

Filaggrin mutations that confer risk of atopic dermatitis confer greater risk for eczema herpeticum

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Background: Loss-of-function null mutations R501X and 2282del4 in the skin barrier gene, filaggrin (*FLG*), represent the most replicated genetic risk factors for atopic dermatitis (AD). Associations have not been reported in African ancestry populations. Atopic dermatitis eczema herpeticum (ADEH) is a rare but serious complication of AD resulting from disseminated cutaneous herpes simplex virus infections.

Objective: We aimed to determine whether *FLG* polymorphisms contribute to ADEH susceptibility.

Methods: Two common loss-of-function mutations plus 9 *FLG* single nucleotide polymorphisms were genotyped in 278 European American patients with AD, of whom 112 had ADEH, and 157 nonatopic controls. Replication was performed on 339 African American subjects.

Results: Significant associations were observed for both the R501X and 2282del4 mutations and AD among European American subjects ($P = 1.46 \times 10^{-5}$, 3.87×10^{-5} , respectively), but the frequency of the R501X mutation was 3 times higher (25% vs 9%) for ADEH than for AD without eczema herpeticum (EH) (odds ratio [OR], 3.4; 1.7-6.8; $P = .0002$). Associations with ADEH were stronger with the combined null mutations (OR, 10.1; 4.7-22.1; $P = 1.99 \times 10^{-11}$). Associations with the R501X mutation were replicated in the African American population; the null mutation was absent among healthy African American subjects, but present among patients

with AD (3.2%; $P = .035$) and common among patients with ADEH (9.4%; $P = .0049$). However, the 2282del4 mutation was absent among African American patients with ADEH and rare (<1%) among healthy individuals.

Conclusion: The R501X mutation in the gene encoding filaggrin, one of the strongest genetic predictors of AD, confers an even greater risk for ADEH in both European and African ancestry populations, suggesting a role for defective skin barrier in this devastating condition. (J Allergy Clin Immunol 2009;124:507-13.)

Key words: Atopic dermatitis, eczema herpeticum, filaggrin, R501X, 2282del4, single nucleotide polymorphisms

Atopic dermatitis (AD) is a common, chronic pruritic, inflammatory skin disease complicated by recurrent bacterial and viral skin infections.^{1,2} A rare but serious complication of AD is atopic dermatitis eczema herpeticum (ADEH), a disseminated infection associated with significant morbidity that had conferred significant mortality before introduction of systemic antiviral therapy. Complications include keratoconjunctivitis, which can result in blindness, as well as multiorgan involvement with meningoencephalitis. The primary predisposing factor for ADEH is herpes simplex virus (HSV)-1 exposure, which is ubiquitous in the general population (ie, ~20% of children and more than 60% of adults are seropositive).³ However, only ~3% of patients with AD develop disseminated cutaneous HSV infections (including HSV-1)⁴ despite the high likelihood of HSV-1 exposure, suggesting this complication is not a function of environmental exposure alone, and genetic susceptibility may be at play. Although there is considerable evidence to support a genetic basis for AD,⁵ virtually nothing is known about the heritability of ADEH.

Two common mutations (R501X and 2282del4) in the gene encoding filaggrin (*FLG*), a critical protein in skin barrier function, have been consistently associated with risk of AD⁶ and related traits, including asthma, hay fever, rhinoconjunctivitis, and allergen-specific IgE.⁷⁻¹³ Full sequencing of the *FLG* gene has revealed multiple, additional polymorphisms with varying frequency across ethnic groups.¹⁴ With a combined allele frequency among patients with AD of 18% and 48% for the R501X and 2282del4 mutations, respectively, these polymorphisms represent the strongest and most compelling genetic risk factors for AD. Little is known regarding the relevance of these mutations in populations of African descent, because nearly all studies to date have been performed in populations of European or Asian descent. We previously reported genotyping 23 African American individuals for these mutations, and all had wild-type genotypes for both mutations,¹⁵ supporting the contention that these mutations may be less common in this ethnic group.¹⁶ As part of the National Institutes of Health/National Institute of

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Abbreviations used

AD:	Atopic dermatitis
ADEH:	Atopic dermatitis eczema herpeticum
ADVN:	Atopic Dermatitis and Vaccinia Network
EASI:	Eczema Area and Severity Index
EH:	Eczema herpeticum
FLG:	Filaggrin
HSV:	Herpes simplex virus
LD:	Linkage disequilibrium
OR:	Odds ratio
SNP:	Single nucleotide polymorphism
tIgE:	Total serum IgE

Allergy and Infectious Diseases (NIH/NIAID)–sponsored Atopic Dermatitis and Vaccinia Network (ADVN) to dissect mechanisms increasing susceptibility of patients with AD to disseminated viral skin infections, we sought to determine whether the R501X and 2282del4 mutations confer risk of ADEH in a European American group, and to test for association of additional markers across the *FLG* gene. Replication of findings was tested in an independent African American group.

METHODS**Study participants and phenotypes**

DNA was isolated from 278 unrelated European American patients with AD (112 with ADEH) and 157 healthy controls participating in the ADVN. The same set of markers was genotyped on 187 African American patients with AD (32 with ADEH) and 152 healthy controls. Baseline characteristics are presented in Table I.

Atopic dermatitis was diagnosed by using the US consensus conference criteria.¹⁷ Patients with ADEH were defined as patients with AD with at least 1 eczema herpeticum (EH) episode documented by an ADVN investigator (or a physician affiliated with the same academic center) or diagnosis by another physician confirmed by HSV PCR, tissue immunofluorescence, Tzanck smear, and/or culture. Nonatopic, healthy controls were defined as having no personal history of chronic disease including atopy.¹⁸ AD severity was defined according to the Eczema Area and Severity Index (EASI), a standardized grading system¹⁹ and total serum IgE was measured as described in this article's Methods in the Online Repository at www.jacionline.org. Clinical characteristics are presented in Table I, and further detail of phenotyping is available in this article's Methods in the Online Repository. The study was approved by the institutional review boards at National Jewish Health in Denver, Johns Hopkins University, the Oregon Health and Science University, the University of California San Diego, the Children's Hospital of Boston, and the University of Rochester. All subjects gave written informed consent before participation.

An additional group of 177 healthy African American controls was included. Participants were recruited from the Baltimore-Washington, DC, metropolitan area and self-reported as African American ethnicity. These subjects made up the consortium for Genomic Research on Asthma in the African Diaspora and represent 8 separate, NIH-funded studies of asthma in pediatric and adult African American populations, plus 1 study on healthy African American subjects. Informed consent was obtained from each study participant, and the study protocol was approved by the institutional review board at either the Johns Hopkins University or Howard University.

