

Simultaneous detection of barrier- and immune-related gene variations in patients with atopic dermatitis by reverse blot hybridization assay

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Summary

Background. Hereditary factors are involved in the pathogenesis of atopic dermatitis (AD). However, AD-related gene variations are significantly different across ethnicities.

Aim. To identify mutations and single-nucleotide polymorphisms (SNPs) in barrier- or immune-related genes from Korean patients with AD and compare the variations with those observed in nonatopic healthy controls (HCs), and to use novel reverse blot hybridization assay (REBA) for AD-related gene variants.

Methods. We carried out REBA to simultaneously detect variations in genes related to barrier or immune function, namely, *FLG*, *SPINK5*, *KLK7*, *DEFB1*, *TNF α* , *KDR*, *FCER1A*, *IL4*, *IL5*, *IL5RA*, *IL9*, *IL10*, *IL12*, *IL12R*, *IL13* and *IL18*, from Korean patients with AD, and compared the variation to that in nonatopic healthy controls.

Results. The homozygous mutants of *KLK7* and *SPINK5*-2475, and the heterozygous mutants of *FLG* 3321delA, *SPINK5*-1156, *DEFB1*, *KDR*, *IL5RA*, *IL9* and *IL12RB1* were significantly more frequent in AD. It has been predicted that the larger the number of gene variants, the higher the odds ratio of AD prevalence; however, we did not find any significant correlation between the number of gene variants and AD severity.

Conclusion. Using REBA, we identified more genetic variants that can predict AD occurrence. We also verified that REBA can be used to easily and accurately detect multiple AD-related gene variants simultaneously. In addition, we identified a correlation between *KLK7* mutation and AD in Koreans, which is the first such report, to our knowledge.

Introduction

Atopic dermatitis (AD) is a common, chronically relapsing, inflammatory skin disease, characterized by pruritic eczematous skin lesions. Recently, there have

been many discoveries involving the genetic and epigenetic studies of AD. Two major biological pathways are responsible for AD aetiology: skin barrier function and innate/adaptive immune responses.^{1,2} The dysfunctional epidermal barrier and immune response reciprocally affect each other and thereby drive development of AD.² In terms of skin barrier function, variations in the filaggrin gene (*FLG*), serine protease inhibitor Kazal-type 5 gene (*SPINK5*) and serine protease Kallikrein 7 gene (*KLK7*) have been identified to play roles.^{3–5} In terms of innate immunity, beta

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defensin 1 (*DEFB1*), kinase insert domain receptor (*KDR*) and tumour necrosis factor alpha (*TNF α*) have been identified as influencing genes.^{6,7} In terms of adaptive immunity, Fc epsilon receptor 1 alpha (*FCER1A*) and interleukin 4 (*IL4*), *IL5*, *IL5RA*, *IL9*, *IL10*, *IL12*, *IL12R*, *IL13* and *IL18* have been reported to be influential.^{8–12} However, gene variation varies according to race, therefore it is important to detect all valuable variations in AD-related genes in patients from the same race in order to develop new clinical and pharmacological strategies for early identification and intervention in AD. We focused on barrier- and immune-related genes using reported data from Koreans and East Asians.^{13–18} For this study, we developed a simple and easy technique using reverse blot hybridization assay (REBA) to simultaneously detect multiple AD gene variants from a small amount of DNA, which can be applied to the clinical diagnosis and prediction of AD.

Methods

This study was approved by the Yonsei University Wonju Campus Institutional Review Board and was performed in accordance with their guidelines. All participants provided written informed consent.

Participants and blood sampling

This study was conducted with outpatients who visited the Departments of Dermatology at Yonsei University Wonju Severance Christian Hospital (Wonju, Korea) and the Department of Dermatology, Chung-Ang University Hospital (Seoul, Korea). Patients with AD aged 1–50 years were divided into three subgroups according to their Eczema Area and Severity Index (EASI): severe ($EASI \geq 25$), moderate ($EASI \geq 15$), and mild ($EASI < 15$) AD. The healthy control (HC) group comprised normal, healthy volunteers without any personal or family history of atopic diseases. To measure serum IgE and perform REBA, blood samples were collected from all participants.

Extraction of genomic DNA, multiplex PCR amplification and sequence analysis

Genomic DNA was extracted from blood samples (QIAamp DNA Mini Kit; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. To design specific primer molecules, the sequences of *KLK7*, *SPINK5*, *FLG*, *DEFB1*, *TNF α* , *KDR*, *FCER1A*, *IL4*, *IL5*, *IL5RA*, *IL9*, *IL10*, *IL12*, *IL12R*, *IL13* and

IL18 were compiled from the GenBank database of the National Center for Biotechnology Information (NCBI). Multiplex PCR was performed in accordance with our previous reports.^{19,20} The sequences of the PCR products were analysed. Direct sequencing of both strands of the PCR product was carried out on an ABI 3100 Genetic Analyzer by COSMO Genetech Co. Ltd. (Seoul, Korea) to verify the accuracy of REBA.

Reverse blot hybridization assay

The genus-specific oligonucleotide probes were designed based on the sequences of the aforementioned genes, obtained from the NCBI database (Bethesda, MD, USA). The REBA membrane was designed to contain a total of 70 probes [35 wild-type (WT) and 35 mutant-type (MT) probes] to detect these genes, and one control probe for colorimetric signal detection (Table S1). REBA was performed in accordance with our previous reports^{19, 20} (Fig. 1).

