

Hint for association of single nucleotide polymorphisms and haplotype in SPINK5 gene with atopic dermatitis in Koreans

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Abstract: Clinical studies, including twin studies, support the concept that the risk of atopic dermatitis (AD) may be mediated through skin-specific genes, rather than simply through systemic immune or atopy risk genes. The SPINK5 gene is expressed on epithelial surfaces and may provide protection against other allergenic serine proteases. Mutations in the SPINK5 gene result in Netherton syndrome, a disorder characterised by AD, ichthyosis, and elevated serum IgE levels. We genotyped 21 single nucleotide polymorphisms (SNPs) from the SPINK5 gene for 1090 case-control samples (631 patients with AD and 459 normal controls) and analysed the SNPs and haplotypes in this gene and also searched for gene-gene interactions between SPINK5 and the DEFB1 gene that we previously reported. Six SNPs [rs17718511 ($P = 0.026$), rs17860502 ($P = 0.024$), KN0001820 ($P = 0.045$), rs60978485 ($P = 0.007$), rs17718737 ($P = 0.02$), and rs1422985 ($P = 0.038$)] and the haplotype TAA (rs60978485, rs6892205,

rs2303064; $P = 0.023$) in the SPINK5 gene showed significant different allelic or genotypic distributions between the AD group and the control group. We also found that four SNPs [rs17718511 ($P = 0.033$), rs17860502 ($P = 0.031$), rs60978485 ($P = 0.005$), rs17718737 ($P = 0.023$)] and the haplotype TAA ($P = 0.02$) in the SPINK5 gene showed associations with the susceptibility of the allergic type of AD (ADe). In addition to this finding, we speculate that the SNPs from DEFB1 and SPINK5 affect the individual susceptibility to development of ADe in an additive manner. This study provides evidence for a significant interaction between allergens and the SPINK5 gene that may contribute to ADe susceptibility.

Key words: atopic dermatitis – haplotype – polymorphisms – SPINK5 gene

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Introduction

Atopic dermatitis (AD), a type of eczema, is defined as a chronic inflammatory skin disease characterised by severe itching and a chronic relapsing clinical course with typical skin lesions (1,2). Atopic dermatitis is the one of the typical multifactorial diseases, which means that both genetic and environmental factors are involved in the development of the disease phenotype. There is substantial evidence of a strong genetic component in AD, such as the concordance rate in twin studies. Also, children whose parents have eczema show a higher risk of developing eczema than do children whose parents have asthma or hay fever, and eczema can occur with increased severity along lines of Blaschko, a condition consistent with genetic mosaicism. These observations support the concept that AD risk may be mediated through skin-specific genes, rather than simply through systemic immunity or an atopy risk gene (3).

Efforts to identify candidate genes for AD through genome-wide linkage screens and DNA microarrays have identified at least 20 genes with a significant association with AD. However, only six of these genetic associations, IL-4, IL-4R, IL-13, mast cell chymase, SPINK5 (serine protease inhibitor Kazal-type 5), and filaggrin genes, have been replicated in two or more independent studies (3).

The SPINK5 gene encodes a serine protease inhibitor proprotein containing two classical Kazal-type and 13 non-Kazal-type domains, which may be crucial for epidermal cell growth and differentiation. The lymphoepithelial Kazal-type inhibitor which

was encoded by SPINK5 gene is a serine protease inhibitor primarily involved in the control of stratum corneum trypsin-like enzyme (SCTE)/stratum corneum chymotrypsin-like enzyme (SCCE) and probably external serine protease such as many allergens which might contribute to the risk of allergic sensitisation. The recessive nonsense mutations in the SPINK5 gene result in Netherton syndrome (4), a disorder characterised by AD, ichthyosis, and elevated serum IgE levels. This result led us to screen SPINK5 for polymorphisms in Korean AD patients. Research from Britain and Japan has shown that SPINK5 polymorphisms are associated with atopic eczema (5–8), but this association has not been replicated in subsequent studies (9–11).

Based on the biological properties involved in AD, we hypothesise that SPINK5 plays important roles in the development of AD. To determine whether the SPINK5 polymorphisms are associated with the disease susceptibility of AD, we performed extensive genotyping for 1090 case-control samples and conducted statistical analysis for single nucleotide polymorphisms (SNP) and haplotypes of the SPINK5 gene. We also analysed the possible gene-gene interactions between SPINK5 and DEFB1, on which we previously reported (12). We found that six SNPs (rs17718511, rs17860502, KN0001820, rs60978485, rs17718737, and rs1422985) and the haplotype TAA (rs60978485, rs6892205, rs2303064) in the SPINK5 gene were associated with AD. Four of these SNPs (rs17718511, rs17860502, rs60978485, rs17718737) and the TAA haplotype significantly interacted with the development of allergic AD.

Materials and methods

Subjects

For this study, 1090 case–control samples, 631 patients with AD, and 459 normal control subjects (NR) were recruited. The AD samples were collected from non-asthmatic atopic patients who were examined at Samsung Medical Center, Seoul, Korea. The AD diagnosis, the criteria for the classification of AD into ADe (allergic type of AD or extrinsic type of AD) or ADi (non-allergic type of AD or intrinsic type of AD), and the blood tests and prick tests for the allergens were as described in our previous report (13). All of the 631 patients with AD (M:F = 351:280, age range: 13.42 ± 9.92 years) meet the criteria of our previous reports (13,14). Among the 631 patients with AD, 417 (M:F = 250:167, age range: 15.6 ± 9.3 years) were classified as ADe and 214 (M:F = 101:113, age range: 9.2 ± 9.7 years) were classified as ADi. The 459 NR subjects were recruited from a group of medical students and volunteers (M:F = 238:221, age range: 23.33 ± 2.19 years) who had no history of AD skin lesions. The demographic information of the study subjects is summarised in Table 1.

This study was conducted according to the Declaration of Helsinki Principles, and written informed consents were obtained from all participants. The Samsung Medical Center Ethics Committee approved this study.

Marker selection

The SNP information was retrieved from the dbSNP (build 119, <http://www.ncbi.nlm.nih.gov/SNP>; accessed 31 May 2007). We included two SNPs (KN0001820 and KN0001823) which were reported as novel SNPs identified in Korean samples, and the detailed information was obtained from the KSNP website (<http://ksnp.ngri.go.kr/>). As a result, 99 SNPs were selected as polymorphic markers in the region between 5 kb upstream and 5 kb downstream of the SPINK5 gene. The SNPs were genotyped from 48 independent samples from the general Korean population (data not shown). Based on these genotype results, we chose SNPs whose minor allele frequencies were >0.1 . Then, the tag SNPs among the chosen SNPs were determined through the linkage disequilibrium (LD) bin approach that was implemented in the Tagger programme (<http://www.broad.mit.edu/mpg/tagger>; accessed March 2009). The LD bin approach defines the bins of SNPs that are in very strong LD with a specified r^2 threshold, and then one SNP is selected to represent the remaining SNPs in each bin (15). We used an r^2 threshold of 0.8. Finally, a total of 21 SNPs from SPINK5 were selected as the markers for the association study (Fig. S1).