FLG genotyping and quality control

Genotyping was performed as described in this article's Methods section in the Online Repository on the 2 *FLG* mutations previously reported as associated with AD (R501X and 2282del4), plus 9 additional polymorphisms (rs12730241, rs2065956, rs11582620, rs3126082, rs6587665, rs11204980,

rs1933063, rs1933064, rs3126091; details of the polymorphisms and minor allele frequencies are presented in this article's Table E1 in the Online Repository at www.jacionline.org). To evaluate genetic structure of the 2 ethnic groups from the 3 continental ancestral populations in the International HapMap project (<http://www.hapmap.org>), we genotyped an additional 74 single nucleotide polymorphisms (SNPs) identified as ancestry informative markers selected for maximal difference between African and European populations and assessed potential confounding caused by population substructure by using genotype data and the STRUCTURE program (v2.2; <http://pritch.bsd.uchicago.edu/software>) to estimate membership in distinct subpopulations (see this article's Fig E1 in the Online Repository at www.jacionline.org). Tests for association were performed by using the Cochran-Armitage trend test and as described in this article's Methods in the Online Repository.

RESULTS

We compared the distribution of observed *P* values to the expected distribution under the null hypothesis of no genetic association, and concordant with findings reported in other European American populations,²⁰ significant associations were observed for both the R501X and 2282del4 mutations and AD, with allele frequencies of the R501X allele among all patients with AD at 7.8% compared with 1.3% among the healthy participants (odds ratio [OR], 7.1; 2.5-20.1; $P = 1.46 \times 10^{-5}$; Table II) and frequencies of the 2282del4 allele at 8.8% compared with 1.6% (OR, 5.6; 2.2-14.6; 3.87×10^{-5} ; Table II). Evidence of associations with the R501X mutation and 2282del4 was significantly strengthened when the analyses were limited to the 112 patients with ADEH. In this group, 25.4% of patients with ADEH carried the R501X null mutation (OR, 13.1; 4.4-38.5; $P = 7.25 \times 10^{-9}$), and 17.8% of patients with ADEH carried the 2282del4 null mutation (OR, 6.5; 2.4-18.1; $P = 3.0 \times 10^{-5}$). When analyses were restricted to the patients with ADEH versus patients with AD without EH, a significant, albeit weaker, association was observed for the R501X mutation (OR, 3.4; 1.7-6.8; $P = .0002$), but not for the 2282del4 mutation (OR, 1.3; 0.7-2.5; $P = .465$). Additional analyses were performed for the combined genotype of the R501X and 2282del4 mutations, and the risks for individuals carrying both null mutations for AD (OR, 6.4; 3.1-13.2; $P = 1.21 \times 10^{-8}$) and ADEH (OR, 10.1; 4.7-22.1; $P = 1.99 \times 10^{-11}$) were remarkably increased compared with the risk for individuals carrying either the R501X mutation or the 2282del4 alone, suggesting that the combination of both R501X and 2282del4 mutations may contribute to an increased risk of both AD and ADEH in this European American group.

In addition to the R501X and 2282del4 mutations, we detected modest associations of 2 SNPs (rs12730241 and rs2065956) in exon 3 localized in the first linkage disequilibrium (LD) block (see this article's Fig E2 in the Online Repository at www.jacionline.org) and a reduced risk of AD (OR, 0.61, 0.40-0.91, $P = .016$; and OR, 0.62, 0.42-0.91, $P = .015$, respectively; see this article's Table E2 in the Online Repository at www.jacionline.org) and 1 SNP (rs1933063) in the promoter region associated with an increased risk of ADEH (OR, 4.19; 1.22-14.44; $P = .015$). Association tests for each mutation among European Americans are summarized in Fig 1. In sliding-window haplotype analyses using all SNPs, we observed several significant 2-marker, 3-marker, and 4-marker haplotypes showing evidence of association for AD ($2.43 \times 10^{-7} < P < .05$; see this article's Fig E3 in the Online Repository at www.jacionline.org), with the strongest association for the haplotype carrying SNP 2065956 and 2 null mutations,

TABLE I. Participant characteristics

Characteristic	European American			African American		
	ADEH+	ADEH-	Healthy	ADEH+	ADEH-	Healthy
Sample size	112	166	157	32	155	152
Male subjects, N (%)	58 (53.7)	49 (29.7)	63 (40.1)	12 (37.5)	36 (23.2)	77 (50.7)
Age (y), mean (SD)	22.9 (22.1)	38.4 (14.6)	36.5 (13.2)	17.9 (17.8)	36.4 (11.0)	41.1 (10.3)
Geometric mean IgE levels (95% CI)*	2083 (1399-3100)	366.7 (261-516)	55.5 (44-69)	2943 (1470-5892)	391.6 (294-522)	142.6 (114-178)
Geometric mean EASI† (95% CI)‡	7.4 (6.0-9.1)	3.5 (2.9-4.3)	ND	9.5 (6.3-14.4)	3.1 (2.5-3.9)	ND

ND, Not done.

*Total serum IgE levels were significantly higher in ADEH+ patients compared with both ADEH- patients and healthy controls.

†EASI determined by the percentage of eczema area on a 7-point ordinal scale: 0 ≤ 10%; 1 = 10% to 29%; 3 = 30% to 49%; 4 = 50% to 69%; 5 = 70% to 89%; and 6 = 90% to 100%.

‡EASI was significantly higher among ADEH+ patients compared with ADEH- patients in both ethnic groups.

TABLE II. Association of *FLG* null mutations with AD and ADEH

TABLE III. Association of FES hair mutations with AD and ADEH										
Mutation	Genotype	No. of subjects (%)			AD vs controls		ADEH vs controls		ADEH vs AD	
		ADEH+	ADEH-	Controls	OR* (95% CI)	P value	OR* (95% CI)	P value	OR* (95% CI)	P value
European American										
R501X	AA	82 (74.6)	151 (91.0)	153 (97.5)	7.1 (2.5-20.1)	1.46×10^{-5}	13.1 (4.4-38.5)	7.25×10^{-9}	3.4 (1.7-6.8)	.0002
	Aa	25 (22.7)	14 (8.4)	4 (2.6)						
	aa	3 (2.7)	1 (0.6)	0						
2282del4	AA	88 (82.2)	142 (85.5)	151 (96.8)	5.6 (2.2-14.6)	3.87×10^{-5}	6.5 (2.4-18.1)	3.00×10^{-5}	1.3 (0.7-2.5)	.4651
	Aa	17 (15.9)	21 (12.7)	5 (3.2)						
	aa	2 (1.9)	3 (1.8)	0						
Combined	AA	66 (61.7)	130 (78.3)	147 (94.2)	6.4 (3.1-13.2)	1.21×10^{-8}	10.1 (4.7-22.1)	1.99×10^{-11}	2.2 (1.3-3.8)	.0029
	Aa	35 (32.7)	33 (19.9)	9 (5.8)						
	aa	6 (5.6)	3 (1.8)	0						
African American										
R501X	AA	29 (90.6)	153 (98.1)	152 (100)		.0351		.0049	5.28 (1.0-27.4)	.0289
	Aa/aa	3 (9.4)	3 (1.9)	0						
2282del4	AA	32 (100)	148 (96.1)	149 (98.7)	2.5 (0.5-12.5)	.2542		1.0		.5919
	Aa/aa	0	6 (3.9)	2 (1.3)						

AA, Wild-type genotype for either R501X or 2282del4; Aa, heterozygous genotype; aa, homozygous mutant genotype.