Statistical analysis

To compare between groups, we performed the two-sample *t*-test, one-way ANOVA, Wilcoxon rank sum test and χ^2 or Fisher exact test) as appropriate. Differences in variation in the aforementioned genes were identified via χ^2 test or Fisher exact test. We performed multiple comparisons using Bonferroni correction and the Scheffé method. We also performed a logistic regression test to calculate the odds ratio (95% CI) for AD prevalence. All statistical analyses were conducted using SAS (v9.4; SAS Inc., Cary, NC, USA) and $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of the participants

This study included 224 (44.5%) HCs and 279 (55.5%) patients with AD. Based on EASI, we classified the subjects as having either mild AD ($EASI < 15$) ($n = 207$ (76.7%)) or moderate/severe AD ($EASI \geq 15$) ($n = 65$ (23.3%)). There was a significant difference in mean age distribution between the AD group and the HC group ($P < 0.001$), as the HCs were adolescents or adults who voluntarily chose to participate. The age of the patients with moderate to severe AD was significantly higher than that of the patients with mild AD ($P = 0.01$) (Table 1). However, because this study was performed to detect any variations of specific genes

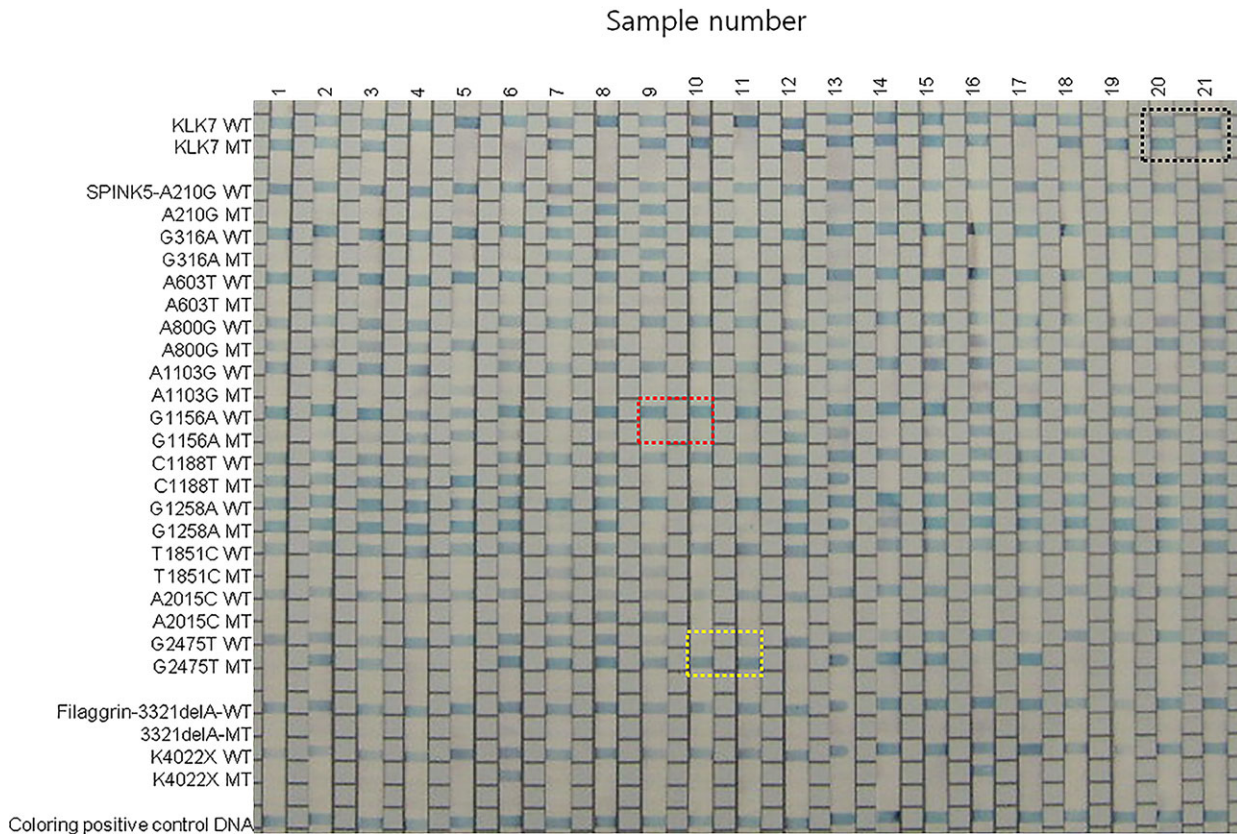


Figure 1 An example of reverse blot hybridization assay (REBA) and its interpretation. After REBA, the band pattern was read and interpreted. The presence of variation was determined either by lack of hybridization signal to the wild-type (WT) probes or by presence of hybridization signal to the mutant-type (MT) probes. Simultaneous signals to the WT probe and the MT probe were defined as the heterozygous type. The black dotted box indicates the heterozygous mutant of *KLK7*; the red dotted box indicates the WT of *SPINK5* 1156; the yellow dotted box indicates the homozygous mutant of *SPINK5* 2475.

composed of DNA from the patient's blood, the results would not be influenced by age.