Genotyping with fluorescence polarisation detection

Genomic DNA was extracted from 5 ml of whole blood using a DNA isolation kit (Gentra Genomic DNA purification kit, Minneapolis, MN, USA) according to the protocol. Genotype identifica-

tion was performed with the GenomeLab SNPstream system [Ultra-high throughput; UHT system (16)], which uses multiplexed PCR in conjunction with tag array single base extension genotyping technology (Beckman Coulter, Fullerton, CA, USA), and its accompanying SNPstream software, as was previously described by Denomme and Van Oene (17).

Statistical analysis

Chi-square tests were used to determine whether individual variants were in Hardy–Weinberg equilibrium at each locus for the normal samples. The allelic (additive) and genotypic effects of the individual SNPs were tested using a logistic regression model where gender and age were used as the adjusting covariates. In this study, we presented nominal P -values and P -values <0.05 were considered to be significant. Because numbers of test were conducted for the same hypothesis, experiment-wise type I error rate can be greater than the significance level. The odds ratios (ORs) were also estimated from the logistic regression model.

To detect the most significantly associated haplotype, the significances of overall haplotype effects were scanned for haplotypes constructed across sliding windows of sizes 2–4 SNPs. For the haplotype scan, haplo.score in R package (<http://www.r-project.org>) was used. The function haplo.score provides a haplotype association test method that enables users to simultaneously model haplotype effects, including various controlling covariates, and statistical significance is obtained by using score test statistics (18). Haplo.score provides global test statistics for a given haplotype locus in addition to the results for individual haplotypes. We used the global test P -value to plot the significance of the haplotype effect for each locus. For the haplotype locus showing the most significant overall effect, we computed ORs using the haplo.glm function in R package. Haplo.glm provides estimates and the significance of the relative effects of the haplotypes on the trait when compared to the effect of the baseline haplotype (19). In this study, the most frequent haplotype was used as a baseline. We grouped the total IgE levels to reduce the effect of extreme values and censored observations. We had several right censored values because of the mechanical limitation at the measurement time. The total IgE levels were categorised into five ordered groups (<40 , 40–200, 200–500, 500–2000, and >2000 U/ml) that was decided based on quantiles of data. The cumulative logistic regression analysis was then conducted to examine the associations between the genotypes and the total IgE levels. The genetic effects on the blood eosinophil counts and eosinophilic cationic protein (ECP) levels in the AD subjects were tested using a linear regression model. The log-transformed eosinophil counts and ECP levels were used in the regression analysis. Age, gender, and the scoring of atopic dermatitis (SCORAD) index were used in the regression analysis model as the adjusted covariates.

Table 1. Demographic features of the three groups. The number of subjects, the mean, and standard deviation for age, the IgE level, the eosinophil count, and the SCORAD index are shown

Group	Number of subjects (F/M)	Age (years)	IgE (U/ml)	Eosinophil	ECP (ng/ml)	SCORAD
ADe	417 (167/250)	15.6 (± 9.3)	1909.2 (± 3284.6)	610.2 (± 651.6)	79 (± 266.2)	33.6 (± 19.7)
ADi	214 (113/101)	9.2 (± 9.7)	51.9 (± 46.2)	386.6 (± 362.7)	39 (± 48.6)	22.5 (± 16.3)
NR	459 (221/238)	23.33 (± 2.19)	241.5 (415.8)			

ADe, allergic type of AD or extrinsic type of AD; ADi, non-allergic type of AD or intrinsic type of AD; ECP, eosinophilic cationic protein; SCORAD, scoring of atopic dermatitis.

To analyse gene–gene interaction multifactor dimensionality reduction (MDR) method (20) was used. MDR has been adopted in about numerous genetic association studies (<http://compugen.blogspot.com/2006/05/mdr-applications.html>) to identify gene–gene interactions associated with complex traits. MDR can effectively handle sparse data that occurs frequently in interaction models, on the contrary to that logistic regression sometimes fails to fit models in the presence of sparse data. In addition, as MDR is a non-parametric method, it avoids large number of tests. Because of these advantages, we used MDR to discover the best interaction/concomitant multilocus effect model. However, because logistic regression provides better interpretable result with odds ratio and significance of effects, we conducted logistic regression analysis for the selected SNP pair. Briefly describing, MDR is a non-parametric and genetic model free method for detecting gene–gene interaction for binary traits such as disease status. MDR classifies multi-locus genotypes into high/low risk groups. This new group variable is evaluated for its ability to classify and predict outcome using cross-validation (CV). For 10-fold CV, MDR splits data into 10 subsets, and nine subsets are used as training sets and the remaining set is used as testing dataset for each CV. Using training datasets, MDR compute training balanced accuracy (BA) of all possible interaction models with given number of SNPs (two SNPs in this study). As a model evaluation measure, BA is defined as the arithmetic mean of sensitivity and specificity. The best model for each CV datasets is selected to maximise training BA and testing BA is computed using the testing dataset. Of the 10 best models selected from 10 CV datasets, the most frequently selected model having the maximum testing BA is the final interaction model. The number of times a particular interaction model is selected across 10 CV datasets is referred as cross-validation consistency (CVC). Because the original MDR does not allow missing values, MDR analysis was performed using the imputeMDR package in R language, which utilises all available information during the MDR analysis by estimating missing information in the presence of missing values (21). Once the most significantly associated gene–gene interaction model was identified, the statistical significance and ORs of each of the multi-locus genotypes were obtained from the logistic regression analysis.

Statistical analysis was performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and R statistical language (<http://www.r-project.org>; accessed 31 March 2009).

Results

A total of 21 SNPs from the SPINK5 gene were genotyped for the 1090 subjects. Information on the SNPs is displayed in Table S1, including the genomic function, chromosomal position, dbSNP id, and minor allele frequency. All of the SNPs were in Hardy–Weinberg equilibrium at the significance level of 0.01. The average genotyping success rate was 97.4%.

We compared the distributions of the allelic and genotypic frequencies for the 21 SNPs between the AD and NR groups. The statistical significances were obtained from logistic regression analysis, while age and gender were used as the adjusting covariates (Table S2). For further analysis, haplotype association tests were conducted using a sliding window approach. Interaction analyses were also conducted for combinations of SPINK5 and DEFB1 polymorphisms.

Differences in the allelic and genotypic distributions of SNPs and haplotypes in SPINK5 between AD subjects and controls

When we tested the associations of the SNPs with AD, six SNPs showed significantly different allelic distributions between the AD and NR groups. Table S3 presents the test results of the six SNPs, rs17718511, rs17860502, KN0001820, rs60978485, rs17718737, and rs1422985. Of the six SNPs, four (rs17718511, rs17860502, rs60978485, and rs17718737) also showed a significant difference between the ADe and NR groups. The remaining two SNPs (KN0001820 and rs1422985) showed only marginally significant results and their effect sizes of risk alleles were least strong (it seems that the larger sample sizes is required to get enough for these SNPs to be significant). None of the SNPs showed a significant result in the comparisons between the ADi and NR groups.