*OR was calculated by the AA versus combined Aa + aa genotypes.

2282del4 and R501X ($P = 2.43 \times 10^{-7}$). Stronger haplotype associations were also observed for the ADEH phenotype, with the strongest association for haplotypes carrying both R501X and 2282del4 null mutations ($P = .00067$; Fig E3).

Replication of findings in African American subjects

We sought replication of *FLG* associations with both ADEH and AD without EH in African American patients with AD and healthy controls. We found the R501X mutation to be completely absent among 152 healthy African American subjects, but 6 patients with AD among 188 successfully genotyped individuals (3.2%) were heterozygotes for the mutation. Using Fisher exact test to account for the absence of the mutation in healthy controls, the R501X mutation was significantly associated with AD among African American subjects ($P = .0351$), as observed in the European Americans. Despite the small number of patients with ADEH (N = 32), 9.4% of this group carried the mutation, and the minor allele frequency was considerably higher (4.7%), resulting in a stronger association for ADEH ($P = .0049$), as observed in the European American subjects. When the analysis was restricted to the 2 case groups (ADEH vs AD without EH), the R501X mutation conferred a greater risk of ADEH (OR,

5.28; 1.0-27.4; $P = .0289$). We did not observe an association between the deletion mutation (2282del4) and AD or ADEH among African American subjects, likely because of the very low frequency of the deletion among African American healthy controls (<1%) and patients with AD (3.2%), and the complete absence of the deletion among the 32 African American patients with ADEH. A significant association was observed for 1 of the same exon 3 markers (rs12730241) associated with a reduced risk of AD in the European American subjects; however, among African American patients with AD, this SNP conferred a greater risk of disease (OR, 1.95; 1.15-3.32; $P = .0127$), and the association was even stronger for ADEH (OR, 4.56; 1.57-13.21; $P = .0029$). Flanking marker rs2065956, also associated with a lower risk of AD among the European American group, was associated with a lower risk of ADEH among African American subjects (OR, 0.24; 0.08-0.75; $P = .0089$). No significant haplotypes were observed among the African American group for any of the markers and risk of AD, and the ADEH group was too small to test for associations independent of AD.

We sought to confirm the absence of the R501X mutation among healthy African American subjects by genotyping these markers in an independent reference population of 177 control individuals participating in an ongoing asthma genetics study (see

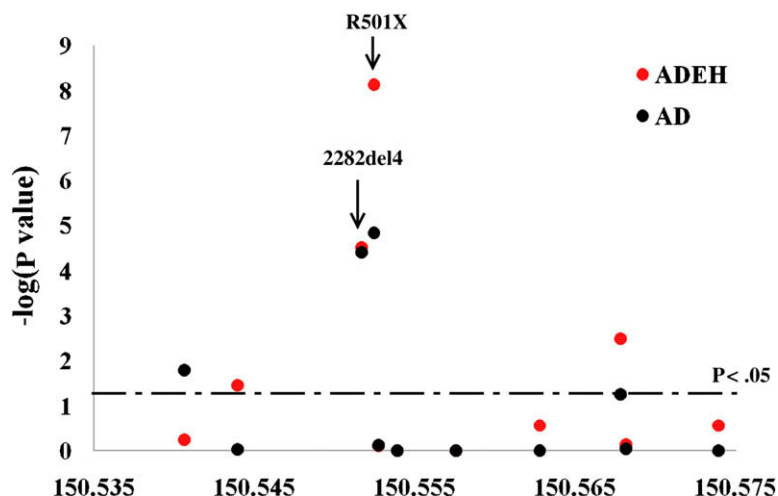


FIG 1. Summary of association tests among European American subjects showing significance for each *FLG* mutation. The $-\log_{10}$ -transformed P values on the y axis for all genotyped markers are plotted against their genomic position in NCBI Build 35 in megabase along the x axis. Red circles indicate associations for ADEH; green circles indicate associations for AD. The horizontal dotted line indicates nominal significance at $P < .05$.

this article's [Methods](#) in the Online Repository). Consistent with our findings in the ADVN healthy African American participants and previous reports of African ancestry populations,^{15,16} the R501X mutation was completely absent, with the exception of 1 individual with a history of AD ([Table III](#)).

DISCUSSION

This study of *FLG* polymorphisms in 2 independent and ethnically diverse populations of patients with AD stratified by the serious complication, ADEH, demonstrated that the functional R501X mutation confers an added risk of ADEH, with an estimated effect size of nearly 13 among European American patients ($P = 7.25 \times 10^{-9}$), and this association was further replicated among African American patients ($P = .0049$). Furthermore, we observed a significant association between the 2282del4 mutation and AD and ADEH *per se* among European American patients, but this association was completely absent among the African American patients with ADEH. Importantly, the combined R501X and 2282del4 mutations further enhanced the association for AD ($P = 1.21 \times 10^{-8}$) and ADEH ($P = 1.99 \times 10^{-11}$) among European American patients, suggesting the combination of these 2 mutations may contribute to an increased risk of AD and ADEH.

