6
Age (median; range)	7.0;0.3-34.0
Family history of atopy (asthma, AD or allergic rhinoconjunctivitis)	49.5
Personal history of asthma or allergic rhinoconjunctivitis	27.1
Early age of onset (<2 years)	65
Keratosis Pilaris / Palmar Hyperlinearity	15.5
Xerosis	99
Mild AD (SCORAD <15)	2
Moderate AD (SCORAD 15-40)	65
Severe AD (SCORAD >40)	33

Table 2	
Phenotype of Ethiopian IV patients (n=7)	%
Female sex	42.8
Age (median; range)	11.0; 0.7-65.0
U.K. Working Party's criteria for AD	16.7
Family history of atopy (asthma, AD or allergic rhinoconjunctivitis)	28.6
Personal history of asthma or allergic rhinoconjunctivitis	0
Keratosis Pilaris / Palmar Hyperlinearity	100
Xerosis	100

Table 3	
Phenotype of Ethiopian healthy controls (n=103)	%
Female sex	53.4
Age (median; range)	9.0; 0.7-60.0
U.K. Working Party's criteria for AD criteria	0
Family history of atopy (asthma, AD or allergic rhinoconjunctivitis)	0
Personal history of asthma or allergic rhinoconjunctivitis	0
Keratosis Pilaris / Palmar Hyperlinearity	0
Xerosis	0

Table 4		
Total and allergen specific IgE-levels	Mild to moderate AD	Severe AD
Number of individuals	40	13
Total serum IgE (kU/L) (median± SD)	77.5 ± 809.9	400 ± 1349.7
Elevated total serum IgE (>100 kU/L) (%)	41	76.9
Serum egg IgE positive (>0.35 kU/L) (%)	10.3	15.5
Serum <i>Der p.</i> IgE positive (>0.35 kU/L) (%)	20.0	30.8
Serum mold mix positive (>0.35 kU/L) (%)	10.0	15.5

Tables 1-4: Phenotypes of included AD patients (Table 1), IV patients (Table 2), healthy controls (Table 3), and total and allergen-specific serum IgE-levels for AD patients (Table 4).