Difference in gene variation between the groups

The homozygous mutant of *KLK7* was significantly more frequent in patients with AD compared with HCs ($P = 0.04$), but there was no significant difference in its frequency between the mild and moderate/severe AD groups ($P = 0.08$).

The heterozygous mutant of *FLG* 3321delA was significantly higher in the moderate/severe AD group compared with HCs ($P = 0.0384$).

The heterozygous mutant of *FLG* pK4022X was more frequent in patients with AD than in HCs, but the difference was not statistically significant ($P = 0.78$).

Both the heterozygous and homozygous mutants of *SPINK5*-1156 were significantly more frequent in patients with AD compared with HCs ($P = 0.00$ for

both), but there was no significant difference between the mild and the moderate/severe AD groups ($P = 0.81$ and $P = 1.0$) (Table S2).

The heterozygous mutant of *DEFB1*, rs5743399, was significantly higher in the patients with AD than in the HCs ($P < 0.002$), but there were no significant differences between the moderate/severe AD and the HC groups ($P = 1.0$) or between the mild and the moderate/severe AD groups ($P = 0.053$).

The heterozygous mutant rs2305948 of *KDR* was more frequent in both AD groups than in the HC group ($P = 0.03$), but the difference did not have a statistically significant association with AD severity ($P = 0.07$).

The heterozygous mutant of *IL5RA*, rs334809, was more frequent in patients with AD than in HCs ($P < 0.001$), and it was significantly more frequent in mild than in moderate/severe AD ($P = 0.01$) (Table S2).

The heterozygous mutant rs31563 of *IL9* was more frequent in the mild AD group than in the HC group

Table 1 Characteristics of participants (N = 503).

	HCs	Whole AD cohort	P*	Mild AD	Moderate/severe AD	P†	P‡	P§
Participants, n (%)	224 (44.5)	279 (55.5)		207 (76.7)	65 (23.3)			
Sex								
Male	112 (50)	163 (58.4)	0.0	118 (57.0)	45 (69.2)	0.02	0.85	0.02
Female	112 (50)	116 (41.6)		96 (43.0)	20 (30.8)			
Age, years	24.6 ± 22.5	13.7 ± 10.7	< 0.001	11.8 ± 10.1	20.3 ± 10.1	< 0.001	< 0.001	0.01
IgE, IU/mL								
Mean ± SD	—	963.7 ± 1219.8	—	808.5 ± 1071.6	1470.3 ± 1515.8	< 0.001	—	—
Median	—	461	—	416	805.5			
Range	—	2–5000	—	2–5000	4.08–5000			
EASI								
Mean ± SD	—	10.9 ± 10.2	—	6.1 ± 4.1	26.3 ± 8.9	< 0.001	—	—
Median	—	7.6	—	5.5	25			
Range	—	0–50.6	—	0–14.8	15–50.6			

AD, atopic dermatitis; EASI, Eczema Area and Severity Index; HC, healthy control. *Comparison between HCs and patients with AD; †comparison of the three groups by ANOVA or χ^2 test; ‡post hoc analysis between HC and mild AD by Bonferroni or Scheffé method; §post hoc analysis between HC and moderate/severe AD by Bonferroni or Scheffé method; ¶post hoc analysis between mild and moderate/severe AD by Bonferroni or Scheffé method.

($P = 0.00$), but there were no statistical differences between mild and moderate/severe AD or between moderate/severe AD and HCs ($P = 1.0$, $P = 0.36$, respectively).

For the *IL12RB1* polymorphism rs393548, the heterozygous mutant was more frequent in patients with AD than in HCs ($P = 0.0201$), but the difference between the three groups was not statistically significant ($P = 0.06$). The heterozygous mutant rs436857 of *IL12RB1* was more frequent in the AD than the HC group ($P = 0.01$) and was significantly more frequent in the mild AD than the HC group ($P = 0.03$). However, there were no significant differences between the mild and the moderate/severe AD groups ($P = 0.47$) or between the HCs and the moderate/severe AD groups ($P = 0.33$).

For the *IL13* polymorphism rs20541, the heterozygous mutation was less frequent in the whole cohort of patients with AD than in the HCs ($P = 0.03$) (Table S2), thus we did not include it for overlapping gene variations.

Overlapping gene variations in each group

When we compared the AD and HC groups, we found that 10 different variations (the 3' untranslated region AACC insertion 5874 of *KLK7*, along with *FLG* 3321delA, *SPINK5*-1156, *SPINK5*-2475, *DEFB1* rs5743399, *KDR* rs2305948, *IL5RA* rs334809, *IL9* rs31563, *IL12RB1* rs393548 and *IL12RB1* rs436857), were statistically significant. We also assessed the heterozygous mutant of *FLG* pK4022X, which was significant in a previous Korean study,²¹ and the homozygous mutant of *SPINK5*-1188, which shows a higher tendency in patients with AD. Thus, we eventually compared 12 SNPs according to overlap of gene variations between the HC, mild AD and moderate/severe AD groups.

The HCs showed fewer overlapping gene variants, while the AD group as a whole showed more frequent overlapping of gene variants compared with HCs. In the HC group, 143 (63.84%) HCs had ≤ 3 gene variants, whereas ≥ 4 gene variants were observed in 188 (67.38%) patients with AD.

