Raman profiles of the stratum corneum define 3 filaggrin genotype–determined atopic dermatitis endophenotypes

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Background: Filaggrin (*FLG*) has a central role in the pathogenesis of atopic dermatitis (AD). *FLG* is a complex repetitive gene; highly population-specific mutations and multiple rare mutations make routine genotyping complex. Furthermore, the mechanistic pathways through which mutations in *FLG* predispose to AD are unclear.

Objectives: We sought to determine whether specific Raman microspectroscopic natural moisturizing factor (NMF) signatures of the stratum corneum could be used as markers of *FLG* genotype in patients with moderate-to-severe AD.

Methods: The composition and function of the stratum corneum in 132 well-characterized patients with moderate-to-severe AD were assessed by means of confocal Raman microspectroscopy and measurement of transepidermal water loss (TEWL). These parameters were compared with *FLG* genotype and clinical assessment.

Results: Three subpopulations closely corresponding with *FLG* genotype were identified by using Raman spectroscopy. The Raman signature of NMF discriminated between *FLG*-associated AD and non–*FLG*-associated AD (area under the curve, 0.94; 95% CI, 0.91-0.99). In addition, within the subset of *FLG*-associated AD, NMF distinguished between patients with 1 versus 2 mutations. Five novel *FLG* mutations were found on rescreening outlying patients with Raman signatures suggestive of undetected mutations (R3418X, G1138X, S1040X, 10085delC, and L2933X). TEWL did not associate with *FLG* genotype subgroups.

Conclusions: Raman spectroscopy permits rapid and highly accurate stratification of *FLG*-associated AD. *FLG* mutations do not influence TEWL within established moderate-to-severe AD. (J Allergy Clin Immunol 2010;126:574-80.)

Key words: *Atopic dermatitis, confocal Raman spectroscopy, eczema, filaggrin, hyperlinearity, natural moisturizing factor, transepidermal water loss, tyrosine*

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Atopic dermatitis (AD) is a complex and heterogeneous inflammatory skin disease driven and modified by immunologic, environmental, and genetic factors.[1,2] The identification of filaggrin (*FLG*) null alleles in up to 50% of patients with moderate-to-severe AD implicates a fundamental role for barrier homeostasis in this disease.[3-6] Although the mechanisms leading to AD in *FLG* mutation carriers are unclear, the deficiency of *FLG* likely facilitates permeability of biologically active allergens and microbial colonization that subsequently trigger inflammatory cascades.[7] The recent identification of a murine model for *FLG* deficiency, with the detection of a homozygous frameshift mutation in the *Flg* gene in *flaky tail* mice, should accelerate our understanding of pathogenic mechanisms and therapeutic intervention points in patients with AD.[8]

Knowledge of the biochemical functions of filaggrin and its breakdown products indicate that a quantitative variation in gene or protein dosage might be relevant in determining phenotype.[9,10] Filaggrin is initially produced as profilaggrin, a large, insoluble, heavily phosphorylated protein consisting of 10 to 12 tandem repeats of filaggrin units separated by short hydrophobic linker peptides.[11,12] In the transitional layer profilaggrin is dephosphorylated and proteolytically processed into its functional filaggrin units, which bind to and collapse the keratin cytoskeleton and other intermediate filaments, acting as a scaffold for the subsequent reinforcement steps of the stratum corneum (SC).[13-15] Subsequently, filaggrin is progressively degraded within the SC into a pool of hygroscopic amino acids, including pyrrolidone carboxylic acid, urocanic acid, and alanine. This composite mixture of amino acids and their derivatives, together with specific salts and sugars, form the natural moisturizing factor (NMF).[16,17]

NMF is highly hygroscopic and plays a central role in maintaining hydration of the SC and is additionally proposed to have a significant role in maintenance of the pH gradient of the skin, cutaneous antimicrobial defense, and regulation of key

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Abbreviations used
AD: Atopic dermatitis
AUC: Area under the curve
FLG: Filaggrin
HLP: Hyperlinear palms
NMF: Natural moisturizing factor
ROC: Receiver operating characteristic
SC: Stratum corneum
TEWL: Transepidermal water loss

enzymatic events in the SC.[18,19] Filaggrin thus has complex functions, with roles in establishing structural and chemical barrier function, hydration, and maintenance of epidermal homeostasis in the face of continuous transformation.[20] Expression of filaggrin and subsequent breakdown of filaggrin into NMF is additionally determined based on properties of the microenvironment, including local pH, relative humidity, and protease activity.[17,21,22] *In vitro* evidence also indicates that filaggrin skin expression might be modulated by the atopic inflammatory response mediated by the cytokines IL-4 and IL-13.[23] Genetically determined modifiers of protein dosage include the copy number of filaggrin repeat units, which vary in the population from 10 to 12 units and segregate by normal Mendelian genetic mechanisms.[11,24] These genetic polymorphisms reflect tandem duplications of *FLG* repeats 8, 10, or both and might be an additional modifier of disease phenotype in heterozygotes who carry longer-sized variants on the unaffected allele.[25] Our early data suggest that NMF levels correlate with *FLG*-null allele status and might therefore directly contribute to the dry skin phenotype seen in both patients with ichthyosis vulgaris and those with AD.[10]

Raman spectroscopy is capable of measuring *in vivo* information regarding the molecular composition of the skin, including quantitative analysis of amino acids and water content. It is based on the inelastic light scattering, or Raman scattering, of monochromatic light when the frequency of photons, usually from a laser source, changes on interaction with a sample, giving rise to characteristic Raman spectra and providing noninvasive real-time signatures of biological samples at a molecular level.