The SNP rs60978485 showed the most difference between the control and the AD and ADe groups, with *P*-values of 0.007 and 0.005, respectively. The odds ratios (95% confidence intervals) of the AD and ADe groups were 0.686 (0.521, 0.903) and 0.651 (0.483, 0.877), respectively. The rs60978485 SNP also resulted in significant *P*-values from the genotypic effect test but the significance and ORs were not greater than those obtained from the allelic effect tests (Table S3). None of the SNPs showed significant associations with ADi.

Additionally, association with the disease was tested for haplotypes constructed along sliding windows of sizes 2–4 SNPs using the score test (18), and the global *P*-value was obtained for each haplotype locus. The haplotype association was tested for AD and the two AD subtypes separately, and the *P*-values of the tests were obtained. The haplotype association tests for AD and ADe resulted in similar patterns of significance across the gene with two significant points. However, ADi did not show a significant result under the null hypothesis of no association between the disease and the haplotypes in the variable-sized window.

The test for AD association was most significant for haplotypes of the three loci rs60978485, rs6892205, rs2303064 (*P*-value = 0.0182) (Fig. S2). For these haplotypes, we further analysed the individual haplotype effect for the susceptibility of AD and AD subtypes (Table 2). Odds ratios (ORs) and the significance of effect differences with respect to the reference haplotype were obtained from the analysis using haplo.glm, with age and gender used as adjusting covariates. The most frequent haplotypes were used as the reference haplotype group. The TAA haplotype showed an OR of 0.716 (95% CI: 0.537–0.955), a *P*-value of 0.023 when compared to the AGG haplotype, and a haplotype frequency of about 18%. This TAA haplotype had an OR of 0.687 (95% CI: 0.501–0.941) in the ADe subtype with a *P*-value of 0.02, and no significant effect in the ADi subtype.

Interaction analysis of SPINK5 and DEFB1

Previously we reported that DEFB1 polymorphisms are associated with ADe (12). To determine if the interactions between polymorphisms from SPINK5 and DEFB1 are associated with ADe susceptibility, we conducted a gene–gene interaction analysis. First, MDR analysis was performed to find the most significantly associated interaction. To minimise the loss of information because of missing genotypes, MDR analysis was performed using the imputeMDR package in R language. ImputeMDR utilises all available information to estimating missing information during the MDR analysis (21). As a result, rs60978485 in SPINK5 and rs5743399 in

Table 2. Result of the analysis of haplotypes of the three loci (rs60978485, rs6892205, rs2303064) in SPINK5 to test for the association with atopic dermatitis (AD) and AD subtypes. Odds ratios and the significances of the effects were obtained from the analysis using haplo.glm and age and gender were used as adjusting covariates. The most frequent haplotypes were used as reference haplotype group.

Haplotype	AD vs NR			Frequency	ADe vs NR			Frequency	ADi vs NR		
	Frequency	OR (95% CI)	P value		Frequency	OR (95% CI)	P value		Frequency	OR (95% CI)	P value
AAG	0.076	1.206 (0.798, 1.825)	0.374	0.075	1.132 (0.725, 1.766)	0.586	0.074	1.493 (0.724, 3.08)	0.278		
AAA	0.303	1.076 (0.834, 1.389)	0.571	0.304	1.084 (0.824, 1.426)	0.564	0.287	1.113 (0.709, 1.748)	0.643		
TAA	0.181	0.716 (0.537, 0.955)	0.023	0.18	0.687 (0.501, 0.941)	0.02	0.2	0.883 (0.53, 1.474)	0.635		
Rare haplotype	0.010	2.766 (0.779, 9.827)	0.116	0.012	2.509 (0.677, 9.3)	0.169	0.007	3.611 (0.311, 41.871)	0.305		
AGG	0.429	Reference		0.429	Reference		0.431	Reference			

ADe, allergic type of AD or extrinsic type of AD; ADi, non-allergic type of AD or intrinsic type of AD. P-values that are significant at the 0.05 level are written in bold type.

DEFB1 were selected as the best models, with a CVC of eight from a 10-fold cross-validation, and the average testing BA of the eight cases was 0.5636.

To determine statistical significances and the effect sizes for the two SNP combinations, we further analysed the logistic regression model using the two SNPs. Genotype combinations of at least one minor allele for the two SNPs showed a protective effect on ADe susceptibility compared to the most common genotype combination of rs60978485 = AA/rs5743399 = CC (Table 3). The ORs were 0.581 (95% CI: 0.472–0.716) and 0.544 (95% CI: 0.438–0.676) for AA/T and T–/CC, respectively. The group having at least one minor allele at both loci, T–/T–, showed the greatest protective effect with an OR of 0.404 (95% CI: 0.305–0.536). In summary, the rs60978485 allele in SPINK5 and the rs5743399 allele in DEFB1 seem to act additively to decrease susceptibility to ADe.

Association of haplotypes in SPINK5 with eosinophil counts

We also tested the SNPs for association with total IgE level, ECP, and peripheral eosinophil counts in the AD group. No significant associations were detected from the individual SNP tests; however, haplotypes of the three loci rs2303067, rs880687, and rs41291431 showed the most significant associations with log-transformed eosinophil counts (P -value = 0.003) in the sliding window haplotype analysis. The P -values of the tests are plotted in Fig. S3. For this haplotype, we further analysed the individual haplotype effects on eosinophil levels (Table 4). Regression coefficients and standard errors were obtained from the analysis using haplo.glm with age, gender, SCORAD index, and AD subtype used as adjusting covariates. The most frequent haplotypes were used as a reference haplotype group. The results showed that eosinophil counts in the GAC haplotype group increased by 1.15-fold compared to those of the ACC haplotype group (at rs2303067, rs880687, and

Table 4. Result of the test for association of three loci haplotype rs2303067, rs880687, and rs41291431 in SPINK5 with log-transformed eosinophil counts. Haplotype association was tested using haplo.glm (37), with sex, age, SCORAD, and the atopic dermatitis subtype adjusted.

Haplotype	Frequency	Coefficient	SE	P value
GAC	0.299	0.136	0.051	0.008
GAT	0.131	–0.045	0.069	0.515
Rare haplotype	0.006	0.329	0.351	0.349
ACC	0.563			

SCORAD, scoring of atopic dermatitis. P-values that are significant at the 0.05 level are written in bold type.

rs41291431 loci; P -value of 0.008), and the haplotype frequency was about 30%.

Discussion

SPINK5 encodes the serine protease inhibitor LEKTI (lympho-epithelial Kazal-type-related inhibitor), which is expressed during terminal differentiation of stratified epithelial tissue. In the epidermis, LEKTI is mainly restricted to the granular layer, where crucial biochemical and morphological changes during terminal differentiation lead to cornification. Using in situ hybridisation in patients with Netherton's syndrome, SPINK5 transcripts were found to be expressed in the uppermost stratum spinosum and stratum granulosum of the epidermis, as well as in hair follicles and sebaceous glands. This localisation strongly suggests that the inhibitor peptides derived from the SPINK5 proprotein may target two known serine proteases in the epidermis, SCTE (kallikrein-related peptidase 7; KLK 7), and SCCE (KLK5) (22). Recently, it was shown that the pH gradient occurring through stratum corneum controls the interaction strength between LEKTI and KLK5, thus allowing the controlled release of active proteinase in the most superficial layers of stratum corneum (23).

