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

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Abstract

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

Objective—To evaluate genetic variation of Filaggrin-2 (*FLG2*) in African-Americans 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 rs rs12568784 and rs16833974 with the respect to the persistence of AD symptoms over time.

Results—Whole exome analysis was conducted on 60 subjects revealing premature stop codon in exon 3 at S2377X (rs12568784), X2392S (rs150529054), and a large exon 3 deletion mutation Q2053del224. Based on *a priori* criteria we then studied rs12568784, rs16833974 (H1249R) and Q2053del224. We noted that S2377X (OR = 0.44; 95% CI: 0.25, 0.46) and H1249R (0.23; 0.12, 0.46) were significantly less likely to be free of symptoms of AD and Q2053del224 (0.54; 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 those of African ancestry with AD.

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

Keywords

Atopic Dermatitis; Filaggrin; Epidemiology; Genetic Epidemiology; Atopy

Introduction

Atopic dermatitis (AD) is a common chronic relapsing disease that usually begins in childhood. It is found worldwide, although its prevalence varies by geographic location^{1;2}. Recent studies have elegantly demonstrated that in some geographic locations more than 20% of individuals 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 people of European and Asian ancestry and these mutations vary by race^{4;10}. Among people of European ancestry, four *FLG* loss-of-function mutations have been consistently associated with the risk of developing AD or the risk of having persistent AD^{4;11}. However, despite extensive evaluation, *FLG* variation has rarely been noted to be associated with AD in people of African ancestry^{4;10–12}.

Why *FLG* loss-of-function mutations are not commonly found in people of African ancestry is currently unknown^{11–13}. We recently 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 counterparts¹¹. Further, 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; so it is possible that important variations are still to be discovered^{15;16}. However, it is also possible individuals of African ancestry with AD do not have inherited defects of skin barrier function due to *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 one (1q21) called the epidermal differentiation complex (EDC). The EDC 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 (SFTP) and contribute to skin barrier function in the stratum corneum¹⁵. 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 a *FLG2* missense variant (rs16833974) was not associated with AD in Europeans^{19;20}. The goal of our study was to evaluate genetic variation of *FLG2* in African-Americans with AD and then determine if *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 post-marketing commitment originally by Novartis and now Valeant to the FDA 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 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 (AIMS) was used to estimate genetic ancestry 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 AIMS¹¹. The percent African ancestry for subjects analyzed in this study was approximately 75% on average.

Outcome

We investigated the self-reported outcome of whether or not a child's skin, without requiring the use of topical medication (e.g., 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 six months by a survey thereby capturing the waxing and waning nature of AD.

Whole exome sequencing

We randomly selected 60 of the 380 eligible African-American individuals 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-Americans with AD. The exome sequencing was conducted by Ambry Genetics (Aliso Viejo, California) utilizing whole exome target enrichment by Agilent SureSelect technology. The libraries were indexed 100 bp paired end and processed using Illumina HiSeq2000 at 100x coverage per exome. The data was then evaluated using two separate pipelines; a proprietary pipeline established by Ambry 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 three exons^{15;21;26}. The tiny first exon is non-coding 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 variants and insertion/deletion variants of exon 3 of *FLG2* present in more than one subject^{4;16;19;20}. Further, we required these variants to be present in more than one individual, in order 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 subjects¹⁹.

Targeted analysis

As noted in the Results section, three variants were analyzed further. As appropriate, SNPs were genotyped via TaqMan® assay using TaqMan® 5' nuclease PCR primers and probes designed by Life Technologies Corporation Assay-By-DesignSM custom oligonucleotide reagent service (Life Technologies, Carlsbad, CA). Each probe consisted of an oligonucleotide with a fluorescent reporter dye, a non-fluorescent quencher and minor groove binder. Allele-specific cleavage of probes was detected using different reporter dyes for each probe (6FAM and VIC fluorophores for each allele) and evaluated using an ABI 7900 HT Sequence Detection System. 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 (i.e., Allele A vs. Allele a).

The *FLG2* deletion mutation was assayed by fragment analysis on ABI 3130XL capillary sequencer. The primers for a PCR product covering the deletion region were designed by Primer 3 software. For generation of fluorescent PCR products the forward primer was labeled in the 5' end with VIC dye. The primer sequences and the PCR protocol are available on request. The PCR products were run on 3130XL using LIZ 600 as size standard and analyzed by GeneMapper v4.1.