Because ADEH manifests as a severe form of AD, it is tempting to attribute the role of the R501X mutation to ADEH susceptibility as it relates to disease severity. Indeed, among both European American and African American patients with ADEH, total serum IgE (tIgE) was substantially higher compared with patients with AD without EH ($P = 6.41 \times 10^{-9}$ and $P = 6.16 \times 10^{-8}$, respectively), as was the more direct measure of severity, the EASI ($P = 1.68 \times 10^{-5}$ and $P = 7.97 \times 10^{-6}$, respectively; [Table I](#)). An association between the R501X mutation and tIgE levels among European American patients with AD has been previously reported,²⁰ and in our own study, as shown in [Table IV](#), we observed the significant association between geometric mean tIgE levels and both the R501X ($P = .023$) and the 2282del4

mutation ($P = 2.90 \times 10^{-5}$) among the European American AD group, with the strongest association between tIgE and haplotypes of the combined R501X and 2282del4 (843.7 ± 7.3 vs 197.8 ± 10.2 ; $P = 5.10 \times 10^{-6}$). Also, we observed significant associations for both mutations and EASI (R501X, $P = 0.0075$; 2282del4, $P = 0.014$), and this association was further enhanced for the combined R501X and 2282del4 null mutation (7.7 ± 3.1 vs 3.9 ± 3.4 , $P = 6.79 \times 10^{-5}$). It is possible that the association between these null mutations and ADEH is a result of confounding associated with the typically higher levels of tIgE and EASI characteristic of patients with ADEH compared with patients with AD without ADEH; unfortunately, it was beyond the scope of the original study design to match adequately patients with ADEH and patients with AD; there were no ADEH controls to address this specifically. However, among all the associations observed, the strongest association was for ADEH, specifically for the combined null mutations ($P = 1.99 \times 10^{-11}$). Furthermore, no associations were observed when analyses were restricted to patients with ADEH, and there were no significant associations at all among African American subjects for the outcomes tIgE and EASI. We therefore speculate that the relationship between this null mutation and disease is most likely related to an increased propensity to disseminated viral skin infection resulting from skin barrier dysfunction, which may be independent of serum IgE and skin severity. It also highlights the importance of skin barrier function in antiviral responses.

The association of *FLG* mutations with ADEH may well be directly causal and have functional consequences relevant for cutaneous viral infectivity. Indeed, it has recently been found that filaggrin-deficient, but not wild-type, mice develop satellite skin lesions when inoculated with vaccinia virus (Geha R, personal communication, December 2008). These data suggest that defects in barrier function may predispose to increased viral penetration and spread in the skin. Because T_H2 responses diminish the innate immune response, *FLG* mutations may play a dual role in allowing increased spread and replication of viruses in AD.^{15,21,22} Furthermore, the elevated serum IgE levels and increased allergen

TABLE III. Minor allele frequencies of the 2 loss-of-function *FLG* variants in ethnically diverse participants in unrelated population-based studies

Population	R501X MAF		2282del4 MAF	
	With AD (N)	Without AD (N)	With AD (N)	Without AD (N)
American				
ADVN European American	0.048 (166)	0.013 (157)	0.082 (166)	0.016 (156)
European American (Rogers, 2007 #3942)	0.039 (179)	0.012 (447)	0.050 (180)	0.023 (449)
ADVN African American	0.010 (156)	<0.001 (151)	0.019 (154)	0.007 (151)
GRAAD African American	NA	0.003 (177)	NA	NA
Other European ancestry				
Irish (Sandilands, 2007 #3920)	0.136 (188)	0.013 (736)	0.098 (188)	0.013 (736)
Scottish (Palmer, 2006 #3636)	NA	0.030 (1008)*	NA	0.019 (1008)*
British (Barker, 2007 #4000)	0.156 (163)	0.029 (1463)	0.117 (163)	0.017 (1463)
French (Hubiche, 2007 #4002)	0.075 (99)	0.025 (102)*	0.101 (99)	0.010 (102)*
German (Marenholz, 2006 #3370)	0.051 (188)	0.008 (319)	0.043 (187)	0.017 (316)
Italian (Giardina, 2008 #3909)	0.006 (178)	<0.001 (210)	0.008 (178)	0.000 (210)
Asian ancestry				
Indian (Palmer, 2006 #3636)	NA	<0.001 (83)	NA	0.014 (111)
Japanese (Nomura, 2007 #4001)	0.000 (143)	<0.001 (156)	<0.001 (143)	<0.001 (156)
Chinese (Palmer, 2006 #3636)	NA	<0.001 (47)	NA	<0.001 (49)
African ancestry				
North African (Palmer, 2006 #3636)	NA	<0.001 (124)	NA	<0.001 (121)

GRAAD, Genomic Research on Asthma in the African Diaspora; MAF, minor allele frequency; NA, not available.

*Control group selected from the general population and not screened for AD.

TABLE IV. Association of null mutations in *FLG* with total serum levels of geometric mean IgE (kU/L) and EASI in the European American subjects

Mutation	Genotype	No. of subjects	Total serum IgE (mean $\pm$ SD, kU/L)*	P value	EASI*	P value
R501X	AA	229	250.1 $\pm$ 10.1	.0234	4.3 $\pm$ 3.3	.0070
	Aa/aa	45	748.8 $\pm$ 7.9		8.2 $\pm$ 3.4	
2282del4	AA	233	245.5 $\pm$ 10.3	2.90×10^{-5}	4.4 $\pm$ 3.5	.0014
	Aa/aa	44	1024.0 $\pm$ 6.2		8.0 $\pm$ 2.9	
Combined	AA	193	197.8 $\pm$ 10.2	5.10×10^{-6}	3.9 $\pm$ 3.4	6.79×10^{-5}
	Aa/aa	77	843.7 $\pm$ 7.3		7.7 $\pm$ 3.1	

aa, Homozygous R501X or 2282del4; Aa, heterozygous genotype for either R501X or 2282del4; AA, wild-type.

*Log-transformed, adjusted for age and sex.

sensitization observed in patients with *FLG* mutations may be a result of greater allergen penetration through the skin.²³ Indeed, a recent study of a newly identified *Flg*-deficient murine model, resulting from a 1-bp deletion that is analogous to the human null mutations, demonstrated that antigen transfer through a disrupted barrier contributes to an elevated IgE sensitization and initiation of cutaneous inflammation in the context of atopic skin disease.²⁴

A novel finding from our study is the association between a SNP in the promoter region (rs1933063, -13289A/G) and a reduced risk of ADEH, lower tIgE levels, and a lower EASI among patients with AD ($P = .0032-.03$). Although this SNP is relatively distant from the transcriptional start site, it is presumably in LD with a mutation or mutations in the promoter region involved in regulation of gene expression or *FLG*-mediated signaling. Although nearly 40 mutations in the *FLG* gene have been reported to date,⁶ none have been found in the *FLG* promoter (McLean I, personal communication, January 2009), suggesting that variants in this region of the *FLG* gene might be uniquely associated with a clinical subset of AD (ie, disease severity, ADEH). Also, 2 synonymous SNPs in pairwise LD (rs12730241 and rs2065956; $D' = 0.93$; $r^2 = 0.64$) were associated with a reduced

risk of AD among European American subjects (OR, 0.61-0.62; $P = .015-.016$). In contrast, these 2 SNPs demonstrated an opposite effect in the African American sample, and were significantly associated with an increased risk of AD and ADEH (OR, 1.95-4.56; $P = .013-.003$). The opposing associations may be a result of variation in allele frequencies according to ethnicity. Interestingly, none of the SNPs were in LD with either R501X or 2282del4 in both European and African American populations (Fig E2). Taken together, these observations suggest there may be a causal variant or variants in LD with these markers (independent of the R501X and 2282del4 mutations) that, together with nongenetic factors (eg, antigens, HSV), may play a role in predicting development of AD and its more serious complication, ADEH.