SPINK5 null mice die within a few hours of birth and have the ichthyosiform phenotype of grafted skin; grafts from the knockout mice have large scales and marked erythema without any erupted hair shafts (24). This finding elucidates the molecular pathology of the severe generalised exfoliation observed in Netherton's syndrome: lack of LEKTI leads to unregulated SCTE and SCCE-like activity and loss of stratum corneum adhesion through degradation of Dsg-1 (desmoglein-1). Reduced granular layer-stratum corneum cohesion results in defective barrier function, which could, in turn, account for dehydration, infections, and increased allergen penetration (25,26).

Moffatt reported (27) that there are two other possibilities with regard to LEKTI's action and the association of its gene with aller-

Table 3. The logistic regression analysis to test for the effect of genotype combinations of the rs60978485 in SPINK5 and rs5743399 in DEFB1. The ADe group was compared to the NR group. Age and gender were included in the model as adjusting covariates.

rs60978485/ rs5743399	Case	Control	OR (95% CI)	P value
AA/CC	150	193	Reference	
AA/T–	109	92	0.581 (0.472, 0.716)	0.009
T–/CC	103	72	0.544 (0.438, 0.676)	0.005
T–/T–	56	36	0.404 (0.305, 0.536)	0.001

gic disease, and both involve LEKTI's potential interaction with external proteases. One possibility is that many allergens are serine proteinases and this proteinase activity may encourage allergenicity in the presence of variable proteinase inhibitors (11). Another possible method for LEKTI's action is the external proteases of *Staphylococcus aureus*. Over 90% of patients with AD are colonised with *S. aureus*, and the degree of colonisation correlates with disease activity. This is in part because of the enterotoxins produced by the *Staphylococcus* species that both exacerbate and prolong the eczema. Briot et al. reported (28) that LEKTI-deficient keratinocytes, KLK5 hyperactivity activates proinflammatory signalling, leading to the recruitment of eosinophilic and mast cells, which is reminiscent of AD-like skin lesion in Netherton's syndrome, independent from skin barrier defect, environmental stimuli, and adaptive immune system.

Through genotyping the SPINK5 gene, we found that six SNPs (rs17718511, rs17860502, KN0001820, rs60978485, rs17718737, and rs1422985) and the haplotype TAA (rs60978485, rs6892205, rs2303064) in the SPINK5 gene show significant differences between the AD group and the control group. Also, four SNPs (rs17718511, rs17860502, rs60978485, rs17718737) and the haplotype TAA showed significant association with the development of the allergic type of AD. Since Walley et al. first reported maternal transmission of the risk allele of the SPINK5 gene in British individuals affected with AD (5), there have been conflicting results of SPINK5 SNP studies. Two studies in French and European groups have since confirmed this original finding (27,29), though two German and one Dutch studies have found no association between SPINK5 SNPs and AD (9–11). On the other hands, three Japanese studies showed significant association between SPINK5 SNP and AD (6–8). Our data in Koreans showed that six SNPs and the haplotype TAA were strongly associated with the AD phenotype and moreover, four of these six SNPs and haplotype TAA showed strong association with the allergic type of AD. Of the genotyped SNPs, two non-synonymous SNPs, D386N (rs2303064) and K420E (rs2306067) were reported to be associated with AD in Japanese population (8). In our study, they did not show significant results in individual SNP association tests. Only in haplotype analysis, D386N (rs2303064) showed significant association with AD. Instead, we found that D106N (rs17860502) significantly associated with AD in our study. This different association pattern may caused by allelic heterogeneity in a gene between Korean and Japanese populations. Also, based on our current results and previous reports, we speculate that there may be ethnic differences between European and Asian populations in regard to AD and its genetic susceptibility. D106N (rs17860502), newly detected AD associated non-synonymous SNP in our study, substitutes negative charged aspartic acid to neutral asparagine and this may affect the protein functionality. Thus, it is prompting to speculate that suspected AD association of SPINK5 could be based on a direct molecular action of LEKTI on the proteinase activity and/or allergenicity of allergens. Thus, LEKTI may be an important candidate protein for the functional studies in AD.

Previous studies have tried to establish a gene–gene interaction between the SPINK5 gene and the KLK 7 gene and/or filaggrin gene in patients with AD; however, no association was found among these genes (29,30). Because external proteases from *S. aureus* could

potentially interact with LEKTI, we tried to determine the relationship between *S. aureus* and LEKTI. It has been known that some antimicrobial peptides, including defensin, are deficient in patients with AD. The defensin gene family is divided into three classes, α , β , and θ , according to the arrangement of disulphide bridges between the cysteine residues. The β -defensins are expressed in a variety of epithelia, and they have been shown to have broad antimicrobial properties, and the β -defensins are considered to be a link between the innate and adaptive immunity by acting as microchemokines. The colonisation of bacteria and the *Candida* species is associated with the β -defensin genes (12). The AD patient's skin usually showed decreased expression of β -defensin; however, human β -defensin 2 was strongly overexpressed in patients with Netherton's syndrome as compensatory mechanism (31). Although we did not verify the bacterial species in patient skin, we previously reported that the SNPs in the defensin beta 1 (DEFB1) gene were strongly associated with AD, and especially with the allergic type of AD (12). Therefore, we sought to analyse the gene–gene interaction between the SPINK5 gene and the DEFB1 gene. We found that the rs60978485 allele of SPINK5 and the rs5743399 allele of DEFB1 seem to act additively to decrease ADe susceptibility. Although this DEFB1 SNP does not directly reflect *S. aureus* infection level, the concomitant effect between the SPINK5 SNP and the DEFB1 SNP indirectly verifies that the bacterial protease is associated with SPINK5, as suggested previously (27).

Although any of the complement 3 (C3) SNPs was not reported to be associated with serum total IgE levels, one of the C3 SNP showed the association with low levels of specific IgE levels to *S. aureus* (32). However, previous reports showed that any of the SNPs in the SPINK5 gene are not associated with serum IgE levels (8,11), and our study confirmed this result. Nevertheless, we found that a certain haplotype in SPINK5 was weakly associated with blood eosinophil counts, although this result is very hard to explain.

In summary, we found that six SNPs (rs17718511, rs17860502, KN0001820, rs60978485, rs17718737, and rs1422985) and the haplotype TAA (rs60978485, rs6892205, rs2303064) in the SPINK5 gene showed marginally significant differences between the AD group and the control group. We also found that four SNPs (rs17718511, rs17860502, rs60978485, rs17718737) and the haplotype TAA in the SPINK5 gene showed significant association with the development of the allergic type of AD, providing evidence of a significant interaction between allergens and SPINK5 that contributes to susceptibility to the allergic type of AD (ADe). In addition to this finding, we speculated that the DEFB1 gene acts together with the SPINK5 gene to aid in the development of ADe in an additive manner. This data may contribute to our understanding of the AD genetic network and account for the high risk of certain patients to develop AD. As a limitation of our results, experiment-wise type I error rate may be greater than the significance level (5%) when accounting for multiple testing.