Individuals in this study were followed longitudinally and surveyed every six 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* (GLLAMM) which accounts for the correlation of the outcomes within patient over time. All analyses assumed an additive genetic model.^{28,29} However, we have also presented results from a dominant model wherever appropriate.

Results

Characteristics of the study subjects

Out of 380 African-American subjects who provided DNA, 81 were excluded from further analysis due to insufficient DNA. The median age (in years) at enrollment into PEER and that of AD onset were 6.8 (interquartile range (IQR) = 4.1, 10.2) and 0.8 (IQR = 0.3, 3) 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 was found in 16 subjects and rs150529054 was noted in only one subject (and therefore not studied further). A deletion mutation was also noted at Q2053del224 in 4 subjects. By 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 1).

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

The minor allele frequencies (MAFs) and results from association testing of rs12568784, rs16833974 and Q2053del224 are presented in Tables 2 and 3 and Figures 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 al* as a loss-of-function mutation¹⁶. Children with this variant, which had a MAF of 0.22, were more likely to have persistent symptoms of AD requiring topical therapy (OR = 0.44; 95% CI: 0.25, 0.46) (Table 3). Rs16833974, is a single base pair substitution of T for C, had a MAF of 0.24, and was also associated with the outcome (0.23 (0.12, 0.46)) (Table 3 & Figure 1). These two loci were highly concordant ($\kappa=0.68$, $R=0.77$, and 82.1% agreement) and were in high linkage disequilibrium (LD) ($D'=0.95$) (As represented in HapMap see Figure 2³⁰). Q2053del224, with a MAF of 0.04, was not statistically associated but did have a trend toward increased persistence (0.54 (0.16, 1.80)). None of the studied variants were associated with an increased risk of asthma, seasonal allergies, or food allergies (Table 3).

Discussion

Mutations in the *FLG* gene have been associated with both risk of developing AD and persistence of AD in European and Asian populations⁴. However, *FLG* mutations have not been consistently or commonly found in African-Americans 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 Europeans, 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 as compared to those without these variants. The magnitude of this finding is similar to previous findings in Europeans with *FLG* loss-of-function mutations¹¹. 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 proven, that diminished *FLG2* production could be associated with the risk of developing AD in a fashion similar to diminished or absent production of *FLG*^{15;19-21}. We hypothesize that 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 or produce diminished amounts of *FLG2*^{4;16}. This is consistent with current knowledge of the pathophysiology of AD. However, the two reported *FLG2* variants are in high LD with each other and other variants of the gene (see Figure 2) so even if they are not loss-of-function mutations it is likely that another variant will be.

The presumption is that *FLG2* protein and the other SFTPs, like *FLG*, ultimately contribute to skin barrier function as part of skin natural moisturizing factor⁹. A recent study examined expression of structural proteins and regulatory molecules in the stratum corneum after experimental disruption of skin barrier in human volunteers with AD and psoriasis and demonstrated that both *FLG* and *FLG2* levels were decreased as a consequence of barrier disruption³¹. The production of both *FLG* and *FLG2* have also been shown to be diminished in lesional as well as non-lesional skin of AD patients^{15;20}. This reduction, which was more

pronounced in the lesional skin and occurred independent of *FLG* genotypes, was presumably due to the influence of pro-inflammatory cytokines. This study failed to find a link between *FLG* null mutations and reduction in protein production³¹. However, it is to be noted that the study investigated only the 4 common mutations found in Europeans; therefore the possibility of a less common, non-genotyped mutation or copy number variation within the *FLG* gene having influenced the results cannot be excluded.

Alterations in the bacterial flora of children with AD have been associated with flares of AD³². Recent studies have implied that *FLG*, *FLG2*, and, another SFTP, *HRNR* may function as part of cutaneous antimicrobial defenses as they are further processed in the stratum corneum to become peptides that make the skin surface inhospitable for pathogenic bacteria³²⁻³⁸. Further, as reported by Broccardo *et al.* 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 TH2 cells playing a bigger part during the acute manifestation of the disease whereas TH1 cells assume greater importance during the chronic phase⁴⁰⁻⁴². The TH1 cells secrete several cytokines including gamma interferon and IL-12⁴¹⁻⁴³. Bacterial colonization propagates immune dysfunction and stimulates TH1 cells to secrete gamma interferon, which eventually leads to macrophage activation thus perpetuating the chronic nature of the disease. Deficiency of *FLG2*, therefore, could encourage bacterial colonization, which could in turn lead to persistence of AD.

