

Filaggrin-2 variation is associated with more persistent atopic dermatitis in African American subjects

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Background: Atopic dermatitis (AD) is a common skin disease characterized by recurrent episodes of itching. Genetic variation associated with the persistence of AD has not been described for African American subjects.

Objective: We sought to evaluate genetic variation of filaggrin-2 (*FLG2*) in African American subjects with AD.

Methods: We evaluated a multiyear prospective cohort study of African American children with AD with respect to *FLG2* variation based on whole-exome sequencing, followed by a targeted analysis. We ultimately evaluated the association of rs12568784 and rs16833974 with respect to the persistence of AD symptoms over time.

Results: Whole-exome analysis was conducted on 60 subjects, revealing a premature stop codon in exon 3 at S2377X (rs12568784) and X2392S (rs150529054) and a large exon 3 deletion mutation, Q2053del224. On the basis of *a priori* criteria, we then studied rs12568784, rs16833974 (H1249R), and Q2053del224. We noted that patients with S2377X (odds ratio [OR], 0.44; 95% CI, 0.25-0.46) and H1249R (OR, 0.23; 95% CI, 0.12-0.46) were significantly less likely to be free of symptoms of AD, and Q2053del224 (OR, 0.54; 95% CI, 0.16-1.80) trended toward this outcome. S2377X and H1249R were in high linkage disequilibrium ($D' = 0.95$).

Conclusions: In an African American cohort with AD, *FLG2* mutations were associated with more persistent AD. This is the first finding of genetic variation of a skin barrier protein in subjects of African ancestry with AD. (J Allergy Clin Immunol 2013;■■■■:■■■-■■■.)

Key words: Atopic dermatitis, filaggrin, epidemiology, genetic epidemiology, atopy

Atopic dermatitis (AD) is a common chronic relapsing disease that usually begins in childhood. It is found worldwide, although

Abbreviations used

AD: Atopic dermatitis
EDC: Epidermal differentiation complex
FLG: Filaggrin
FLG2: Filaggrin-2
LD: Linkage disequilibrium
MAF: Minor allele frequency
OR: Odds ratio
PEER: Pediatric Eczema Elective Registry
RPTN: Repetin
SFTP: S100-fused type protein

its prevalence varies by geographic location.^{1,2} Recent studies have elegantly demonstrated that in some geographic locations more than 20% of patients afflicted with AD have a genetic predisposition for this disorder.³⁻⁹ The most common gene associated with AD is filaggrin (*FLG*), which initially produces the profilaggrin protein that is processed in the stratum granulosum into the FLG protein. FLG contributes to the barrier function of the stratum corneum of the skin. An intact skin barrier is presumed to protect the skin from penetration by environmental irritants and allergens. *FLG* loss-of-function mutations have been extensively described in persons of European and Asian ancestry, and these mutations vary by race.^{4,10} Among persons of European ancestry, 4 *FLG* loss-of-function mutations have been consistently associated with the risk of AD or the risk of persistent AD.^{4,11} However, despite extensive evaluation, *FLG* variation has rarely been noted to be associated with AD in persons of African ancestry.^{4,10-12}

Why *FLG* loss-of-function mutations are not commonly found in persons of African ancestry is currently unknown.¹¹⁻¹³ Recently, we noted that in a cohort of US children with AD, nearly 30% of the white children had a common European *FLG* loss-of-function mutation, whereas the proportion was less than 6% in their African American counterparts.¹¹ Furthermore, attempts by our team and others to discover new *FLG* mutations in the African American population have not been fruitful.^{4,11,14} The *FLG* gene is in a technically difficult section of the genome to sequence because of highly redundant base sequences, and therefore it is possible that important variations are still to be discovered.^{15,16} However, it is also possible that subjects of African ancestry with AD do not have inherited defects of skin barrier function caused by *FLG* and that the defect is associated with another “skin barrier” gene.