We sought to determine whether specific Raman NMF signatures of the SC could be used as markers of *FLG* genotype in patients with moderate-to-severe AD. We examined the association of NMF estimation with clinical evidence of hyperlinear palms (HLP), a clinical sign that has been shown to be associated with *FLG* mutations in previous studies. We also sought to examine the effects, within patients with moderate-to-severe AD, of *FLG* genotype on transepidermal water loss (TEWL) as a measure of an inside-out barrier defect.

METHODS

Following standard genetic practice, in this article $FLG^{-/-}$ designates a patient homozygous for null alleles (ie, 2 null alleles), $FLG^{+/-}$ designates a heterozygote null allele/wild-type (ie, 1 null allele), and $FLG^{+/+}$ designates a homozygote wild-type (ie, 0 null alleles). A further abbreviation describes patients with AD with *FLG* mutations ($FLG^{+/-}$ and $FLG^{-/-}$) as AD_{FLG} and those without *FLG* mutations (ie, $FLG^{+/+}$) as $AD_{NON\text{-}FLG}$.

One hundred thirty-five unrelated Irish children with a history of moderate-to-severe AD were recruited from dedicated tertiary referral AD clinics. Diagnosis was made by experienced pediatric dermatologists according to the United Kingdom diagnostic criteria.[26] Exclusion criteria from the study were patients who had received systemic therapy, such as corticosteroids or immunosuppressants, in the preceding 3 months and patients whose ancestry was not exclusively Irish (4/4 grandparents). Detailed phenotypic data were collected. The Nottingham Eczema Severity Score[27] was selected as an estimate of disease severity. Given previous publications and our own clinical experience of noting palmar hyperlinearity, this clinical sign was scored by using an investigator assessment of 0 (no hyperlinearity), 1 (mild/subtle hyperlinearity), and 2 (severe hyperlinearity).

Genetic screening

All patients were screened for the 6 most prevalent *FLG* mutations in the Irish population (R501X, 2282del4, R2447X, S3247X, 3702delG, and Y2092X), as previously described.[28] Full sequencing of *FLG* was performed as detailed previously.[28] Based on screening for these 6 prevalent *FLG* mutations, 58.3% were carriers of 1 or more *FLG* mutations (15.1% $FLG^{-/-}$, 43.2% $FLG^{+/-}$, and 41.7% $FLG^{+/+}$). An additional rare mutation was known to be present in 1 subject in a heterozygous state (R1474X). Additional screening was performed by means of complete sequencing of the *FLG* gene in selected subjects, as previously described.[29] The entire collection was then rescreened for these 5 novel mutations found on complete sequencing.

Biophysical analysis of the SC

Skin biophysical measurements were performed under standardized conditions (room temperature, 22 °C-25 °C; humidity levels, 30% to 35%). Before measurements, patients were acclimatized for a minimum of 10 minutes. All measurements were performed by one of 2 investigators (G.M.O. and P.M.J.H.K.). Topical therapies, including emollients, were withheld from the measurement sites for 48 hours preceding the study. TEWL was measured on nonlesional skin of the extensor forearm (Tewameter 300; Courage and Khazaka Electronic GmbH, Cologne, Germany).

NMF was measured in the SC of the thenar eminence by using confocal Raman microspectroscopy (model 3510 Skin Composition Analyzer; River Diagnostics, Rotterdam, The Netherlands). The principles of this method and the procedure have been described elsewhere.[30,31] Depth profiles of Raman spectra were measured at 5-μm intervals from the skin surface at 30, 35, 40, 45 and 50 μm below the skin surface. An average of 8 profiles (totaling to 40 Raman spectra) from different areas of the thenar eminence were measured per patient. Raman spectra were recorded in the spectral region at 400 to 1,800 cm^{-1} with a 785-nm laser. Laser power on the skin was 25 mW. Levels of skin constituents relative to keratin were determined from the Raman spectra by means of classical least-squares fitting. Details of the method have been described elsewhere.[30,31] Briefly, reference spectra of keratin, NMF, urocanic acid, lactate, urea, ceramide, and cholesterol were fitted to the individual Raman spectra from the skin. The combination of these reference spectra provides an adequate model for *in vivo* Raman spectra of normal human SC. A spectrum of reagent-grade L-tyrosine (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to this set of reference-fit spectra to enable determination of increased tyrosine levels. The resulting fit coefficients represent the relative proportions in which the skin constituents contribute to the total Raman skin spectrum. The reference spectrum of NMF had been constructed from the weighted sum of the spectra of its dominant constituents (pyrrolidone carboxylic acid, ornithine, serine, proline, glycine, histidine, and alanine). All concentrations of skin constituents relative to keratin were calculated from the recorded Raman spectra by using SkinTools 2.0 (River Diagnostics B.V., Rotterdam, The Netherlands); NMF levels derived from the individual Raman spectra were used to assess intrapatient variation in NMF. These NMF levels were then averaged to obtain the mean NMF level per patient. Of the 135 subjects who participated in the study, 3 patients were unable to fully cooperate with the Raman measurement, and their results were excluded because of insufficient spectra collection.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Our Lady's Children's Hospital, Dublin. Written informed consent was obtained from all patients or their parents.

Statistical methods

Patients were characterized, *a priori*, into 3 genotypes ($FLG^{+/+}$, $FLG^{+/-}$, and $FLG^{-/-}$), as described in the methods. The designation *FLG-associated*