An increase in the number of valid SNPs can help predict a higher probability of both mild and moderate/severe AD, but is not predictive of disease severity (Table 2). OR of AD occurrence showed significant increases when the number of variants was > 3 (adjusted OR = 3.75) and especially when it was > 5 (adjusted OR = 10.30) (Table 3).

Table 2 Overlap of gene variations in healthy control, mild atopic dermatitis and moderate/severe atopic dermatitis groups (N = 503).

Genes	Variations, n	HC, n (%)	Whole AD, n (%)	P*	Mild AD, n (%)	Moderate/ severe AD, n (%)	P†	P‡	P§	P¶
<i>FLG</i> 3321delA, <i>FLG</i>	≤ 1	43 (19.20)	4 (1.43)	< 0.001	2 (0.93)	2 (3.08)	< 0.001	< 0.001	< 0.001	0.71
pK4022X, <i>KLK7</i> , <i>SPINK5</i> -1156, <i>SPINK5</i> -1188,	2	50 (22.32)	26 (9.32)		16 (7.48)	10 (15.38)				
<i>SPINK5</i> -2475, <i>DEFB1</i>	3	50 (22.32)	61 (21.86)		48 (22.43)	13 (20.00)				
(rs5743399), <i>KDR</i>	4	42 (18.75)	61 (21.86)		51 (23.83)	10 (15.38)				
(rs2305948), <i>IL5RA</i>	5	20 (8.93)	62 (22.22)		48 (22.43)	14 (21.54)				
(rs334809), <i>IL9</i> (rs31563), <i>IL12RB1</i> (rs393548), <i>IL12RB1</i> (rs436857)	≥ 6	19 (8.48)	65 (23.30)		49 (22.90)	16 (24.62)				

AD, atopic dermatitis; HC, healthy control. *Comparison between HCs and whole AD cohort; †comparison of the three groups by χ^2 test; ‡*post hoc* analysis between HC and mild AD by Bonferroni method; §*post hoc* analysis between HC and moderate/severe AD by Bonferroni method; ¶*post hoc* analysis between mild and moderate/severe AD by Bonferroni method.

Table 3 Prevalence of atopic dermatitis.

Genes	Variations, n	Groups		OR (95% CI)	
		HC, n (%)	Whole AD, n (%)	Crude	Adjusted
<i>FLG</i> 3321delA, <i>FLG</i> pK4022X,	≤ 2	93 (41.52)	30 (10.75)	1 (reference)	1 (reference)
<i>KLK7</i> , <i>SPINK5</i> -1156,	3–4	92 (41.07)	122 (43.73)	4.11 (2.51–6.73)	3.75 (2.24–6.26)
<i>SPINK5</i> -1188, <i>SPINK5</i> -2475, <i>DEFB1</i> (rs5743399), <i>KDR</i> (rs2305948), <i>IL5RA</i> (rs334809), <i>IL9</i> (rs31563), <i>IL12RB1</i> (rs393548), <i>IL12RB1</i> (rs436857)	≥ 5	39 (17.41)	127 (45.52)	10.10 (5.85–17.43)	10.30 (5.79–18.32)

*Adjusted variables: age, sex.

Discussion

Recently, there have many reports involving genetic and epigenetic studies of AD, especially skin barrier function and innate/adaptive immune responses.² In the current study, we aimed to determine the known variations in the genes *FLG*,^{13–15,21} *SPINK5*,^{16–18} *KLK7*,^{5,22} *DEFB*,⁷ *TNF α* ,⁶ *KDR*,⁶ *FCER1A*,²³ *IL4*,¹¹ *IL5*,⁸ *IL5RA*,⁸ *IL9*,⁹ *IL10*,²⁴ *IL12*, *IL12R*,²⁵ *IL13*,^{10,11} and *IL18*,¹² using data reported for East Asian patients with AD.

The *KLK7* gene (encoding stratum corneum chymotryptic enzyme; SCCE) plays a crucial role in epidermal homeostasis by influencing cornification and desquamation.²³ The homozygous mutant of *KLK7* with only one AACC insertion has been shown to have a significant correlation with AD.^{5,22} In the current study, the homozygous mutant of *KLK7* was more frequent in patients with AD compared with HCs. The *KLK7* mutation can predict the occurrence but not the severity of AD.

FLG mutations are very different depending on race. Although pK4022X (5%) and c3321delA (1.8%) were

almost the only *FLG* mutations (about 7% of all mutations) detected in 1100 Korean patients with AD in a previous study,²¹ we found that the heterozygous mutant of *FLG* 3321delA was more frequent in AD than in HCs, and significantly more frequent in moderate/severe AD compared with HCs. However, we did not observe any difference in *FLG* pK4022X between patients with AD and HCs. We think that the small sample size was the main reason for the *FLG* pK4022X result observed in this study.

SPINK5 encodes lymphoepithelial Kazal-type-related inhibitor (LEKTI), which affects SCCE activity. Loss-of-function mutations in the *SPINK5* gene are related to defective cornification, AD, allergic rhinitis, asthma, markedly elevated serum IgE and hypereosinophilia.²⁶ In the current study, the heterozygous mutants of *SPINK5*-1156 and the homozygous mutant of *SPINK5*-2475 were more frequent in patients with AD, while the homozygous mutant of *SPINK5*-1188 also showed a strong tendency to AD occurrence.