Most genetic studies of AD to date have evaluated the risk of developing AD; however, very few have studied the *persistence* of AD as an outcome. Often when persistence was measured, it was measured as time to when the child first noted that he/she was symptom free⁴⁴. 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 AD¹¹. In fact, we believe that our definition of outcome is clinically more meaningful since it takes into account the chronicity as well as the intermittently remitting and relapsing character of AD^{11;44}.

The two *FLG2* mutations associated with the persistence of AD in African-Americans are either absent or only infrequently found in people 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 reference MAF in American of African Ancestry in the South West (ASW) = 0.26; browser. 1000genomes.org). Why *FLG* loss-of-function mutations that are prevalent in whites are not found consistently in people of African ancestry and while the opposite appears true with respect to *FLG2* is not clear. However, all these mutations appear to be functionally similar; i.e., 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 due to a multitude of factors that could include natural selection, genetic drift or a population bottleneck effect⁴⁵. 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 to 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 for *developing* AD.

In conclusion, we found that two 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 could lead to absence of or diminished ability to produce *FLG2*. While mutations in *FLG* that are common in Europeans and Asians have not been consistently found in individuals of African ancestry, the results of our study indicate that in African-Americans, AD could be associated with mutations in another skin barrier protein like *FLG2*.

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Abbreviations

AD	Atopic Dermatitis
AIMS	Ancestral informative markers
CI	Confidence interval
CRNN	Cornulin
EDC	Epidermal Differentiation Complex
FLG	Filaggrin
FLG2	Filaggrin-2
GLLAMM	Generalized linear latent and mixed models
HRNR	Hornerin
MAF	Minor allele frequency
OR	Odds ratio
PEER	Pediatric Eczema Elective Registry
RPTN	Repetin
SFTP	S100-fused type protein
TCHH	Trichohyalin
TCHHL1	Trichohyalin-like 1

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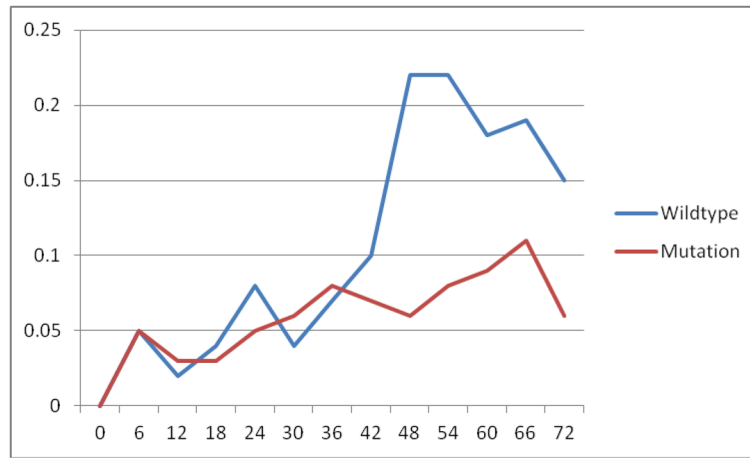


Figure 1. Percent individuals reporting skin symptom free off of medication by rs16833974 over the first 6 years of follow up