Ours is the first study to genotype these 2 purportedly common loss-of-function mutations in a group of African American subjects, and concurrent with our preliminary studies,¹⁵ we found a complete absence of the R501X mutation and very low frequency (1%) of the 2282del4 mutation among healthy individuals from this ethnic group. As further confirmation for our findings on the R501X mutation in the 152 healthy African American subjects participating in the ADVN, we examined an additional reference population (177 African Americans recruited in the

Baltimore-Washington, DC, metropolitan area) and observed the mutation only in a single individual, who on further examination had reported a history of AD. The low frequency and even absence of this mutation is not novel; elsewhere, the prevalence of the R501X mutation among individuals without AD has ranged from 0.8% to 3.0% among European populations, and has been found to be absent in southern European (ie, Italian²⁵) and Asian^{16,26} groups (Table III). In the only summary data available on the frequency of this mutation in African populations, it was absent in a cohort of 124 North Africans.¹⁶ Although we observed a minor allele frequency of 1.0% among 153 African American patients with AD without EH (higher among patients with ADEH, at 4.7%), this was considerably less than the observed frequency among European American patients with AD (including the findings in this study, 4.8%; Table III). This is lower than their European counterparts with AD, ranging from 5% to 16%, and suggests a latitude-dependent distribution with a decreasing north-south gradient of frequency. Although we cannot speculate at this time about the past selective pressures among Northern Europeans potentially to account for this distribution of *FLG* genotype, we hypothesize that the considerably lower frequencies of both mutations among the European American subjects in our study compared with Northern Europeans in previous reports¹⁶ probably result from the admixture of the African (6%) and Asian (9.5%) ancestry represented in this group (Fig E2). Conversely, the relatively high frequency of the R501X mutation among African American subjects with AD, especially those with ADEH, is likely owed to the European (17.7%) and Asian (7.6%) admixture observed in this sample.

Palmer et al¹⁶ have previously noted these differences in mutation frequencies and have suggested that different populations will have different *FLG* mutation profiles. Indeed, this group and others have demonstrated that, in populations where the R501X and 2282del4 mutations are not present, other mutations are prevalent and confer risk of AD—for example, the 3321delA and S2554X mutations among Japanese patients.²⁶ Although it is therefore tempting to dismiss the significance of the 2 mutations common among individuals of European ancestry as important risk factors among patients of African descent, the observation that 14% of African American subjects with the R501X mutation had ADEH is very intriguing given that the prevalence was also high (25%) among the European American ADEH group. The significance of this finding—and the importance of the R501X mutation in diseases complicated by AD—is underscored by a recent report²⁷ in which compound heterozygous R501X and 3321delA *FLG* mutations were present in a Japanese family with ichthyosis vulgaris, despite the fact that the R501X mutation failed to be detected in previous studies of both healthy Japanese individuals and Japanese patients with AD.^{16,26} Eliminating any possibility of European admixture in this Japanese family, Hamada et al²⁷ concluded that the R501X mutation could therefore occur as a *de novo* mutation in any population regardless of ethnicity. Although the number of African American patients with ADEH here was too small to permit full replication of results in European American patients, our findings strongly suggest the R501X mutation, but not 2282del4, confers a substantial risk for ADEH regardless of ethnicity. Moreover, we observe, for the first time, a significant association between the R501X mutation and AD among African American subjects, which is particularly relevant given that minority groups, including African American subjects, have eczema disproportionately.²⁸ We acknowledge

that the ADEH samples in this study, especially the African American sample, are small, which is reflective of the rarity of the ADEH phenotype. Although we appreciate the potential for false-positive associations, the association between the R501X mutation and ADEH was observed in both (independent) ethnic groups, and was sustained after analyses were repeated by using the Fisher exact tests and permutation tests in the African American patients. Thus, we are encouraged that the observations are likely to be real.

In summary, our findings confirm that both the R501X mutation and the 2282del4 mutation are the strongest predictors of AD, and for the first time, this association is reported in a population of African descent. Importantly, the R501X mutation confers a greater risk for ADEH in both European and African ancestry populations. The higher frequency of this mutation among patients with ADEH than patients with AD strongly implicates its role in controlling the severity of AD. Novel findings of association with several additional SNPs in *FLG* suggest there may be additional causal variants, independent of the null mutations, contributing to a risk of AD and ADEH. A clearer understanding of genetic risk factors associated with ADEH may improve the ability of identifying patients at greatest risk for ADEH, which may ultimately lead to early intervention and improved surveillance in this vulnerable population.

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Clinical implications: The *FLG* R501X mutation, a major risk factor for AD, confers a greater risk of the severe, HSV-associated complication, ADEH, in diverse ethnic groups.

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METHODS

Study participants and phenotypes

For the diagnosis of AD, patients were required to have disease for at least 3 months before enrollment, and in children younger than 4 years, the disease must have been present for at least 6 months to minimize the likelihood of recruiting children with other eczematous disorders that mimic AD. For the ADEH group, the age requirement was 1 to 80 years, and for the ADEH-negative group and healthy controls, age was restricted to 18 to 80 years to provide greater assurance that these populations had been exposed to HSV and to minimize the possibility that the difference between ADEH-positive and ADEH-negative subjects did not simply reflect viral exposure.^{E1} Consequently, the ADEH group was significantly younger than the AD and healthy control group ($P = 7.26 \times 10^{-13}$; Table E1), likely reflecting the fact that most ADEH episodes occur early in life. There was no bias in sex selection, although considerably more of the patients with ADEH were female compared with the AD group ($P = .0003$; Table E1). Severity of AD was defined according to EASI.^{E2} EASI is a standardized grading system (range of score, 0-72) that permits distinction between mild, moderate, and severe atopic dermatitis by means of a score summation using the following parameters: (1) extent (by rule of 9), (2) course (via history), and (3) intensity (disturbance of night's sleep by itching). All study participants were further evaluated by a detailed history and physical examination, as well as a questionnaire to assess history of cutaneous viral infections and concomitant medication use. Total IgE levels were determined from serum samples by using the UniCap 250 system (Pharmacia and Upjohn, Kalamazoo, Mich); all samples were measured in duplicate.

Genotyping and quality control

We performed genotyping on genomic DNA extracted from blood samples by using the MagAttract DNA blood Mini M48 kit (QIAGEN, Gaithersburg, Md) on a Biorobot M48, according to the manufacturer's instructions. DNA quantification was performed by using Pico-Green (Molecular Probes, Eugene, Ore).