Several other genes, identified through candidate gene and genome-wide association studies, have been associated with AD, although many have not been replicated in additional investigations.^{4,7,8,16-18} These discoveries include genes that are found in close proximity to *FLG* in a section of chromosome 1 (1q21) called the epidermal differentiation complex (EDC). The EDC

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Supported by R01-AR0056755 from the National Institute of Arthritis Musculoskeletal and Skin Diseases and from a grant from Valeant Pharmaceuticals for the Pediatric Eczema Elective Registry study.

Disclosure of potential conflict of interest: D. J. Margolis has received grants from the National Institutes of Health (NIH) and Valent. A. J. Apter has received grants from the NIH, AstraZeneca, and Bristol-Myers Squibb; is a board member for the American Academy of Allergy, Asthma & Immunology; and has consultant arrangements with UpToDate. T. Ganguly, M. Papadopoulos, and N. Mitra have received a grant from the NIH. The rest of the authors declare that they have no relevant conflicts of interest. Received for publication July 1, 2013; revised September 5, 2013; accepted for publication September 14, 2013.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2013.09.015>

contains several genes that encode proteins important for late epidermal differentiation. Six genes located within the EDC and close to *FLG* have structures similar to *FLG*.^{15,19,20} Proteins coded for by these genes are characterized as S100-fused type proteins (SFTPs) and contribute to skin barrier function in the stratum corneum.¹⁵ The SFTPs include profilaggrin (*FLG*), hornerin (*HRNR*), filaggrin-2 (*FLG2*), repetin (*RPTN*), cornulin (*CRNN*), trichohyalin (*TCHH*), and trichohyalin-like 1 (*TCHHL1*).

The structure and function of *FLG2* protein has recently been described.^{20,21} The *FLG2* protein appears to have a role similar to *FLG* with respect to skin barrier function.^{20,21} A previous study found that an *FLG2* missense variant (rs16833974) was not associated with AD in European subjects.^{19,20} The goal of our study was to evaluate genetic variation of *FLG2* in African American subjects with AD and then determine whether *FLG2* variation was associated with the persistence of AD.

METHODS

Population

Subjects for this study were enrolled in the Pediatric Eczema Elective Registry (PEER; www.thepeerprogram.com), which is an ongoing, prospective, 10-year observational registry that is part of a postmarketing commitment originally by Novartis and now Valeant to the US Food and Drug Administration and the European Medicines Agency. The enrollment criteria and goals of the PEER study have been described in detail elsewhere.^{11,22} The diagnosis of AD for each child was made by the enrolling physicians, the majority of whom were pediatricians, allergists, and dermatologists.^{11,23} The diagnosis was confirmed based on the UK Working Party criteria.^{22,24} Children/parents in the PEER study who also enrolled in our current study completed an additional informed consent form approved by the Institutional Review Board of the University of Pennsylvania and provided a saliva sample from which DNA was extracted. Our study included subjects who self-described as African American and provided saliva/DNA ($n = 380$). A panel of ancestry informative markers was used to estimate genetic ancestry by using clustering techniques, as implemented in the program STRUCTURE.^{11,25} Self-reported African ancestry was highly correlated with genetically derived ancestry based on these ancestry informative markers.¹¹ The percentage of African ancestry for subjects analyzed in this study was approximately 75% on average.

Outcome

We investigated the self-reported outcome of whether a child's skin, without requiring the use of topical medication (eg, steroids or calcineurin inhibitors to treat their AD), was AD symptom free for the previous 6 months. Information about this outcome was collected longitudinally every 6 months by using a survey, thereby capturing the waxing and waning nature of AD.

Whole-exome sequencing

We randomly selected 60 of the 380 eligible African American subjects for whole-exome sequencing. For this study, the primary goal of whole-exome analysis was the discovery of new variants of *FLG2* that were likely to be loss-of-function mutations in African American subjects with AD. The exome sequencing was conducted by Ambry Genetics (Aliso Viejo, Calif) using whole-exome target enrichment with Agilent SureSelect technology (Agilent, Santa Clara, Calif). The libraries were 100 base paired ends and were processed with the Illumina HiSeq 2000 (Illumina, San Diego, Calif) at 100× coverage per exome. The data was then evaluated by using 2 separate pipelines: a proprietary pipeline established by Ambry Genetics and a pipeline created at the University of Pennsylvania based on the best practices guidelines of the Broad Institute. The SFTP genes, including *FLG*, have 3 exons.^{15,21,26} The tiny first exon is noncoding, whereas the small second