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References

- 1 Hanifin J M. *J Am Acad Dermatol* 1982; **6**: 1–13.
- 2 Leung D Y. *J Allergy Clin Immunol* 1999; **104**: S99–S108.
- 3 Brown S J, McLean W H I. *J Invest Dermatol* 2009; **129**: 543–555.
- 4 Chavanas S, Bodemer C, Rochat A *et al.* *Nat Genet* 2000; **25**: 141–142.
- 5 Walley A J, Chavanas S, Moffatt M *et al.* *Nat Genet* 2001; **29**: 175–178.
- 6 Kusunoki T, Okafuji I, Yoshioka T *et al.* *J Allergy Clin Immunol* 2005; **115**: 636–638.
- 7 Nishio Y, Noguchi E, Shibasaki M *et al.* *Genes Immun* 2003; **4**: 515–517.
- 8 Kato A, Fukai K, Oiso N *et al.* *Br J Dermatol* 2003; **148**: 665–669.
- 9 Folster-Holst R, Stoll M, Koch W A *et al.* *Br J Dermatol* 2005; **152**: 1365–1367.
- 10 Jongepier H, Koppelman G H, Nolte I M *et al.* *J Allergy Clin Immunol* 2005; **115**: 486–492.
- 11 Kabesch M, Carr D, Weiland S K *et al.* *Clin Exp Allergy* 2004; **34**: 340–345.
- 12 Kim E, Lee J-E, Namkung J-H *et al.* *J Dermatol Sci* 2009; **54**: 25–30.
- 13 Jung C-W, Ahn K-S, Rho N-K *et al.* *Clin Exp Allergy* 2003; **33**: 1717–1724.
- 14 Namkung J H, Lee J E, Kim E *et al.* *Allergy* 2007; **62**: 934–942.
- 15 Carlson C S, Eberle M A, Rieder M J *et al.* *Am J Hum Genet* 2004; **74**: 106–120.
- 16 Bell P A, Chaturvedi S, Gelfand C A *et al.* *Bio-Techniques* 2002; **570–2**, **74**: 76–77.
- 17 Denomme G, Van Oene M. *Transfusion* 2005; **45**: 660–666.
- 18 Schaid D, Rowland C, Tines D *et al.* *Am J Hum Genet* 2002; **70**: 425–434.
- 19 Lake S, Lyon H, Silverman E *et al.* *Hum Hered* 2002; **55**: 56–65.
- 20 Ritchie M D, Hahn L W, Roodi N *et al.* *Am J Hum Genet* 2001; **69**: 138–147.
- 21 Namkung J, Elston R C, Yang J M *et al.* *Genet Epidemiol* 2009; **33**: 646–656.
- 22 Komatsu N, Takata M, Otsuki N *et al.* *J Invest Dermatol* 2002; **118**: 436–443.
- 23 Deraison C, Bonnart C, Lopez F *et al.* *Mol Biol Cell* 2007; **18**: 3607–3619.
- 24 Descargues P, Deraison C, Bonnart C *et al.* *Nat Genet* 2005; **37**: 56–65.
- 25 Komatsu N, Suga Y, Saijoh K *et al.* *J Invest Dermatol* 2006; **126**: 2338–2342.
- 26 Yang T, Liang D, Koch P J *et al.* *Genes Dev* 2004; **18**: 2354–2358.
- 27 Moffatt M F. *Clin Exp Allergy* 2004; **34**: 325–327.
- 28 Briot A, Deraison C, Lacroix M *et al.* *J Exp Med* 2009; **206**: 1135–1147.
- 29 Hubiche T, Ged C, Benard A *et al.* *Acta Derm Venereol* 2007; **87**: 499–505.
- 30 Weidinger S, Baurecht H, Wagenpfeil S *et al.* *J Allergy Clin Immunol* 2008; **122**: 560–568.
- 31 Raghunath M, Tontsidou L, Oji V *et al.* *J Invest Dermatol* 2004; **123**: 474–483.
- 32 Purwar R, Langer K, Werfel T. *Exp Dermatol* 2009; **18**: 30–34.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Map of SPINK5 on chromosome 5q32 (81.2 kb).

Figure S2. Haplotype association scan results.

Figure S3. Haplotype association scan results on log-Eosinophil counts.

Table S1. The SNP markers genotyped for the case-control samples (dbSNP build 126).

Table S2. Summary result for all SNPs.

Table S3. Comparison of the allelic and genotypic frequencies of the SNPs in SPINK5 between the two AD subtypes and the normal control group.

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Supplementary materials

Figure S1. Map of SPINK5 on chromosome 5q32 (81.2kb). Coding exons are marked by black blocks, and the 5' and 3' UTRs are marked by gray blocks. The first base of the translation start site is denoted as nucleotide +1. Genotyped polymorphisms in the four genes are marked. Minor allele frequencies of the polymorphisms in 48 samples are distinguished as over 10% or less. Dots indicate the polymorphisms that were genotyped in a larger population (n=1,090). LD among the tag SNPs and their tagged SNPs are presented in r^2 and the haplotypes are listed.

