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CONFLICT OF INTEREST

The authors state no conflict of interest.

Tomonori Motokawa[1], Tomomi Kato[1], Yuki Hashimoto[2], Hiroyuki Takimoto[1], Hiroaki Yamamoto[3] and Takayuki Katagiri[1]

[1]Cutaneous Drug Research Department, POLA Chemical Industries Inc., Yokohama, Japan; [2]First Department of Dermatology, Toho University School of Medicine, Tokyo, Japan and [3]Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, Japan
E-mail: motchy@pola.co.jp

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Prevalent and Low-Frequency Null Mutations in the Filaggrin Gene Are Associated with Early-Onset and Persistent Atopic Eczema

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TO THE EDITOR

Eczema (Johansson *et al.*, 2004) is a common inflammatory skin disorder associated with epidermal barrier dysfunction (Jakasa *et al.*, 2007). The finding that null mutations within the filaggrin gene (*FLG*) are strong predisposing factors for atopic eczema has been demonstrated and replicated in multiple independent case–control and association studies from different European populations, as recently reviewed by Irvine (2007). *FLG* is located within the epidermal differentiation complex on chromosome 1q21, a cluster of genes involved in the terminal differentiation of keratinocytes (Mischke *et al.*, 1996). *FLG* is expressed in the granular layer of the stratum corneum during terminal epidermal differentiation. It aggregates the keratin cytoskeleton to facilitate the collapse and flattening of keratinocytes to produce squames (Manabe *et al.*, 1991); filaggrin may also contribute to epidermal barrier function following its degradation to release hygroscopic amino acids (Rawlings and Harding, 2004).

Detailed study of the structure and function of the *FLG* gene has revealed five prevalent null mutations within the European population, as well as multiple low-frequency null mutations, some of which may be family specific (Sandilands *et al.*, 2007b). These mutations have been found in 47% of one eczema case series and together they make a significant contribution to the genetic susceptibility to atopic eczema (Sandilands *et al.*, 2007a).

Following on from a previous report where the two most common *FLG* mutations (R501X and 2282del4) were strongly associated with early-onset eczema persisting into adulthood (Barker *et al.*, 2007), we have extended that case–control study to investigate the significance of all five of the recently described and most prevalent *FLG* mutations. We studied a total of 186 adult eczema patients from London and Newcastle-upon-Tyne, UK, whose eczema had begun in early childhood. Their phenotypic characteristics are as reported previously (Barker *et al.*, 2007). We similarly screened an unselected population birth cohort of 1,035 individuals from the northwest of England, of unknown phenotype, for use as a control population.

The study was carried out in accordance with the Declaration of Helsinki

Abbreviation: FLG, *filaggrin gene*

Table 1. *FLG* genotypes in English adult cases of early-onset persistent atopic eczema and an unselected population control group

	R501X		2282del4		R2447X		S3247X		3702delG		3673delC		Combined null genotype	
Genotype	Control population	Atopic eczema cases	Control population	Atopic eczema cases	Control population	Atopic eczema cases	Control population	Atopic eczema cases	Control population	Atopic eczema cases	Control population	Atopic eczema cases	Control population	Atopic eczema cases
AA	943	138	899	150	1,014	174	791	183	880	183	881	183	661	100
Aa	52	39	52	32	8	10	4	1	1	1	0	1	84	58
aa	1	9	0	4	0	0	0	0	0	0	0	0	2	26
Total	996	186	951	186	1,022	184	795	184	881	184	881	184	747	184
	$P=3.2\times10^{-17}$		$P=7.1\times10^{-10}$		$P=7.8\times10^{-5}$		$P=1.0$		$P=0.3$		$P=0.2$		$P=1.3\times10^{-28}$	