Some antimicrobial peptides such as defensins are deficient in the skin of patients with AD. The β -defensins are expressed in a variety of epithelia and have

broad antimicrobial properties.²⁷ A previous Korean study showed that a single nucleotide polymorphism (SNP) in *DEFB1* was more frequent in moderate/severe AD than in mild AD.⁷ Our study showed that the heterozygous mutant of *DEFB1* (rs5743399) was significantly more frequent in the AD than the HC group.

KDR is the human gene encoding the kinase insert domain receptor known as vascular endothelial growth factor receptor 2 (VEGFR-2). Previous studies have demonstrated that V297I, which causes amino acid change in regions of the *KDR* gene, was significantly associated with the prevalence of atopy,²⁶ and *KDR* variation contributed to the sensitization to antigens and the development of atopy.^{6,28} Similar to previous reports, our results demonstrated that the frequency of the heterozygous mutant of *KDR* was significantly higher in patients with AD compared with HCs. Our study showed that SNPs in *IL5RA*, *IL9* and *IL12RB1* are significantly more frequent in AD than in HCs. In *IL13*, the heterozygous mutant rs20451 was more frequent in the HCs compared with AD, similar to results from a previous report.^{8–12}

Regarding the overlapping effect of the selected 12 gene variants, we demonstrated that overlapping gene variations could result in a higher prevalence of AD (Table 2). ORs for AD prevalence indicated that a higher likelihood of AD can be predicted by high-frequency gene variation on the REBA membrane (Table 3).

This study is also significant in that we simultaneously assessed a total of 35 gene variants using REBA with only a small amount of tissue sample. REBA, along with DNA chip assays, has been used not only to identify genotypes of human papilloma virus, mycobacteria and dermatophytes, but also to identify mutations within specific genes.^{19,20} This trial is significant because it is the first application of REBA to identify human disease-related gene variations, especially in patients with AD with complex gene variations.

Conclusion

We found that AD has a higher prevalence in patients who harbour more genetic variants related to AD. We also verified that REBA can be used to simultaneously, simply and accurately detect multiple variants in barrier- and immune response-related genes in patients with AD or in people with a predisposition to AD.

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What's already known about this topic?

- Variations in genes related to barrier or immune function have been reported in AD; however, AD-related gene variations are significantly different between ethnicities.
- There are few methods currently available to detect these gene variations simultaneously with only a small amount of blood.

What does this study add?

- We developed a novel assay, REBA, which can be applied to easily and accurately detect multiple AD-related gene variants simultaneously.
- Using REBA, we identified more genetic variants that can predict AD occurrence.
- Statistical analysis predicted that the larger the number of gene variants, the higher the OR of AD prevalence.

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Supporting Information

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

Table S1. Wild type (WT) and mutant type (MT) probes for reverse blot hybridization assay.

Table S2. Differences in gene mutations between the three groups (N = 503).

Genes	Position	Probes	Sequences (5'-3')
<i>KLK 7</i>	3'UTR AACC ins 5874	WT	ATTTAAACCTCATGCCCTGTTGAT
		MT	TATTTAAACCA A CCTCATGCCCTG
<i>SPINK5</i>	210-139A>G (His246Arg)	WT	TCTGTGTCC A CTTCTGAACATGCTAC
		MT (A → G)	TTCTGTGTCC G CTTCTGAACATG
	316G>A (Asp106Asn)	WT	AAAGGAGAAAGAGATGGGGATTTTATC
		MT (G → A)	AAAGGAGAAAGAA A TGGGGATTTTATC
	603-49A>T (Glu335Val)	WT	AGGACTGAGGATACTG A AAATATGATTGAGT
		MT (A → T)	GACTGAGGATACTG T AAATATGATTGAGT
	800A>G (Gln267Arg)	WT	CAGCAAGC A GCGTTTTTCAGAG
		MT (A → G)	CAGCAAGC G GCGTTTTTCA
	1103A>G (Asn368Ser)	WT	AGGAGCTTTGCA A TGAATATCGA
		MT (A → G)	CAGAGCTTTGC A GTGAATATCGA
	1156G>A (Asp386Asn)	WT	CCAGAGAGAACGATCCTATCCA
		MT (G → A),	GCACCAGAGAGAA A ATCCTATCCA
	1188T>C (His396His)	WT	GGAAAGTGCAT T GGCAACAC
		MT (T → C)	AAAGTGC A CGGCAACACCTG
	1258G>A (Glu420Lys)	WT	GAAAAAGAAGGAAGGT G AATCAAGAAACAA
		MT (G → A)	AGAAGGAAGGT A AATCAAGAAACAAAAG
	1851T>C (Ala617Ala)	WT	GAAAGAGCT T GAACCCAGAGCAA
		MT (T → C)	AAAGAGCCGAACCCAGAGCAA
	2015A>C (Gln672Arg)	WT	CCTTACAGTAATAGAA A CAGAAGGTTATCTGTA
		MT (A → C)	CCTTACAGTAATAGA A CCAGAAGGTTATCTGTA
	2475G>T (Glu825Asp)	WT	AAGAAAGAG G GAGGAAGACAGGAGCA
		MT (G → T)	AAGAAAGAT T GATGAAGACAGGAGCA
<i>FLG</i>	c3321delA	WT	ACTGGTCAGGGGGAAGGTCTG

pK4022X	MT	ACTGGTC GGGGGAAGGTCTG
	WT	CGTTTGGTAAAGATCATCCAAGGT
	MT (A → T)	CGTTTGGTTAAGATCATCCAAGGT

Table S1. Wild type (WT) and mutant type (MT) probes for reverse blot hybridization assay