Genotyping of the R501X null mutation was determined by using custom-designed primers and probes for the TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, Calif). The primer pair was CCCTCTTGGGACGCTGAA and CACTGGAGGAAGACAAGGATCG, and the probe pair was VIC-AGCTGTCTCATGCCT and 6FAM-AGCTGTCTCGTGCCT. Reactions were set up in 5 μ L on 96-well plates in TaqMan Universal Master Mix (Applied Biosystems, Foster City, Calif) with 5 ng DNA, 1 μ M of each primer, and 0.2 μ M of each probe. The thermal cycling reactions (50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) were run and analyzed on a 7900HT Sequence Detection System (Applied Biosystems) with Applied Biosystems Genotyper software (SDS system, version 2.2).

For the 2282del4 mutation, genotyping was performed with an Applied Biosystems 3700 DNA Sequencer with the addition of a size standard and data analysis using Genotyper (Applied Biosystems), an allele calling software program.^{E3} In brief, a 152-bp PCR fragment of genomic DNA was amplified with primers 5'-6-FAM-CCAGTGGTAGTCAGGCCACT and 5'-AAAGACCCTGAACGTCGAGA. PCR was performed in a total volume of 20 μ L containing 1 $\times$ PCR buffer (including 1.5 mM MgCl₂), 12 pmol primers, 2 mM deoxynucleotide-

triphosphate (dNTP), 20 ng genomic DNA, and 1.0 U DNA polymerase (Qiagen, Gaithersburg, Md). Amplification was performed as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 5 minutes. Fragments were diluted 1:20 and sized according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 152 bp, and the 2282del4 allele was 148 bp. For quality control, genotyping was repeated on 155 samples, including all samples called heterozygotes by Genotyper and a sampling of variant homozygotes; 5% of samples were repeated in every PCR run to determine genotype reproducibility rates.

Tagging SNPs were selected to provide coverage 10 kb upstream and 10 kb downstream of the *FLG* gene in both the European American and African American groups in accordance with design score validations based on Illumina (Illumina, San Diego, Calif) in-house measurements and the 60-bp limitation (a SNP cannot be closer than 60 bp to another SNP on an oligonucleotide pool assay [OPA]). All available *FLG* SNPs were initially selected from the HapMap (<http://www.hapmap.org/>) to tag the LD blocks in each ethnic group (European American and African American). Tagging was based on the LDSelect algorithm,^{E4,E5} with a minor allele frequency $\geq 10\%$ and an r^2 threshold of 0.80 (as reported in HapMap) to ensure nearly perfect LD to infer information on all SNPs captured by the tag set. Of the 9 tagging SNPs selected, 5 (rs12057226, rs1048534, rs17670505, rs7524281, and rs12023196) qualified as tagging SNPs from both the HapMap CEPH (Centre d'Etude du Polymorphisme Humain) Utah (CEU, with European ancestry) and the HapMap Yoruba (YRI, with African ancestry) samples; an additional 4 tagging SNPs (rs11204980, rs6587665, rs3126082, and rs3126091) were genotyped only in the African American subjects.

The 9 SNPs were genotyped by using the custom-designed Illumina OPA for the BeadXpress Reader System and the GoldenGate Assay with VeraCode Bead technology (Illumina, San Diego, Calif) according to the manufacturer's protocol.^{E6} An additional 74 SNPs identified as ancestry informative markers (AIMs) were selected for maximal difference between African American and European American populations and genotyped to assess potential confounding caused by population substructure using the same Illumina platform. Briefly, the GoldenGate assay uses 3 primers designed for each locus. Two are specific to each allele at the SNP site, and a third hybridizes at a downstream locus from the site. All 3 primers have regions complementary to both genome and universal PCR primer sites. A total of 250 ng high quality genomic DNA was plated and then activated. The activated DNA, paramagnetic particles, assay oligos, and hybridization buffer were combined in a hybridization step to allow DNA to bind to the particles. After hybridization of primers, plates were washed to reduce noise, and allele specific oligos were extended and ligated to the downstream locus specific primer. This mix then served as a PCR template using the universal primers, P1, P2, and P3. P1 and P2 are cyclophilin (Cy) Cy3 and Cy5-labeled. After downstream processing, the single-stranded dye-labeled PCR products were hybridized to their complement VeraCode bead type. Plates were then scanned in the BeadXpress Reader for fluorescence and code identification. Scanned data and oligo assignments were uploaded into Illumina's BeadStudio software for downstream genotype cluster analysis.

Genotyping quality was high, with an average completion rate of 97% for the TaqMan genotyping and 97.2% to 98.2%

(384-SNP and 96-SNP OPA, respectively) for the BeadXpress genotyping. For the TaqMan genotyping, each plate contained 8 duplicated samples that were randomly selected, as well as 4 blank wells as negative controls, with an average error rate of 0.7%. In addition, approximately 8% of randomly selected samples were further validated by direct sequencing on the 7900HT Sequence Detection System. There were no significant departures from Hardy-Weinberg equilibrium within each ethnic group ($P > .01$).

Statistical analyses

We assessed potential confounding caused by population substructure using genotype data from the 74 AIMs and the STRUCTURE program (v2.2; <http://pritch.bsd.uchicago.edu/software>) to estimate membership in distinct subpopulations (Fig E1). Because individual African admixture was highly left skewed, the nonparametric Wilcoxon rank sum test was used to test for a difference in distribution between cases and controls. The test statistic (W) was 12,334 with a corresponding P value of .7956, indicating that the medians of both distributions are essentially the same. Among African American subjects, we found the proportion of African ancestry to be very similar for both AD cases and controls (76.4% and 77.2%, respectively), suggesting little possibility of confounding in tests for association. Among European American subjects, the proportion of European ancestry was nearly identical for AD cases and controls (88.4% and 88.0%, respectively).

The Cochran-Armitage trend test was used to test for association between each individual marker (under an additive model) and disease status with PLINK software (<http://pngu.mgh.harvard.edu/~purcell/plink/to>). A Fisher exact test was used to test for

association in African American subjects where the R501X mutation was absent in controls. Analyses were performed for subjects of European and African ancestry separately to minimize confounding caused by ethnic differences. We tested for association between genetic markers and total serum levels of log-adjusted IgE using recessive logistic regression models adjusted for confounding variables including age and sex, and log-adjusted EASI score adjusting for age and sex. Departures from Hardy-Weinberg equilibrium at each locus were tested with χ^2 tests separately for cases and controls by using PLINK. Haplotype analyses were performed with PLINK using sliding windows of 2 to 4 SNPs and empiric P values for haplotype frequency differences established over 10,000 permutations.