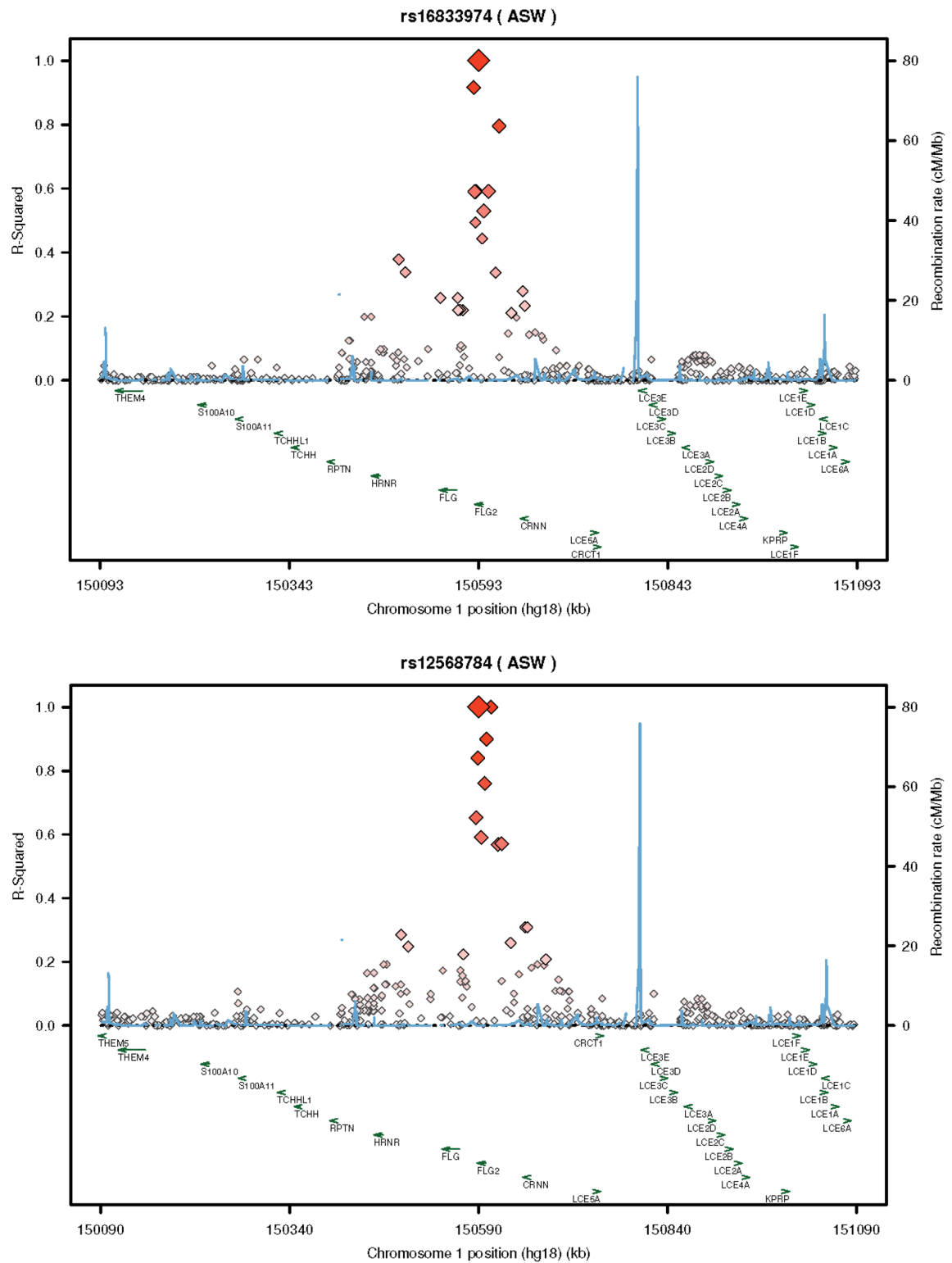


Figure 2. LD plots for rs16833974 (A.) and rs12568784 (B.) utilizing the ASW population demonstrating LD relationships (brightness and size of diamond is proportional to R-

squared) for these variants with respect to the *FLG2* as well as other nearby genes of the EDC.

Table 1

General information about FLG2 variants in African-Americans 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_6161delp.A1979_Q2053del	N/A	Deletion of 224 base pairs

Table 2

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

Variant	Minor allele frequency	Genotype frequency		
	African - American PEER Cohort (n = 299)	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

Table 3

Association of *FLG2* variants in the African-American PEER cohort with the persistence of AD, asthma, seasonal allergy and food allergy at study entry.
OR = odds ratio; CI = confidence intervals

Variant	OR (95% CI) ADD p-value				
	Additive genetic model *	Dominant genetic model*	Asthma ⁺	Seasonal allergy ⁺	Food allergy ⁺
S2377X (rs12568784)	0.44 (0.25, 0.77) p=0.004	0.43 (0.21, 0.88) p=0.022	1.05 (0.70, 1.57) p=0.8010	1.01 (0.66, 1.56) p=0.625	0.96 (0.84, 1.11) p=0.928
H1249R (rs16833974)	0.23 (0.12, 0.46) p=0.00002	0.23 (0.11, 0.46) p=0.00004	1.12 (0.66, 1.90) p=0.677	0.89 (0.48, 1.44) p=0.391	0.79 (0.42, 1.47) p=0.451
Q2053del1224	0.54 (0.16, 1.80) p=0.234	----	0.88 (0.34, 2.32) p=0.804	0.58 (0.23, 1.52) p=0.220	1.42 (0.71, 2.81) p=0.319

* GLLMM model;

⁺ Logistic regression model