Gene (SNP)	Position	Probe	Sequence (5'-3')
<i>DEFB1</i>	C2266T	WT	GAGACGGAGTGCGGTTTTGTC
rs5743399		MT (C → T)	GAGACGGAGTG T GGTTTTGT C AC
<i>DEFB1</i>	G1241T	WT	ATCTCTGTTTCTCT G TGTCTCTCTACTGTC
rs5743409		MT (G → T)	ATCTCTGTTTCTCT T TGTCTCTCTACTGTC
<i>IL 4</i>	C590T	WT	GAGAACATTGT C CCCCAGTGCT
rs2243250		MT (C → T)	GAGAACATTGT T CCCCAGTGCT G
<i>IL 4</i>	C33T	WT	TAAACTAATTG C CTCACATTGTC A CTGC
rs2070874		MT (C → T)	ATAAACTAATTG T CTCACATTGTC A CTG C A
<i>IL 5</i>	T4597A	WT	ATTTATTTATTTATTTGAAATGGAATCTCGCT
rs2522411		MT (T → A)	ATTTATTTA A TTATTTGAAATGGAATCTCGCT
<i>IL 5RA</i>	T26838A	WT	CCAACATCTAGCTCTATG T TATAAGCTAGAGATG
rs334809		MT(T → A)	CCAACATCTAGCTCTATG A TATAAGCTAGAGATG
<i>IL 9</i>	G4091A	WT	AAACTGATGTGAC G GGAGGGTAGT
rs31563		MT (G → A)	GAAACTGATGTGAC A GGAGGGTAGTA T
<i>IL 9R</i>	T6944C	WT	AACCCATCC C TACCCCTGTCAG
rs3093467		MT (T → C)	CCATCCCCA C CCCTGTCAG
<i>IL 10</i>	A592C	WT	AACCGCCTGT A CTGTAGGAAGCC
rs1800872		MT (A → C)	ACCGCCTGT C CTGTAGGAAG C
<i>IL 10</i>	T819C	WT	TGTACAGGTGATGTA A TATCTCTGTGCCT
rs1800871		MT(T → C)	TTGTACAGGTGATGTA A CATCTCTGTGC T

<i>IL 12RB1</i>	A17016T	WT	CTTTTTTTT C AGTCTTTTCTCCTTGCTCA
rs393548		MT(A → T)	CTTTTTTTT T GTCTTTTCTCCTTGCTCAG
<i>IL 12RB1</i>	C119T	WT	TCTACGTGGAT C CGATGGAGCC
rs436857		MT(C → T)	TACGTGGAT T CGATGGAGCCG
<i>IL 13</i>	T1055C	WT	TCAGTTGAA T TGTCCCTCGCG
rs20541		MT (T → C)	TCAGTTGAA C TGTCCCTCGC
<i>IL 13RA1</i>	G4763T	WT	GTGGAAGAAGTTTT G TTTCGGTCAGA
rs 6603441		MT (G → T)	GTGGAAGAAGTTTT T TTTCGGTCAGAG
<i>IL 13RA1</i>	C15529A	WT	GCAATAGGAATT C TTTGTTTCCACAAG
rs2247035		MT (C → A)	TGCAATAGGAATT A TTTGTTTCCACAAG
<i>IL 13RA1</i>	C52737G	WT	TTGGCCAAC C ACATCACGC
rs 2265753		MT (C → G)	TTGGCCAAC G ATCACGCA
<i>IL 13RA1</i>	T70223G	WT	CCTTTCAAAGCCATT T TAGGCTGTT
rs2254672		MT (T → G)	CTTTCAAAGCCATT G TAGGCTGTTAG
<i>IL 18</i>	C3549T	WT	AGCTGGGAGTTACGGGATGAAAGAT
rs795467		MT (C → T)	AGCTGGGAGTTATGGGATGAAAGAT
<i>TNF</i>	G308A	WT	TTTTGAGGGGCATG G GGAC
rs1800629		MT (G → A)	TTTTGAGGGGCATG A GGACG
<i>KDR</i>	17205G>A	WT	AACTATAGATGGT G TAACCCGGAGTGA
rs2305948		MT (G → A)	AACTATAGATGGT A TAACCCGGAGTGAC
<i>FCER1a</i>	-66T>C	WT	GGGTTAACCAGATATGATACAGAAAACATT
rs2251746		MT (T → C)	GGTTAACCAGATATGACACAGAAAACATTT

Table S2. Differences in gene mutations between the three groups ($N = 503$).