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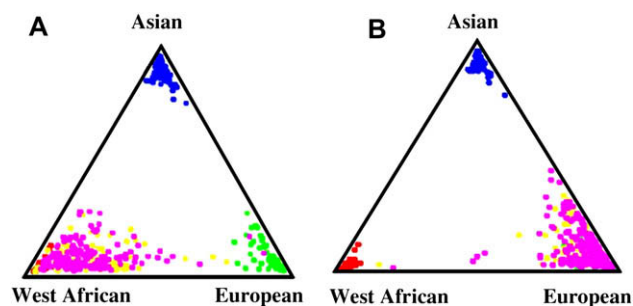


FIG E1. Triangle plots showing estimated admixture in 2 populations. Admixture estimates were generated using 74 AIMs in STRUCTURE (v2.2; <http://pritch.bsd.uchicago.edu/software>). The International HapMap Project (<http://www.hapmap.org>) samples (or 60 Yoruba [YRI], 60 Utah European American [CEU], 90 Han Chinese/Tokyo Japanese [CHB/JPT] founders) were used as reference populations. **A**, A total of 176 African American AD patients (*pink circles*) and 152 nonatopic controls (*yellow circles*) compared with 60 YRI (*red circles*), 60 CEU (*green circles*), and 90 CHB/JPT (*blue circles*) founders. **B**, A total of 241 European American AD patients (*pink circles*) and 153 nonatopic controls (*yellow circles*) compared with 60 YRI (*red circles*), 60 CEU (*green circles*), and 90 CHB/JPT (*blue circles*) founders.

Panel A

Panel B

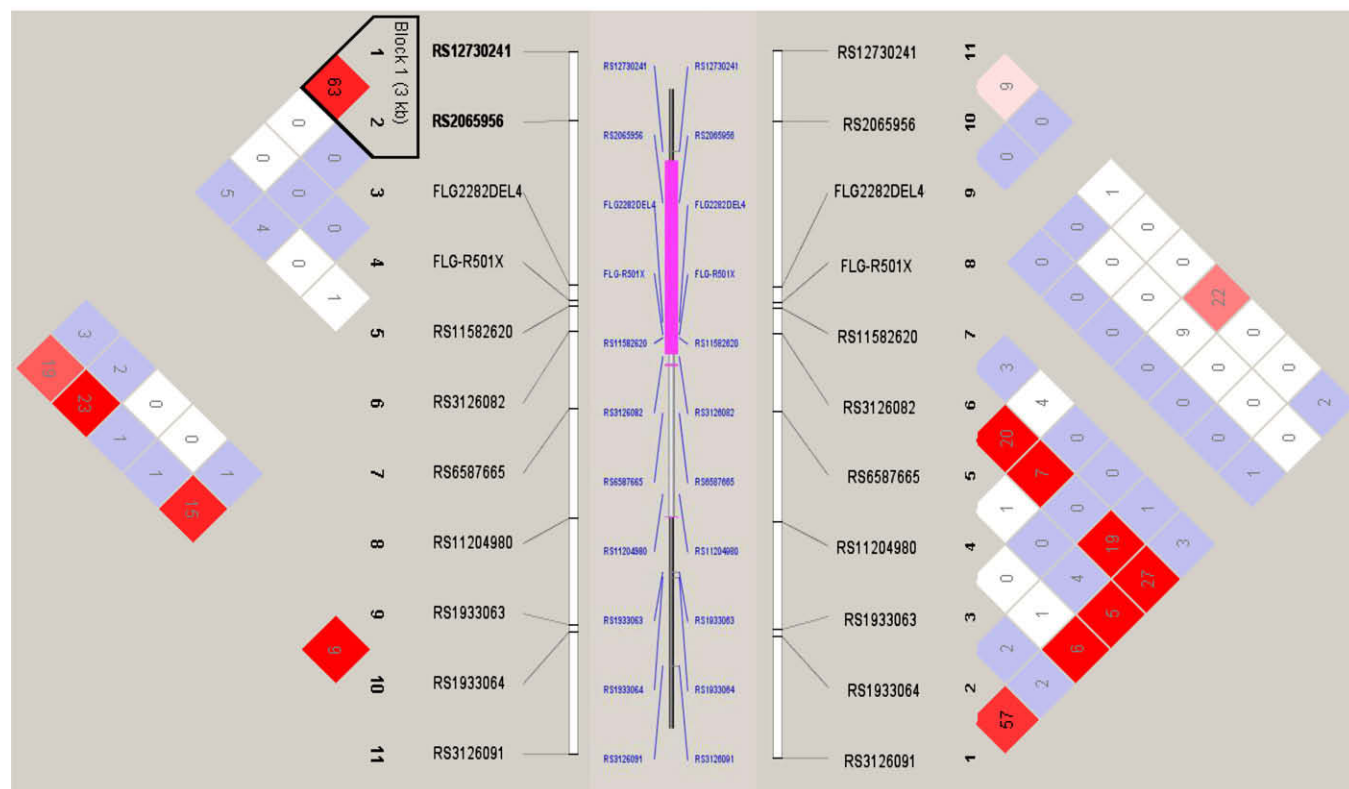


FIG E2. Gene structure and LD estimation for 11 *FLG* variants spanning 33.3 kb on chromosome 1q31. The value within each diamond is the pairwise r^2 statistic in 432 European American founders (A) and 339 African American founders (B). Diamonds without a number correspond to $D' = 1$; shading represents the magnitude and significance of pairwise LD with a red to white gradient reflecting higher to lower LD values (<http://www.broad.mit.edu/haploview>). The middle panel shows the *FLG* gene structure, with exons in pink and introns in light gray.