exon is responsible for initiating translation. The large third exon contains sequence information for the precursor protein.^{15,21,26} It has been hypothesized that stop-gain (nonsense) mutations of SFTP genes in exon 3 are loss-of-function mutations.^{12,15,16,19,20,27} Because of the structural similarity between *FLG* and *FLG2*, we targeted stop-gain and insertion/deletion variants of exon 3 of *FLG2* present in more than 1 subject.^{4,16,19,20} Furthermore, we required these variants to be present in more than 1 subject to minimize false discovery, to avoid further study of sporadic mutations, and to achieve our goal of investigating more common variants (minor allele frequency [MAF], >0.05). We also decided *a priori* to pursue rs12568784, which was a nonsense mutation of exon 3 previously studied in European subjects.¹⁹

Targeted analysis

As noted in the Results section, 3 variants were analyzed further. As appropriate, single nucleotide polymorphisms were genotyped by using the TaqMan assay with TaqMan 5' nuclease PCR primers and probes designed by Life Technologies Corporation's Assay-By-Design custom oligonucleotide reagent service (Life Technologies, Carlsbad, Calif). Each probe consisted of an oligonucleotide with a fluorescent reporter dye, a nonfluorescent quencher, and a minor groove binder. Allele-specific cleavage of probes was detected by using different reporter dyes for each probe (6FAM and VIC fluorophores for each allele) and evaluated with an ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif). PCR end point fluorescence levels were measured automatically by using the SDS 2.3 manufacturer's custom software (Life Technologies). Allelic discrimination results were then graphed on a scatter plot contrasting reporter dye fluorescence (ie, allele A vs allele a).

The *FLG2* deletion mutation was assayed by means of fragment analysis on an ABI 3130xl capillary sequencer. The primers for a PCR product covering the deletion region were designed by using Primer3 software (www.primer3.sourceforge.net). For generation of fluorescent PCR products, the forward primer was labeled in the 5' end with VIC dye. The primer sequences and PCR protocol are available by request. The PCR products were run on the 3130xl by using LIZ 600 as a size standard and analyzed with GeneMapper version 4.1 (Life Technologies).

Subjects in this study were followed longitudinally and surveyed every 6 months. Hence we assessed the statistical association between the repeated measures of the binary outcome (presence or absence of AD) and each variant using a mixed-effects framework called a generalized linear latent and mixed model, which accounts for the correlation of the outcomes within patients over time. All analyses assumed an additive genetic model.^{28,29} However, we have also presented results from a dominant model, where appropriate.

RESULTS

Characteristics of study subjects

Of 380 African American subjects who provided DNA, 81 were excluded from further analysis because of insufficient DNA. The median age (in years) at enrollment into the PEER study and that of AD onset were 6.8 years (interquartile range, 4.1-10.2 years) and 0.8 years (interquartile range, 0.3-3 years), respectively. Male subjects accounted for 56.5% ($n = 169$) of the cohort. At enrollment, 47.5% ($n = 142$) had limited or uncontrolled AD, 55.5% ($n = 166$) had a history of asthma or wheezing, 68.2% ($n = 204$) had a history of seasonal allergies, and 25.4% reported food allergies ($n = 76$). The mothers of 21.7% ($n = 65$) had a history of eczema, and the fathers of 15.4% ($n = 46$) had eczema. At enrollment, no child had experienced skin free of symptoms of AD while not using topical medications in the last 6 months.