	HCs			Whole AD				Mild AD			Moderate/severe AD	
	(n = 224)			(n = 279)				(n = 214)				
Gene	WT	HeteroT	HomoT	WT	HeteroT	HomoT	P*	WT	HeteroT	HomoT	WT	HeteroT
KLK7	71	134	19	89	146	44	0.04	71	112	31	18	34
	(31.70%)	(59.82)	(8.48)	(31.90)	(52.33)	(15.77)		(33.18)	(52.34)	(14.49)	(27.69)	(52.31)
Filaggrin	223	1	0	269	10	0	0.03	206	8	0	63	2
3321delA	(99.55)	(0.45)	(0)	(96.42)	(3.58)	(0)		(96.26)	(3.74)	(0)	(96.92)	(3.08)
Filaggrin	217	7	0	269	10	0	0.78	209	5	0	60	5
K4022X	(96.88)	(3.13)	(0)	(96.42)	(3.58)	(0)		(97.66)	(2.34)	(0)	(92.31)	(7.69)
SPINK5_210	146	75	3	197	79	3	0.40	150	62	2	47	17
	(65.18)	(33.48)	(1.34)	(70.61)	(28.32)	(1.08)		(70.09)	(28.97)	(0.93)	(72.31)	(26.15)
SPINK5_316	147	69	8	200	73	6	0.29	153	58	3	47	15
	(65.63)	(30.80)	(3.57)	(71.68)	(26.16)	(2.15)		(71.50)	(27.10)	(1.40)	(72.31)	(23.08)
SPINK5_603	149	70	5	179	98	2	0.26	140	72	2	38	26
	(66.52)	(31.25)	(2.23)	(64.16)	(35.13)	(0.72)		(65.42)	(33.64)	(0.93)	(60.00)	(40.00)
SPINK5_800	81	113	30	83	147	49	0.22	64	112	38	19	35
	(36.16)	(50.45)	(13.39)	(29.75)	(52.69)	(17.56)		(29.91)	(52.34)	(17.76)	(29.23)	(53.85)
SPINK5_1103	205	11	8	237	27	15	0.07	182	24	8	55	3
	(91.52)	(4.91)	(3.57)	(84.95)	(9.68)	(5.38)		(85.05)	(11.21)	(3.74)	(84.62)	(4.62)

Genetic Association Study Results: SNPs and Associated Genes												
SNP ID	Gene	Chromosome	Position (kb)	Allele 1	Allele 2	Frequency (%)	P-value	Effect Size (beta)	95% CI (beta)	OR	95% CI (OR)	LD (r^2)
rs1234567	SPINK5	5	1156	A	G	0.15	0.001	0.12	(0.05, 0.19)	1.13	(1.02, 1.25)	0.85
				G	A	0.85						
rs2345678	SPINK5	5	1188	C	T	0.22	0.06	0.08	(-0.02, 0.18)	1.08	(0.98, 1.19)	0.72
				T	C	0.78						
rs3456789	SPINK5	5	1258	G	A	0.18	0.63	0.05	(-0.05, 0.15)	1.05	(0.95, 1.16)	0.68
				A	G	0.82						
rs4567890	SPINK5	5	1851	T	C	0.12	0.48	0.03	(-0.03, 0.09)	1.03	(0.93, 1.14)	0.55
				C	T	0.88						
rs5678901	SPINK5	5	2015	A	G	0.25	0.44	0.07	(-0.03, 0.17)	1.07	(0.97, 1.18)	0.61
				G	A	0.75						
rs6789012	SPINK5	5	2475	C	T	0.19	0.00	0.15	(0.08, 0.22)	1.16	(1.05, 1.28)	0.88
				T	C	0.81						
rs5743409	DEFB1	5	2475	A	G	0.15	0.11	0.09	(-0.01, 0.19)	1.09	(0.99, 1.20)	0.75
rs5743399	DEFB1	5	2475	G	A	0.85	0.00	0.12	(0.05, 0.19)	1.13	(1.02, 1.25)	0.85
rs1800629	TNF	6	2475	C	T	0.22	0.22	0.08	(-0.02, 0.18)	1.08	(0.98, 1.19)	0.72
rs2305948	KDR	12	2475	G	A	0.18	0.04	0.05	(-0.05, 0.15)	1.05	(0.95, 1.16)	0.68
rs2251746	FcER1a	1	2475	A	G	0.25	0.61	0.07	(-0.03, 0.17)	1.07	(0.97, 1.18)	0.61
rs2070874	IL-4	2	2475	T	C	0.12	0.28	0.03	(-0.03, 0.09)	1.03	(0.93, 1.14)	0.55
rs1234567	SPINK5	5	1156	A	G	0.15	0.00	0.12	(0.05, 0.19)	1.13	(1.02, 1.25)	0.85
rs2345678	SPINK5	5	1188	C	T	0.22	0.06	0.08	(-0.02, 0.18)	1.08	(0.98, 1.19)	0.72
rs3456789	SPINK5	5	1258	G	A	0.18	0.63	0.05	(-0.05, 0.15)	1.05	(0.95, 1.16)	0.68
rs4567890	SPINK5	5	1851	T	C	0.12	0.48	0.03	(-0.03, 0.09)	1.03	(0.93, 1.14)	0.55
rs5678901	SPINK5	5	2015	A	G	0.25	0.44	0.07	(-0.03, 0.17)	1.07	(0.97, 1.18)	0.61
rs6789012	SPINK5	5	2475	C	T	0.19	0.00	0.15	(0.08, 0.22)	1.16	(1.05, 1.28)	0.88
rs5743409	DEFB1	5	2475	A	G	0.15	0.11	0.09	(-0.01, 0.19)	1.09	(0.99, 1.20)	0.75
rs5743399	DEFB1	5	2475	G	A	0.85	0.00	0.12	(0.05, 0.19)	1.13	(1.02, 1.25)	0.85
rs1800629	TNF	6	2475	C	T	0.22	0.22	0.08	(-0.02, 0.18)	1.08	(0.98, 1.19)	0.72
rs2305948	KDR	12	2475	G	A	0.18	0.04	0.05	(-0.05, 0.15)	1.05	(0.95, 1.16)	0.68
rs2251746	FcER1a	1	2475	A	G	0.25	0.61	0.07	(-0.03, 0.17)	1.07	(0.97, 1.18)	0.61
rs2070874	IL-4	2	2475	T	C	0.12	0.28	0.03	(-0.03, 0.09)	1.03	(0.93, 1.14)	0.55