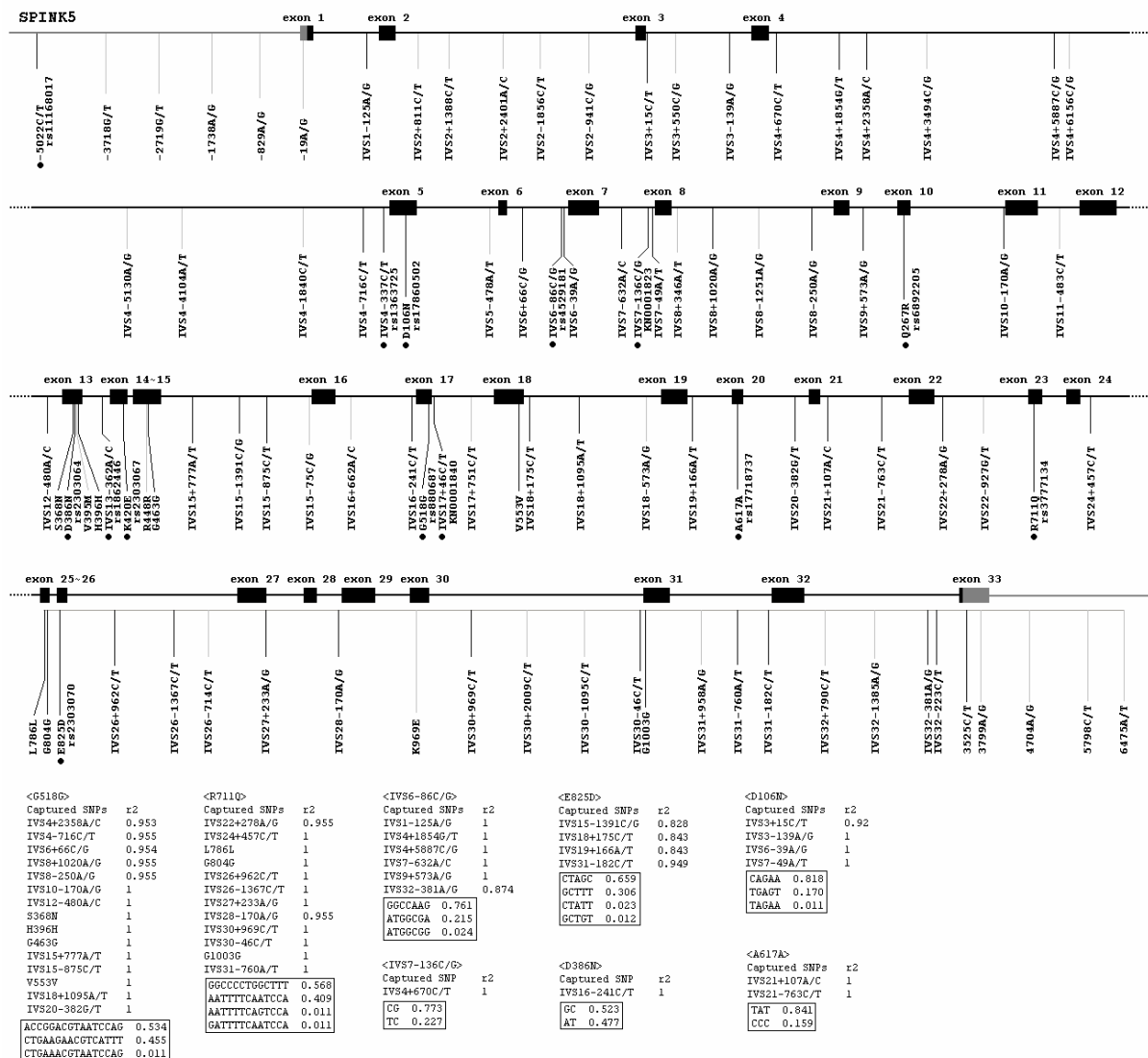
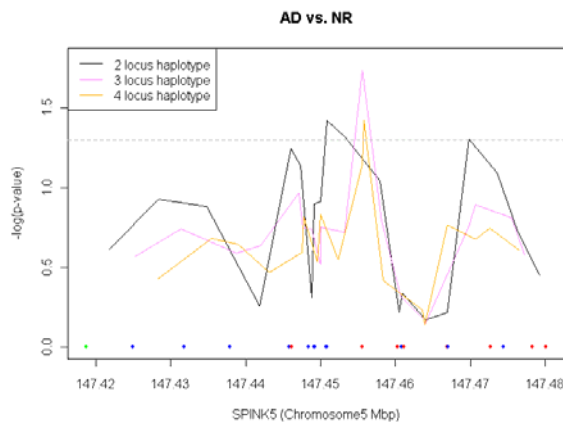


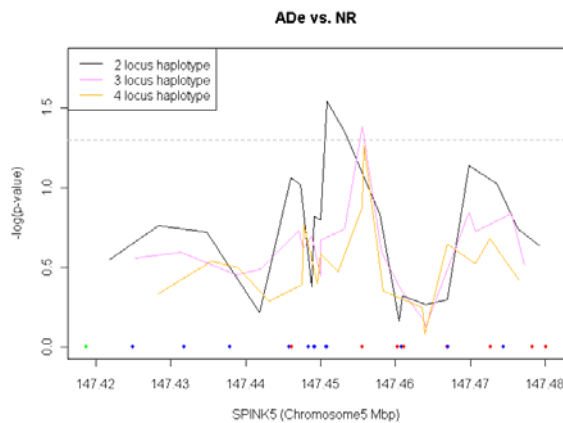
Figure S2. Haplotype association scan results. The results from the test for the haplotype association with AD, (b) ADe, and (c) ADi are shown. Two to four-locus haplotypes were tested. The inverse negative of the base 10 logarithm of p-values are presented. The results are pointed at the center of the tested haplotypes and draw lines from point to point. Dashed line designates pre-transformed p-value of 0.05.

Genotyped positions are presented as dots at the bottom lines and the functions of variation are presented as different colors of dots : Promotor, intron (including upstream untranslated region) and exon as green, blue and red respectively.

(a)



(b)



(c)

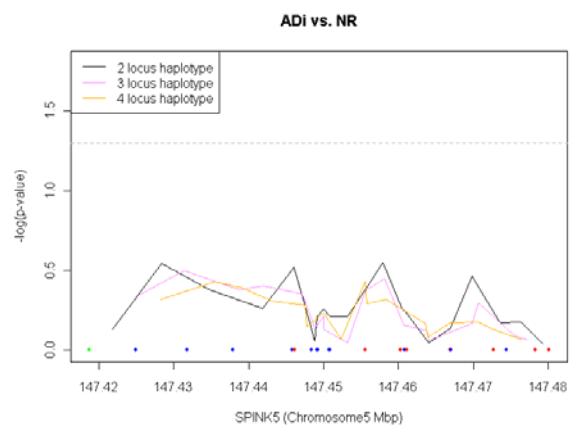


Figure S3. Haplotype association scan results on log-Eosinophil counts. Two to four loci haplotypes were tested. The inverse negative of the base 10 logarithm of p-values are presented. The results are pointed at the center of the tested haplotypes and draw lines from point to point. Dashed line designates pre-transformed p-value of 0.05. The most significant haplotype is three locus haplotype (s2303067 (K420E), rs880687 (G518G), and rs41291431;n p=0.003), which includes polymorphism.

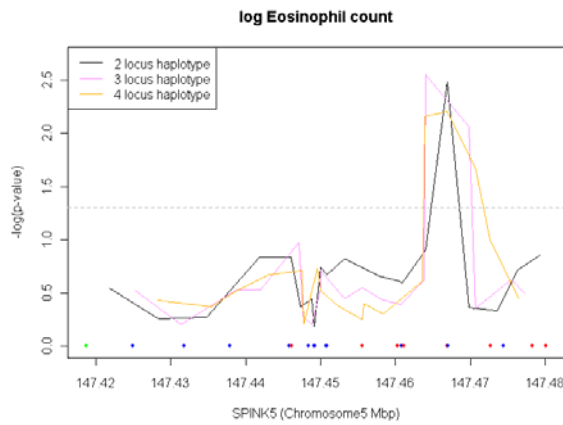


Table S1. The SNP markers genotyped for the case-control samples (dbSNP build 126)