FLG2 exome analysis to identify potential loss-of-function mutations ($n = 60$)

A premature stop codon was noted in exon 3 at S2377X (rs12568784) and another at X2392S (rs150529054). rs12568784

TABLE I. General information about *FLG2* variants in African American subjects noted in a whole-exome analysis (n = 60)

Type of variation	Location	dbSNP	Variant allele
Stop-gain	NM_001014342:exon3:c.C7130A:p.S2377X	rs12568784	G>T
Nonsynonymous	NM_001014342:exon3:c.A3746G:p.H1249R	rs16833974	T>C
Deletion mutation	NM_001014342:exon3:c.5937_6161del p.A1979_Q2053del	NA	Deletion of 224 bp

NA, Not applicable.

was found in 16 subjects, and rs150529054 was noted in only 1 subject (and therefore not studied further). A deletion mutation was also noted at Q2053del224 in 4 subjects. By using exome sequencing, all variants were heterozygotes. As per our *a priori* decision to evaluate premature stop codon and deletion variants that occurred in more than 1 subject, S2377X and Q2053del224 were further followed up in the full cohort. H1249R (rs16833974) on exon 3, which was present in 14 subjects, was also included in the next phase of the study because it was a nonsynonymous variant of prior interest (Table I).

***FLG2* loss-of-function mutations in the African American PEER cohort (n = 299)**

The MAFs and results from association testing of rs12568784, rs16833974, and Q2053del224 are presented in Tables II and III and Figs 1 and 2. rs12568784, which is a single-base-pair substitution of T for G resulting in a premature stop codon, fulfilled the previously defined criteria for *FLG* by Sandilands et al¹⁶ as a loss-of-function mutation. Children with this variant, which had an MAF of 0.22, were more likely to have persistent symptoms of AD requiring topical therapy (odds ratio [OR], 0.44; 95% CI, 0.25-0.46; Table III). rs16833974 is a single-base-pair substitution of T for C, had an MAF of 0.24, and was also associated with the outcome (OR, 0.23; 95% CI, 0.12-0.46; Table III and Fig 1). These 2 loci were highly concordant ($\kappa = 0.68$, $R = 0.77$, 82.1% agreement) and were in high linkage disequilibrium (LD; $D' = 0.95$), as represented in HapMap (Fig 2).³⁰ Q2053del224, with an MAF of 0.04, was not statistically associated but did have a trend toward increased persistence (OR, 0.54; 95% CI, 0.16-1.80). None of the studied variants were associated with an increased risk of asthma, seasonal allergies, or food allergies (Table III).

DISCUSSION

Mutations in the *FLG* gene have been associated with both the risk of AD and the persistence of AD in European and Asian populations.⁴ However, *FLG* mutations have not been consistently or commonly found in African American subjects with AD.^{4,11,12} Investigators have postulated that other proteins involved in skin barrier function could, like *FLG*, be associated with AD.^{15,19,20} However, this hypothesis, which has been tested mainly in European subjects, has not been fully substantiated.^{15,19-21} Like *FLG*, *FLG2* is located within the EDC.^{15,19,20} To the best of our knowledge, our study is the first report showing that a common loss-of-function mutation of *FLG2* is associated with the persistence of AD in African American children. Children with the *FLG2* loss-of-function mutation rs12568784 or the nonsynonymous variant rs16833974, which was in high LD with rs12568784, were more than 50% more likely to have persistent AD compared with those without these variants. The magnitude of this finding is similar to previous findings in

TABLE II. Minor allele and genotype frequencies for the African American PEER cohort

Variant	MAF African American PEER cohort (n = 299)	Genotype frequency		
		AA	Aa	aa
S2377X (rs12568784)	0.22	0.55	0.39	0.06
H1249R (rs16833974)	0.24	0.58	0.37	0.05
Q2053del224	0.04	0.92	0.08	0

European subjects with *FLG* loss-of-function mutations.¹¹ The presence of *FLG2* variants was not associated with asthma, seasonal allergies, or food allergies. Finally, the presence of a large deletion mutation of *FLG2* showed a trend toward an association with persistent AD.