AA, homozygous wild type for *FLG* null mutation; Aa, heterozygous for any one of the *FLG* null mutations; aa, homozygous for each of the six *FLG* null mutations studied and/or compound heterozygous for any of the null mutations in the ''combined null genotype'' data. The rationale for generating this combined genotype is based on the fact that each mutation results in premature termination of the profilaggrin molecule and hence absence of processed filaggrin (Sandilands *et al.*, 2007b).
Each variant was in Hardy–Weinberg equilibrium in the control population with the exception of 3673delC, because there is insufficient data to allow this calculation.
In the control population, there was one homozygote (R501X) and one compound heterozygote (R501X/2282del4). Among the eczema cases, there were 12 homozygotes (eight for R501X and four for 2282del4) and 13 compound heterozygotes (eight R501X/2282del4, three R501X/R2447X, one R501X/3673delC, and one 2282del4/R2447X).
The total figures vary because of incomplete genotyping results; all available data has been used for optimal analysis of each individual variant, but the combined null genotype data include only those individuals for whom all six genotype results are available.
P-values are calculated using the Fisher's exact test.

Principles and approved by the local Research Ethics Committees in London and Newcastle. All subjects or the subjects' guardians gave informed consent. Mutation analysis was performed using a TaqMan-based allelic discrimination assay (Applied Biosystems, Foster City, CA) for R501X, 2282del4, R2447X, and S3247X and size analysis of fluorescently labeled PCR products using an Applied Biosystems 3100 DNA sequencer for 3702delG, as described previously (Palmer *et al.*, 2006; Sandilands *et al.*, 2006). Homozygote and heterozygote results were confirmed by restriction enzyme digest (Palmer *et al.*, 2006; Sandilands *et al.*, 2006) or sequencing (Sandilands *et al.*, 2007b). Allele and genotype frequencies were compared by Fisher's exact test and logistic regression, using the statistical analysis package Stata (StataCorp LP, College Station, TX). Exploratory haplotype analysis was performed using the software package UNPHASED (Dudbridge, 2007).

In this study population, three of the common *FLG* variants are strongly and independently associated with early-onset persistent eczema: R501X ($P=3.2\times10^{-17}$, odds ratio (OR) 5.6, 95% confidence interval (95% CI) 3.7–8.3), 2282del4 ($P=7.1\times10^{-10}$, OR 4.1, 95% CI 2.7–6.4), and R2447X ($P=7.8\times10^{-5}$, OR 7.3, 95% CI 2.8–18.7). Sequencing of the PCR fragment produced by the assay for 3702delG identified a novel, previously unreported single base-pair deletion, 3673delC, within the third filaggrin repeat. This mutation was found in one of the eczema cases (a compound heterozygote with R501X) but was not identified in any of the controls.

The rarer S3247X, 3702delG, and 3673delC variants were not significantly associated with eczema when analyzed individually. However, the combined null genotype of all six mutations showed a highly significant association ($P=1.3\times10^{-28}$, OR 5.6, 95% CI 4.1–7.8). As the control population consists of individuals with unknown phenotype and an estimated prevalence of 23–25% atopic eczema (Shamssain, 2007), the true association may in fact be even more highly significant. In total, 45.7% of cases carried one or more of the filaggrin mutations, compared with 11.5% of the control population. This is therefore very comparable to the recent study of these mutations in an Irish pediatric eczema case–control study, where 47% of cases carried *FLG* mutations (Sandilands *et al.*, 2007b).

The figures shown in Table 1 result from statistical calculations assuming a genotype-based model of disease, testing to what extent the genotypes (AA, Aa, or aa) can predict the disease status (case or control). An alternative statistical model may also be used, based on alleles, assuming a multiplicative model, that is the presence of two copies of the null allele (aa) will give twice the effect of one copy on the log odds scale (Aa; Table 2). Comparison of the two models using a likelihood ratio test shows that there is no significant difference (for example, $P=0.7$ for the R501X variant), indicating that either model can appropriately be used.