Gene	ID	RS number	Chr	Position	Function	MA F ^a	HW E ^a	Succes s rate
5 SPINK	-5022C/T	7 rs1116801	5q32	14741877	promote	0.30	0.06	0.961
5 SPINK	IVS1-125A/G	rs2287772	5q32	14742497	intron 1	0.26	0.06	0.983
5 SPINK	IVS3-139A/G	1 rs1771851	5q32	14743176	intron 3	0.19	1	0.988
5 SPINK	IVS4+5887C/ G	rs4333302	5q32	14743786	intron 4	0.27	0.11	0.939
5 SPINK	IVS4-337C/T	rs1363725	5q32	14744582	intron 4	0.49	0.39	0.977
5 SPINK	D106N	2 rs1786050	5q32	14744619	exon 5	0.19	1	0.987
5 SPINK	IVS6+66C/G	rs7711953	5q32	14744842	intron 6	0.46	0.21	0.978
5 SPINK	IVS6-86C/G	rs4529181	5q32	14744916	intron 6	0.27	0.07	0.993
5 SPINK	IVS6-39A/G	0 ^b KN000182	5q32	14744921	intron 6	0.19	0.65	0.994
5 SPINK	IVS7-136C/G	3 ^b KN000182	5q32	14745078	intron 7	0.22	0.14	0.989
5 SPINK	IVS7-49A/T	5 rs6097848 (KN000182 3 ^b)	5q32	14745087	intron 7	0.21	0.88	0.93
5 SPINK	Q267R	rs6892205	5q32	14745557	exon 10	0.43	0.70	0.967
5 SPINK	D386N	rs2303064	5q32	14746027	exon 13	0.49	0.44	0.964
5 SPINK	IVS13- 362A/C	rs1862446	5q32	14746074	intron 13	0.07	0.49	0.979
5 SPINK	K420E	rs2303067	5q32	14746114	exon 14	0.42	0.84	0.983
5 SPINK	G518G	rs880687	5q32	14746687	exon 17	0.42	0.7	0.967
5 SPINK	IVS17+46C/T	1 rs4129143 (KN0001840 b)	5q32	14746696	intron 17	0.13	0.07	0.988
5 SPINK	A617A	7 rs1771873	5q32	14747265	exon 20	0.19	0.76	0.99
5 SPINK	IVS21+107A/ C	rs1422985	5q32	14747435	intron 21	0.19	0.88	0.986

5	SPINK	R711Q	rs3777134	5q32	14747821	exon 23	0.41	0.92	0.977
					2		4	3	
5	SPINK	E825D	rs2303070	5q32	14748008	exon 26	0.30	0.82	0.963
					4		3		

^aMAF: Minor Allele Frequency

HWE: P-values from chi-square testing for Hardy-Weinberg equilibrium

The MAF and HWE are calculated from 459 normal control samples

^b KSNP ID (<http://www.ngri.go.kr/SNP/>)

Table S3. Comparison of the allelic and genotypic frequencies of the SNPs in SPINK5 between the two AD subtypes and the normal control group. Odds ratios and the significances of the effects were obtained from the analysis using a logistic regression model where age and gender were used as adjusting covariates.

rs number (allele A/allele B)	group	Genotype Frequency			MAF	Allele test		Genotype test		
		AA	AB	BB		P value	OR (95% CI)	P value ^a	OR1 (95% CI)	OR2 (95% CI)
rs17718511 A/G	NR	293 (0.644)	144 (0.316)	18 (0.04)	0.198					
	AD	439 (0.706)	165 (0.265)	18 (0.029)	0.162	0.026	0.739 (0.565,0.965)	0.08	0.718 (0.52,0.99)	0.601 (0.266,1.358)
	ADe	296 (0.724)	100 (0.244)	13 (0.032)	0.154	0.033	0.728 (0.543,0.974)	0.091	0.695 (0.49,0.987)	0.624 (0.255,1.524)
	ADi	143 (0.671)	65 (0.305)	5 (0.023)	0.176	0.413	0.821 (0.512,1.316)	0.708	0.805 (0.459,1.411)	0.727 (0.167,3.161)
rs17860502 G/A	NR	291 (0.641)	145 (0.319)	18 (0.04)	0.199					
	AD	437 (0.703)	167 (0.268)	18 (0.029)	0.163	0.024	0.736 (0.564,0.961)	0.075	0.716 (0.519,0.986)	0.598 (0.265,1.351)
	ADe	298 (0.72)	103 (0.249)	13 (0.031)	0.156	0.031	0.725 (0.542,0.97)	0.086	0.695 (0.491,0.984)	0.615 (0.251,1.504)
	ADi	139 (0.668)	64 (0.308)	5 (0.024)	0.178	0.406	0.817 (0.508,1.315)	0.693	0.793 (0.45,1.397)	0.747 (0.172,3.25)
KN0001820 A/G	NR	295 (0.648)	141 (0.31)	19 (0.042)	0.197					
	AD	441 (0.702)	168 (0.268)	19 (0.03)	0.164	0.045	0.763 (0.586,0.994)	0.124	0.736 (0.534,1.016)	0.655 (0.297,1.444)
	ADe	298 (0.718)	103 (0.248)	14 (0.034)	0.158	0.059	0.757 (0.568,1.01)	0.139	0.713 (0.503,1.012)	0.699 (0.296,1.653)
	ADi	143 (0.671)	65 (0.305)	5 (0.023)	0.176	0.439	0.83 (0.518,1.33)	0.737	0.819 (0.467,1.436)	0.726 (0.167,3.153)
rs60978485 A/T	NR	260 (0.619)	142 (0.338)	18 (0.043)	0.212					
	AD	416 (0.7)	162 (0.273)	16 (0.027)	0.163	0.007	0.686 (0.521,0.903)	0.023	0.652 (0.468,0.907)	0.559 (0.244,1.281)
	ADe	290 (0.727)	98 (0.246)	11 (0.028)	0.15	0.005	0.651 (0.483,0.877)	0.014	0.604 (0.423,0.864)	0.553 (0.224,1.365)
	ADi	126 (0.646)	64 (0.328)	5 (0.026)	0.19	0.533	0.852 (0.514,1.412)	0.805	0.822 (0.449,1.504)	0.822 (0.18,3.751)
rs17718737 T/C	NR	292 (0.643)	146 (0.322)	16 (0.035)	0.196					
	AD	445 (0.712)	162 (0.259)	18 (0.029)	0.158	0.02	0.726 (0.554,0.951)	0.055	0.685 (0.495,0.947)	0.65 (0.282,1.5)
	ADe	303 (0.734)	97 (0.235)	13 (0.031)	0.149	0.023	0.709 (0.527,0.954)	0.054	0.655 (0.46,0.934)	0.671 (0.268,1.68)
	ADi	142 (0.67)	65 (0.307)	5 (0.024)	0.177	0.416	0.821 (0.51,1.321)	0.699	0.793 (0.452,1.393)	0.77 (0.175,3.397)
rs1422985 A/C	NR	293 (0.651)	141 (0.313)	16 (0.036)	0.192					
	AD	446 (0.714)	161 (0.258)	18 (0.029)	0.158	0.038	0.751 (0.573,0.984)	0.104	0.72 (0.52,0.997)	0.652 (0.283,1.504)
	ADe	301 (0.731)	98 (0.238)	13 (0.032)	0.15	0.051	0.745 (0.555,1.002)	0.128	0.707 (0.496,1.007)	0.674 (0.27,1.683)
	ADi	145 (0.681)	63 (0.296)	5 (0.023)	0.171	0.384	0.807 (0.498,1.308)	0.653	0.77 (0.434,1.367)	0.778 (0.176,3.445)

^a p-value of the type III effect of the genotype

p-values that are significant at the 0.05 level are written in bold type

Table S2. Summary result for all SNPs. Genotype and allele frequencies for each group and association test p-values are presented.