We did not have direct access to study participants because of the design of the PEER study. Previous studies have shown that the *FLG2* protein, which resides in the stratum corneum, is an important component of skin barrier function and is diminished as a consequence of skin inflammation.^{15,19-21} Thus it has been conjectured, but not proved, that diminished *FLG2* production could be associated with the risk of AD in a fashion similar to diminished or absent production of *FLG*.^{15,19-21} We hypothesize that the rs12568784 variant is a loss-of-function mutation because, like the *FLG* loss-of-function mutations, rs12568784 represents a stop codon in exon 3. If true, children with this variant are unable to produce *FLG2* or produce diminished amounts of *FLG2*.^{4,16} This is consistent with current knowledge of the pathophysiology of AD. However, the 2 reported *FLG2* variants are in high LD with each other and other variants of the gene (see Fig 2), and therefore even if they are not loss-of-function mutations, it is likely that another variant will be.

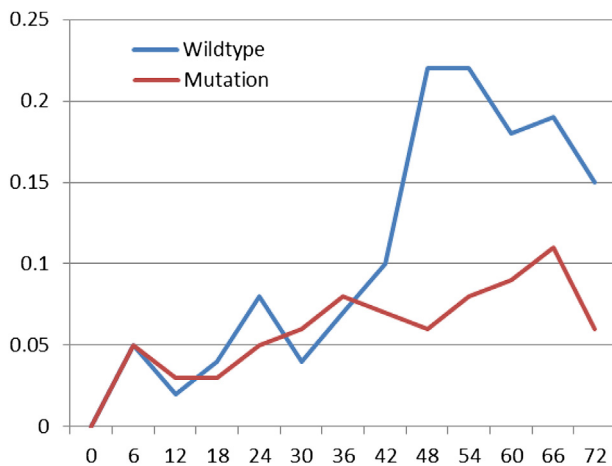
The presumption is that the *FLG2* protein and the other SFTPs, like *FLG*, ultimately contribute to skin barrier function as part of the natural skin moisturizing factor.⁹ A recent study examined the expression of structural proteins and regulatory molecules in the stratum corneum after experimental disruption of the skin barrier in human volunteers with AD and psoriasis and demonstrated that both *FLG* and *FLG2* levels were decreased as a consequence of barrier disruption.³¹ The production of both *FLG* and *FLG2* has also been shown to be diminished in the lesional and nonlesional skin of patients with AD.^{15,20} This reduction, which was more pronounced in lesional skin and occurred independent of *FLG* genotypes, was presumably caused by the influence of proinflammatory cytokines. This study did not find a link between *FLG* null mutations and reductions in protein production.³¹ However, it is to be noted that the study investigated only the 4 common mutations found in European subjects, and therefore the possibility of a less common, nongenotyped mutation or copy number variation within the *FLG* gene having influenced the results cannot be excluded.

TABLE III. Association of *FLG2* variants in the African American PEER cohort with the persistence of AD, asthma, seasonal allergy, and food allergy at study entry

Variant	OR (95% CI), <i>P</i> value				
	Additive genetic model *	Dominant genetic model*	Asthma†	Seasonal allergy†	Food allergy†
S2377X (rs12568784)	0.44 (0.25-0.77), <i>P</i> = .004	0.43 (0.21-0.88), <i>P</i> = .022	1.05 (0.70-1.57), <i>P</i> = .8010	1.01 (0.66-1.56), <i>P</i> = .625	0.96 (0.84-1.11), <i>P</i> = .928
H1249R (rs16833974)	0.23 (0.12-0.46), <i>P</i> = .00002	0.23 (0.11-0.46), <i>P</i> = .00004	1.12 (0.66-1.90), <i>P</i> = .677	0.89 (0.48-1.44), <i>P</i> = .391	0.79 (0.42-1.47), <i>P</i> = .451
Q2053del224	0.54 (0.16-1.80), <i>P</i> = .234	—	0.88 (0.34-2.32), <i>P</i> = .804	0.58 (0.23-1.52), <i>P</i> = .220	1.42 (0.71-2.81), <i>P</i> = .319

*Generalized linear latent and mixed model.

†Logistic regression model.

**FIG 1.** Percentage of subjects reporting symptom-free skin while not receiving medication stratified by rs16833974 over the first 6 years of follow-up.