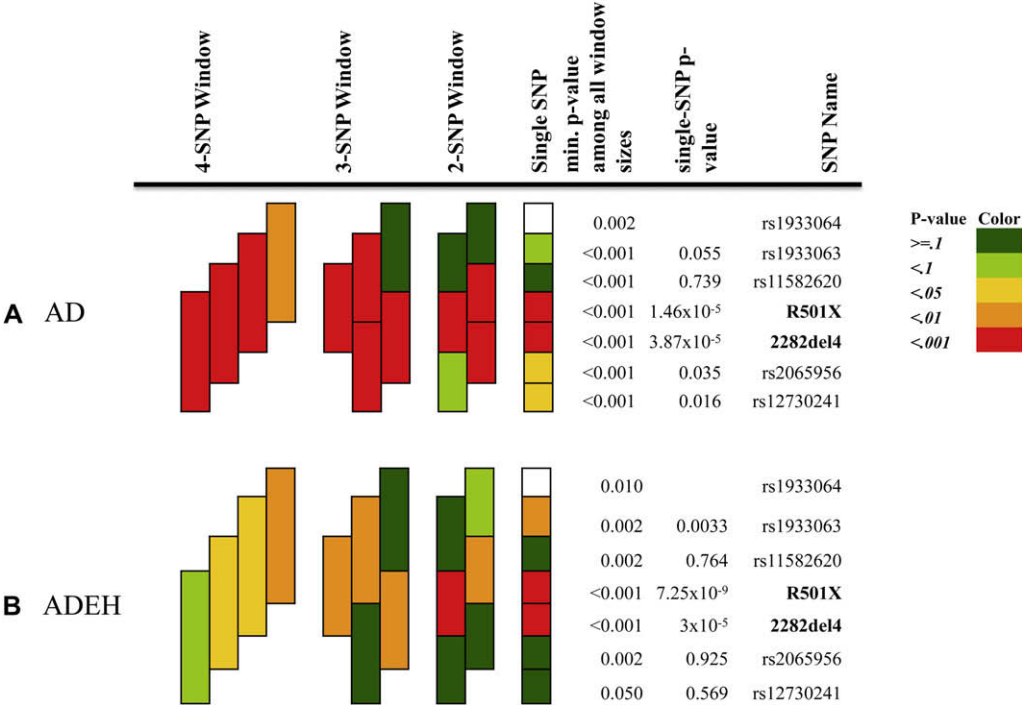


FIG E3. Summary of association tests between *FLG* haplotypes and AD and the subphenotype, ADEH. PLINK results showing *P* values from haplotypes constructed across sliding windows of sizes 2 to 4 for subjects **(A)** with AD versus controls and ADEH-positive subjects **(B)** versus ADEH-negative subjects in European Americans. *P* value of the test is represented by the color of the box, and the color coding of *P* values is as presented in the figure.

TABLE E1. Chromosome 1q21.3 location, type, and minor allele frequency of *FLG* polymorphisms

dbSNP ID (build 129)	Location	Inter-SNP distance (bp)	Type of variant	Risk allele	Allele frequency			
					European American* (N = 156)	African American* (N = 152)	CEU† (N = 60)	YRI† (N = 60)
rs12730241	150,540,640	0	Downstream	A	0.24	0.09	0.114	0.034
rs2065956	150,543,969	3329	Coding exon (Gln/Gln)	A	0.19	0.07	0.058	NR
2282del4	150,551,703	7734	Deletion	del	0.016	0.007	NR	NR
R501X	150,552,483	780	Coding exon	T	0.013	0.00	NR	NR
rs11582620	150,552,750	267	Coding exon (Arg/Arg)	G	0.15	0.02	0.133	NR
rs3126082	150,553,931	1181	Intron	G	NT	0.36	NR	0.375
rs6587665	150,557,595	3664	Intron	G	NT	0.10	NR	0.108
rs11204980	150,562,815	5220	Intron	C	NT	0.12	0.15	0.10
rs1933063	150,567,845	5030	Promoter	A	0.09	0.01	0.08	NR
rs1933064	150,568,200	355	Promoter	G	0.50	0.27	0.558	0.192
rs3126091	150,573,962	5762	Promoter	A	NT	0.33	0.850	0.217

NR, No reported frequencies in the public database; NT, not genotyped.

*Minor allele frequencies from healthy controls participating in the ADVN study.

†Allele frequencies from HapMap (<http://www.hapmap.org>) for 60 Yoruba (YRI) and 60 Utah European American (CEU) founders.

TABLE E2. Summary of associations between *FLG* variants and AD and ADEH

SNP	European American			African American		
	AD vs controls	ADEH ⁺ vs controls	ADEH ⁺ vs ADEH ⁻	AD vs controls	ADEH ⁺ vs controls	ADEH ⁺ vs ADEH ⁻
	P value (OR, 95% CI)	P value (OR, 95% CI)	P value (OR, 95% CI)	P value (OR, 95% CI)	P value (OR, 95% CI)	P value (OR, 95% CI)
rs12730241	.0159 (0.60, 0.40-0.91)	.5690 (0.85, 0.50-1.47)	.0533 (1.73, 0.99-3.02)	.0127 (1.95, 1.15-3.32)	.0029 (4.56, 1.57-13.21)	.0608 (2.64, 0.93-7.49)
rs2065956	.0346 (0.51, 0.27-0.96)	.9247 (0.95, 0.31-2.90)	.9497 (0.98, 0.51-1.88)	.6067 (1.18, 0.62-2.25)	.0108 (3.98, 1.30-12.21)	.0089 (4.11, 1.34-12.59)
2282del4	3.87×10^{-5} (5.6, 2.4-14.6)	3.0×10^{-5} (6.5, 2.4-18.1)	.4651 (1.3, 0.70-2.5)	.2542 (2.5, 0.5-12.5)	ND (ND)	ND (ND)
R501X	1.46×10^{-5} (7.1, 2.5-20.1)	7.25×10^{-9} (13.1, 4.4-38.5)	.0002 (3.4, 1.7-6.8)	.0351* —	.0049* —	.0289 (5.28, 1.0-27.4)
rs11582620	.7385 (0.92, 0.58-1.47)	.7635 (1.10, 0.61-1.98)	.3351 (1.34, 0.74-2.44)	.1900 (2.05, 0.69-6.12)	.1347 (3.56, 0.62-20.59)	.4428 (1.90, 0.36-9.93)
rs3126082	ND (ND)	ND (ND)	ND (ND)	.5386 (0.85, 0.51-1.41)	.3841 (0.61, 0.20-1.86)	.5095 (0.69, 0.23-2.08)
rs6587665	ND (ND)	ND (ND)	ND (ND)	.9729 (1.01, 0.55-1.86)	.5730 (0.64, 0.13-3.05)	.5918 (0.65, 0.14-3.11)
rs11204980	ND (ND)	ND (ND)	ND (ND)	.9307 (1.03, 0.57-1.84)	.3093 (1.83, 0.57-5.93)	.2686 (1.92, 0.60-6.21)
rs1933063	.0552 (0.56, 0.31-1.02)	.0032 (0.19, 0.05-0.63)	.0149 (0.24, 0.07-0.82)	.5482 (0.58, 0.10-3.51)	.5654* —	.6565* —
rs1933064	.890 (0.96, 0.15-1.75)	.733 (1.15, 0.51-2.63)	.722 (1.16, 0.52-2.58)	.944 (0.96, 0.32-2.87)	.797 (1.34, 0.15-12.31)	.741 (1.46, 0.16-13.70)
rs3126091	ND (ND)	ND (ND)	ND (ND)	.656 (0.89, 0.54-1.47)	.669 (0.78, 0.26-2.38)	.799 (0.87, 0.29-2.61)

Significant values are in boldface.

ND, Analyses not done because the variant was not present in 1 or more comparison groups.

*The Fisher exact test was used to test for association in the African American group because of the small dataset and because the R501X mutation was absent in controls.