The genotype-based analysis allows us to compare the estimated effect of carrying one mutation with the estimated effect of carrying two mutations, where homozygotes are present (Table 2). The CIs are large and overlapping, but it can be seen that homozygote mutants for R501X tend to have a higher OR of disease than heterozygotes when compared with wild-type homozygotes. More strikingly, although the CIs are again wide, the combined null genotype shows an OR of 85.9 (95% CI 20.1–367.6) for homozygotes and compound heterozygotes, as opposed to 4.6 (3.1–6.8) for hetero-

Table 2. Results of logistic regression analysis to calculate ORs using the genotype and allele-based models

	R501X	2282del4	R2447X	S3247X	3702delG	3673delC	Combined null genotype
OR calculated using the allele model (95% CIs)	5.6 (3.7–8.3)	4.1 (2.7–6.4)	7.3 (2.8–18.7)	1.1 (0.1–9.7)	4.8 (0.3–77.2)	NA	5.6 (4.1–7.8)
OR calculated using the genotype model (95% CIs)							
Heterozygotes vs wild type	5.1 (3.3–8.1)	3.7 (2.3–5.9)	7.3 (2.8–18.7)	1.1 (0.1–9.7)	4.8 (0.3–77.2)	NA	4.6 (3.1–6.8)
Homozygote mutants vs wild type	61.5 (7.7–489.2)	NA	NA	NA	NA	NA	85.9 (20.1–367.6)

CI, confidence interval; NA, not analyzed, because of absence or insufficient numbers of homozygotes (and heterozygotes for delC); OR, odds ratio.

zygotes. In other words, an individual carrying one of these six null mutations has an approximately four times greater chance of having early-onset persistent eczema, whereas carriage of two *FLG* null mutations increases the risk about 80 times, when compared with an individual who does not carry any of these variants. These data support the clinical observations reported in the original cohort studies of ichthyosis vulgaris and atopic eczema, suggesting that *FLG* mutations are semidominant (Palmer *et al*., 2006; Smith *et al*., 2006). The genotype-based analysis (Table 2) also demonstrates a significant association between heterozygote status and eczema, independent of the homozygotes and compound heterozygotes, a group likely to contain a large proportion of individuals with ichthyosis vulgaris and severe eczema.

Comparison of the ORs associated with different mutations has been used as an estimate of their relative strength of effect in influencing the skin phenotype. In this way, it has been suggested that the S3247X mutation (which occurs in repeat 9 of exon 3) may be less penetrant than other mutations that occur in earlier repeats (Sandilands *et al*., 2007b). However, a more sophisticated statistical technique can be used—the test of equality of haplotype risks—to test the risk of disease associated with different mutations. Analyzing our data in this way has shown that there is no significant difference in the risk associated with R501X and 2282del4 ($P=0.2$) or R501X and R2447X ($P=0.6$). However, there is a trend towards significance when comparing the risks associated with R501X and S3247X ($P=0.07$). This is in keeping with different ORs associated with these variants in the original report (Sandilands *et al*., 2007b). This may be explained either by the fact that S3247X allows partial expression of profilaggrin or because it is in linkage disequilibrium with a protective factor nearby in the epidermal differentiation complex (Sandilands *et al*., 2007b). As it has been shown biochemically both in mouse (Presland *et al*., 2000) and humans (Sandilands *et al*., 2007b) that distal *FLG* null mutations are equivalent in terms of preventing profilaggrin-to-filaggrin processing as well as destabilizing the truncated proprotein, the latter hypothesis is perhaps more likely. However, in this study, it is difficult to draw firm conclusions, as the S3247X mutation may not have shown significant association with the eczema phenotype owing to its low frequency and the presence of eczema cases within the unphenotyped control group.

It has been reasoned from first principles that a compound heterozygote individual is likely to carry one *FLG* mutation on each chromosome. However, it would theoretically be possible for two mutations to occur on the same chromosome, although clearly the mutation occurring downstream of a null mutation would not have any additional effect on protein expression. This possibility was tested statistically by haplotype analysis (Dudbridge, 2007). In our group of cases and controls, the probability of any two mutations occurring in the same phase (that is, on the same chromosome) is vanishingly small, with estimated haplotype frequencies $<10^{-10}$.