Alterations in the bacterial flora of children with AD have been associated with flares of AD.³² Recent studies have implied that *FLG*, *FLG2*, and another *SFTF*, *HRNR*, might function as part of the cutaneous antimicrobial defense because they are further processed in the stratum corneum to become peptides that make the skin surface inhospitable for pathogenic bacteria.³²⁻³⁸ Furthermore, as reported by Broccardo et al,³⁹ skin barrier proteins, including *FLG2*, can act as powerful antimicrobial agents against *Staphylococcus aureus*, which is known to be associated with exacerbations of AD.^{32,39} It is now well accepted that AD is a disorder resulting at least partially from immune dysfunction, with T_H2 cells playing a bigger part during acute manifestations of the disease, whereas T_H1 cells assume greater importance during the chronic phase.⁴⁰⁻⁴² The T_H1 cells secrete several cytokines, including IFN- γ and IL-12.⁴¹⁻⁴³ Bacterial colonization propagates immune dysfunction and stimulates T_H1 cells to secrete IFN- γ , which eventually leads to macrophage activation, thus perpetuating the chronic nature of the disease. Therefore *FLG2* deficiency could encourage bacterial colonization, which could in turn lead to persistence of AD.

To date, most genetic studies of AD have evaluated the risk of AD; however, very few have studied the persistence of AD as an outcome. Often when persistence was measured, it was measured as the time to when the child first noted that he or she was symptom free.⁴⁴ This might not be the appropriate definition of

persistence for a chronic waxing and waning illness like AD. In our study, as well as in a previous report, we focused on the persistence of AD over years of observation, which mimics the natural history of AD.¹¹ In fact, we believe that our definition of outcome is clinically more meaningful because it takes into account both the chronicity and the intermittently remitting and relapsing character of AD.^{11,44}

The 2 *FLG2* mutations associated with the persistence of AD in African American subjects are either absent or only infrequently found in persons of European origin. The variant allele of rs16833974 is not found in the HapMap database of Utah residents with Northern and Western European Ancestry (CEU); as a reference, the MAF in Americans of African Ancestry in the South West (ASW) is 0.26 (browser: 1000genomes.org). Why *FLG* loss-of-function mutations that are prevalent in white subjects are not found consistently in persons of African ancestry and the opposite appears true with respect to *FLG2* is not clear. However, all these mutations appear to be functionally similar: they diminish or stop the production of skin barrier proteins irrespective of their location in the EDC. It is tempting to speculate that these mutations arose in different populations during different time periods and were propagated selectively because of a multitude of factors that could include natural selection, genetic drift, or a population bottleneck effect.⁴⁵ Whether these mutations confer any survival advantage is a matter of ongoing debate.

An important limitation of our study was that we were not able to evaluate the presence or absence of *FLG2* protein in the skin of our study subjects. As a result, we can only hypothesize (as others have also done) that based on previous reports of a structurally related *FLG*, the mutations noted in our study would diminish the production of *FLG2*.^{15,19,21,26} Also, it is important to note that our study was limited to subjects with mild-to-moderate AD, which is the approved patient population to receive pimecrolimus. Our results are also not directly comparable with those of other studies that have focused on genetic factors that are associated with the onset of AD. Our study design did not allow us to explore whether children with rs12568784 or rs16833974 were at increased risk of AD.

In conclusion, we found that 2 mutations in *FLG2*, rs12568784 and rs16833974, were associated with more persistent AD in African American children. This is the first report of this finding. Of particular interest is that rs12568784 is a stop-gain mutation in exon 3 and is in a high degree of LD with rs16833974. Ultimately, functional studies will be needed to confirm that these mutations