	NR				AD				allele test p.value	genotype test p.value	ADe				allele test p.value	genotype test p.value	AA	AB
	AA	AB	BB	MAF	AA	AB	BB	MAF			AA	AB	BB	MAF			AA	AB
rs11168017	205 (0.467)	202 (0.46)	32 (0.073)	0.303	257 (0.423)	289 (0.475)	62 (0.102)	0.34	0.166	0.375	170 (0.42)	198 (0.489)	37 (0.091)	0.336	0.216	0.397	87 (0.429)	91 (0.448)
rs2287772	253 (0.558)	161 (0.355)	39 (0.086)	0.264	364 (0.588)	219 (0.354)	36 (0.058)	0.235	0.132	0.322	250 (0.61)	134 (0.327)	26 (0.063)	0.227	0.12	0.298	114 (0.545)	85 (0.407)
rs17718511	293 (0.644)	144 (0.316)	18 (0.04)	0.198	439 (0.706)	165 (0.265)	18 (0.029)	0.162	0.026	0.08	296 (0.724)	100 (0.244)	13 (0.032)	0.154	0.033	0.091	143 (0.671)	65 (0.305)
rs4333302	236 (0.549)	156 (0.363)	38 (0.088)	0.27	342 (0.576)	213 (0.359)	39 (0.066)	0.245	0.243	0.505	232 (0.595)	128 (0.328)	30 (0.077)	0.241	0.292	0.556	110 (0.539)	85 (0.417)
rs1363725	118 (0.263)	215 (0.48)	115 (0.257)	0.497	155 (0.251)	308 (0.499)	154 (0.25)	0.499	0.662	0.442	106 (0.259)	207 (0.506)	96 (0.235)	0.488	0.483	0.244	59 (0.284)	101 (0.485)
rs17860502	291 (0.641)	145 (0.319)	18 (0.04)	0.199	437 (0.703)	167 (0.268)	18 (0.029)	0.163	0.024	0.075	298 (0.72)	103 (0.249)	13 (0.031)	0.156	0.031	0.086	139 (0.668)	64 (0.308)
rs7711953	135 (0.302)	209 (0.468)	103 (0.23)	0.464	177 (0.286)	309 (0.499)	133 (0.215)	0.464	0.599	0.621	114 (0.279)	206 (0.505)	88 (0.216)	0.468	0.502	0.397	63 (0.299)	103 (0.488)
rs4529181	249 (0.548)	164 (0.361)	41 (0.09)	0.271	361 (0.575)	227 (0.361)	40 (0.064)	0.244	0.179	0.403	247 (0.594)	139 (0.334)	30 (0.072)	0.239	0.149	0.351	114 (0.538)	88 (0.415)
KN0001820	295 (0.648)	141 (0.31)	19 (0.042)	0.197	441 (0.702)	168 (0.268)	19 (0.03)	0.164	0.045	0.124	298 (0.718)	103 (0.248)	14 (0.034)	0.158	0.059	0.139	143 (0.671)	65 (0.305)
KN0001823	268 (0.586)	171 (0.374)	18 (0.039)	0.226	334 (0.538)	252 (0.406)	35 (0.056)	0.259	0.348	0.577	225 (0.546)	165 (0.4)	22 (0.053)	0.254	0.446	0.739	109 (0.522)	87 (0.416)
KN0001824	260 (0.619)	142 (0.338)	18 (0.043)	0.212	416 (0.7)	162 (0.273)	16 (0.027)	0.163	0.007	0.023	290 (0.727)	98 (0.246)	11 (0.028)	0.15	0.005	0.014	126 (0.646)	64 (0.328)
rs6892205	143 (0.325)	212 (0.482)	85 (0.193)	0.434	196 (0.319)	297 (0.484)	121 (0.197)	0.439	0.401	0.676	129 (0.316)	197 (0.483)	82 (0.201)	0.442	0.325	0.615	67 (0.325)	100 (0.485)
rs2303064	108 (0.244)	229 (0.518)	105 (0.238)	0.497	155 (0.255)	307 (0.504)	147 (0.241)	0.493	0.384	0.684	105 (0.259)	203 (0.5)	98 (0.241)	0.491	0.406	0.705	50 (0.246)	104 (0.512)
rs1862446	380 (0.854)	64 (0.144)	1 (0.002)	0.074	529 (0.85)	90 (0.145)	3 (0.005)	0.077	0.528	0.303	349 (0.853)	57 (0.139)	3 (0.007)	0.077	0.788	0.175	180 (0.845)	33 (0.155)
rs2303067	146 (0.324)	223 (0.494)	82 (0.182)	0.429	199 (0.321)	302 (0.487)	119 (0.192)	0.435	0.383	0.613	131 (0.317)	202 (0.489)	80 (0.194)	0.438	0.279	0.556	68 (0.329)	100 (0.483)
rs880687	144 (0.324)	221 (0.498)	79 (0.178)	0.427	195 (0.32)	296 (0.485)	119 (0.195)	0.438	0.305	0.52	129 (0.318)	198 (0.488)	79 (0.195)	0.438	0.258	0.524	66 (0.324)	98 (0.48)
KN0001840	342 (0.753)	99 (0.218)	13 (0.029)	0.138	467 (0.75)	142 (0.228)	14 (0.022)	0.136	0.716	0.359	308 (0.751)	96 (0.234)	6 (0.015)	0.132	0.884	0.103	159 (0.746)	46 (0.216)
rs17718737	292 (0.643)	146 (0.322)	16 (0.035)	0.196	445 (0.712)	162 (0.259)	18 (0.029)	0.158	0.02	0.055	303 (0.734)	97 (0.235)	13 (0.031)	0.149	0.023	0.054	142 (0.67)	65 (0.307)
rs1422985	293 (0.651)	141 (0.313)	16 (0.036)	0.192	446 (0.714)	161 (0.258)	18 (0.029)	0.158	0.038	0.104	301 (0.731)	98 (0.238)	13 (0.032)	0.15	0.051	0.128	145 (0.681)	63 (0.296)
rs3777134	155 (0.344)	217 (0.482)	78 (0.173)	0.414	205 (0.333)	298 (0.485)	112 (0.182)	0.424	0.257	0.523	133 (0.325)	202 (0.494)	74 (0.181)	0.428	0.156	0.304	72 (0.35)	96 (0.466)
rs2303070	211 (0.483)	187 (0.428)	39 (0.089)	0.303	277 (0.452)	272 (0.444)	64 (0.104)	0.326	0.773	0.862	181 (0.446)	181 (0.446)	44 (0.108)	0.331	0.846	0.961	96 (0.464)	91 (0.44)