In conclusion, this study has demonstrated that the three most prevalent null mutations in *FLG* (R501X, 2282del4, and R2447X) are significantly and independently associated with the phenotype of early-onset atopic eczema that recurs or persists into adulthood. Together with three lower-frequency null mutations (S3247X, 3702delG, and 3673delC), they make a significant contribution to the genetic susceptibility to this severe eczema phenotype.

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene. The other authors state no conflict of interest.

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Sara J. Brown[1,6], Aileen Sandilands[2], Yiwei Zhao[2], Haihui Liao[2], Caroline L. Relton[3], Simon J. Meggitt[1], Richard C. Trembath[4], Jonathan N.W.N. Barker[5], Nick J. Reynolds[1,7], Heather J. Cordell[6] and W.H. Irwin McLean[2]

[1]Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne, UK; [2]Epithelial Genetics Group, Human Genetics Unit, University of Dundee, Ninewells Hospital and Medical School, Dundee, UK; [3]School of Clinical Medical Sciences (Child Health), Newcastle University, Newcastle upon Tyne, UK; [4]Department of Medical and Molecular Genetics, King's College, London, UK; [5]St John's Institute of Dermatology, King's College London, St Thomas' Hospital, London, UK; [6]Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, UK and [7]Institute of Cellular Medicine,

Newcastle University, Newcastle upon Tyne, UK
E-mail: sara.brown@ncl.ac.uk

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Filaggrin Expression in Oral, Nasal, and Esophageal Mucosa

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TO THE EDITOR

Allergic diseases typically develop in mucosal surfaces such as the skin, airway and gastrointestinal tract. The best described of these are atopic dermatitis (AD), food allergy, allergic rhinitis (AR), and asthma. More recently, eosinophilic esophagitis (EE) has also been characterized as an atopic disorder. Genetic studies have highlighted the importance of epithelial-derived genes such as dipeptidyl peptidase 10, serine protease inhibitor–kazal-type 5, stratum corneum chymotryptic enzyme, and filaggrin (*FLG*) in either asthma or AD or both (Morar *et al.*, 2006). Specific loss-of-function mutations in the *FLG* gene recently described in subjects with AD are thought to explain, in part, the cutaneous barrier defects long observed in AD (Palmer *et al.*, 2006). Stratum granulosum cells produce filaggrin as they differentiate into stratum corneum cells. Filaggrin aggregates keratin filaments within the cells resulting in the cornified envelope, which is critical for barrier function. This work has led many to hypothesize that the skin is a key portal of entry for relevant allergens and irritants that can drive both skin and airway disease (Cork *et al.*, 2006). This is further supported by the evidence that *FLG* mutations are associated with extrinsic AD, early onset of eczema that persists into adulthood, and the coexistence of atopic asthma or AR and AD (Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Weidinger *et al.*, 2007). This latter association has led to the speculation that *FLG* mutations may directly impact barrier function at other mucosal surfaces such as airway or gastrointestinal tracts. The mucosal surfaces where filaggrin is expressed have not been well characterized. An online textbook and previous published studies state that filaggrin immunoreactivity has been observed in orthokeratinized and parakeratinized areas of human oral epithelium (Smith and Dale, 1986; Reibel *et al.*, 1989), cervix, endometrium, and vagina (http://www.proteinatlas.org/tissue_profile.php?antibody_id=2210). Whether mutations in *FLG* would have a direct impact on barrier function in other mucosal surfaces such as upper airway and esophagus has not been well studied. To address the lower airway, Ying *et al.* (2006) recently reported the complete absence of filaggrin immunoreactivity in bronchial biopsies obtained from both asthmatic and nonatopic (NA) control subjects. Bronchial staining ($n=3$) conducted in our laboratory have confirmed Ying's findings (data not shown). This data suggests that filaggrin has no direct role in lower

Abbreviations: AD, atopic dermatitis; AR, allergic rhinitis; EE, eosinophilic esophagitis; FLG*, filaggrin; IRB, Institutional Review Board; NA, nonatopic*