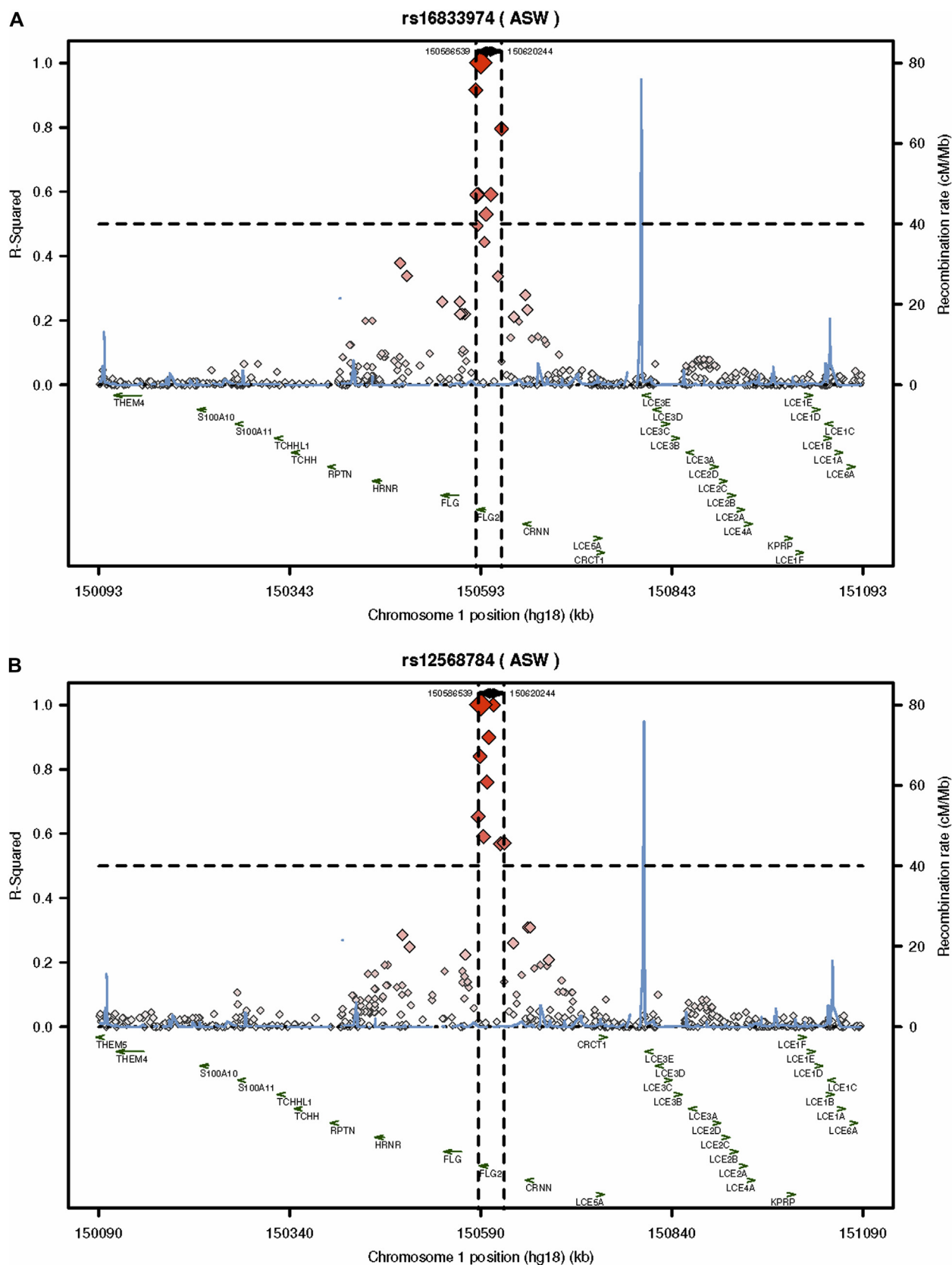


FIG 2. LD plots for rs16833974 (**A**) and rs12568784 (**B**) using the Americans of African Ancestry in the South West (ASW) population, demonstrating LD relationships (the brightness and size of the *diamond* is proportional to the R^2 value) for these variants with respect to the *FLG2* gene, as well as other nearby genes of the EDC.

could lead to the absence of or a diminished ability to produce FLG2. Although mutations in *FLG* that are common in European and Asian subjects have not been consistently found in subjects of African ancestry, the results of our study indicate that in African American subjects AD could be associated with mutations in another skin barrier protein, such as *FLG2*.

Clinical implications: *FLG2* variation is associated with more persistent AD in children of African ancestry.

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