rs2243250	8	62	154	7	89	183	< 0.50	5	69	140	2	20
<i>IL-4</i>	(3.57)	(27.68)	(68.75)	(2.51)	(31.90)	(65.59)		(2.34)	(32.24)	(65.42)	(3.08)	(30.77)
rs2522411	58	68	98	79	84	116	0.82	62	63	89	17	21
<i>IL-5</i>	(25.89)	(30.36)	(43.75)	(28.32)	(30.11)	(41.58)		(28.97)	(29.44)	(41.59)	(26.15)	(32.31)
rs334809	124	100	0	59	220	0	< 0.00	37	177	0	22	43
<i>IL-5Ra</i>	(55.36)	(44.64)	(0)	(21.15)	(78.85)	(0)	1	(19.29)	(82.71)	(0)	(33.85)	(66.15)
rs31563	180	40	4	187	87	5	0.00	142	69	3	45	18
<i>IL-9</i>	(80.36)	(17.86)	(1.79)	(67.03)	(31.18)	(1.79)		(66.36)	(32.34)	(1.40)	(69.23)	(27.69)
rs3093467	25	105	94	23	136	120	0.54	18	102	94	5	34
<i>IL-9R</i>	(11.16)	(46.88)	(41.96)	(8.24)	(48.75)	(43.01)		(8.41)	(47.66)	(43.93)	(7.69)	(52.31)
rs1800872	114	110	0	133	146	0	0.47	101	113	0	32	33
<i>IL-10</i>	(50.89)	(49.11)	(0)	(47.67)	(52.33)	(0)		(47.20)	(52.80)	(0)	(49.23)	(50.77)
rs1800871	109	115	0	126	153	1	0.43	97	117	0	29	36
<i>IL-10</i>	(48.66)	(51.34)	(0)	(45.16)	(54.84)	(0.29)		(45.33)	(54.67)	(0)	(44.62)	(55.38)
rs393548	138	86	0	143	136	0	0.02	108	106	0	35	30
<i>IL-12RB1,2</i>	(61.61)	(38.39)	(0)	(51.25)	(48.75)	(0)		(50.47)	(49.53)	(0)	(53.85)	(46.15)
rs436857	150	69	5	150	121	8	0.01	114	96	4	36	25
<i>IL-12RB1,2</i>	(66.96)	(30.80)	(2.23)	(53.76)	(43.37)	(2.87)		(53.27)	(44.86)	(1.87)	(55.38)	(38.46)
rs20541	18	145	61	41	151	87	0.03	33	109	72	8	42
<i>IL-13</i>	(8.04)	(64.73)	(27.33)	(14.70)	(54.12)	(31.18)		(15.42)	(50.93)	(33.64)	(12.31)	(64.62)
rs6603441	63	55	106	87	57	135	0.51	66	46	102	21	11
<i>IL-13RA1</i>	(28.13)	(24.55)	(47.32)	(31.18)	(20.43)	(48.39)		(30.84)	(21.50)	(47.66)	(32.31)	(16.92)
rs2247035	102	59	63	121	70	88	0.71	91	56	67	30	14
<i>IL-13RA1</i>	(45.54)	(26.34)	(28.13)	(43.37)	(25.09)	(31.54)		(42.52)	(26.17)	(31.31)	(46.15)	(21.54)

rs2265753	100	56	68	113	63	103		85	50	79	28	13
							0.30					
<i>IL-13RA1</i>	(44.64)	(25.00)	(30.36)	(40.50)	(22.58)	(36.92)		(39.72)	(23.36)	(36.92)	(43.08)	(20.00)
rs2254672	104	54	66	125	52	102		94	44	76	31	8
							0.16					
<i>IL-13RA1</i>	(46.43)	(24.11)	(29.46)	(44.80)	(18.64)	(36.56)		(43.93)	(20.56)	(35.51)	(47.69)	(12.31)
rs795467	161	62	1	189	90	0		145	69	0	44	21
							0.24					
<i>IL-18</i>	(71.88)	(27.68)	(0.45)	(67.74)	(32.26)	(0)		(67.76)	(32.24)	(0)	(67.69)	(32.31)

AD, atopic dermatitis; HC, healthy control; WT, wild/wild type, HeteroT, heterozygous mutant,

HomoT, homozygous mutant. *Comparison between HCs and whole AD cohort; ‡comparison of the

three groups by χ^2 test; †*post hoc* analysis between HC and mild AD by Bonferroni method; §*post*

hoc analysis between HC and moderate/severe AD by Bonferroni method; ¶*post hoc* analysis

between mild and moderate/severe AD by Bonferroni method.