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Association of Genetic Polymorphisms with Atopic Dermatitis, Clinical Severity and Total IgE: A Replication and Extended Study

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Purpose: Atopic dermatitis (AD) is a common and chronic inflammatory skin disease affecting up to 20% of children and 3% of adults worldwide. Although previous reports including genome-wide association study (GWAS) approaches have identified several risk factors that appear to be associated with AD development, replication studies are lacking. In our current study, we replicated the associations between candidate susceptibility loci and AD. **Methods:** A total of 885 Korean subjects (425 AD patients and 460 unaffected controls) were genotyped for 17 single nucleotide polymorphisms (SNPs) from previous GWASs and meta-analyses of AD and from immune-related genes. **Results:** Several SNPs showed significant associations with AD in the case-control analysis (minimum $P = 0.005$ at rs17389644), suggesting that these polymorphisms may be related to this disease. In addition, several SNPs showed significant signals (minimum $P = 0.004$ at rs6473227) in severe AD compared to unaffected controls. In additional linear regression analysis, a few genotypes appeared to have potential effects on the SCORing AD (SCORAD) values (minimum $P = 0.003$ at rs13361382 on *TMEM232*) and immunoglobulin E (IgE) levels (minimum $P < 0.0001$ at rs4713555 near *HLA-DRB1* and *HLA-DQA1*) in AD patients. **Conclusions:** Our replication and extended study provide additional supporting information on the genetic associations (especially, variants in *TMEM232* and nearby to *IL21* and *HLA-DRB1/HLA-DQA1*) related to AD, its clinical severity and IgE involvement.

Key Words: Atopic dermatitis; single nucleotide polymorphism, severity

INTRODUCTION

Atopic dermatitis (AD) is a common, chronic and relapsing inflammatory skin disease characterized by itchiness, pruritis, erythema, scaling and papulovesicles.[1] AD is the most common skin disease worldwide, affecting up to 30% of children and 3% of adults.[2] A number of familial studies and twin studies have also demonstrated that AD is a highly heritable disease.[3]

AD belongs to a group of atopic diseases with common characteristics including allergen sensitization, epithelial barrier abnormalities and Type 2 immune responses.[4] It is widely assumed that atopy is related to AD in children because immunoglobulin E (IgE) plays a pivotal role in its pathogenesis. Hence, measurements of the total IgE levels have been used to evaluate allergic subjects in clinical practice and to determine the risk factors for severe AD.[5]

It is now known that AD is a complex disease affected by interactions among multiple genetic factors and environmental components. Several genome-wide association studies (GWASs) on AD in European, Chinese and Japanese populations have identified numerous potential susceptibility loci.[6-9] The first meta-analysis of AD in subjects of European descent identified 3 additional risk loci (rs479844 at 11q13.1, rs2164983 at 19p13.2, rs2897442 on *KIF3A* at 5q31).[10] More recently, a multi-ancestry meta-analysis in the largest number of samples studied to date from European, African, Japanese and Latino populations identified 31 susceptibility loci, including 10 new risk loci, for AD.[11] Studies of immune-related candidate genes (such as *IL23R*, *IL-*

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5RA, and *IL2*) have also reported the associations between genetic variants and immune-mediated diseases including atopic eczema.[12-14] For instance, a promoter single nucleotide polymorphism (SNP; rs2069762) of *IL2* was found to be significantly associated with allergic disease through the mediation of the type 1 T helper (Th1)/type 2 T helper (Th2) cells balance.[14] However, although they are important for substantiating previously identified GWAS and candidate genes, replication studies in different AD cohorts are lacking, and therefore the need for a comprehensive etiology of AD still remains.

The aim of our present study was to investigate whether the genetic variants previously identified by genome-wide and candidate genes studies would be replicated in our own AD cohort and to perform an extended analysis of the association of these variants with the clinical severity and IgE in AD.

MATERIALS AND METHODS

Subjects

AD subjects (n = 425) were recruited solely from our tertiary referral hospital, having been examined at the Childhood Asthma Atopy Center of Asan Medical Center in Seoul, Korea and diagnosed according to the criteria of Hanifin and Rajka.[15] The severity of AD was assessed in these cases using the SCOring AD (SCORAD) classification as follows: mild < 15, $15 \leq$ moderate < 40, severe ≥ 40.[16] Patients with moderate to severe AD were included in the AD group. A control group (n = 460) was also recruited from 9 primary schools and 16 kindergartens in the Seoul area. Subjects in the control group had no history of AD, food allergy, allergic rhinitis, asthma or any parental AD history, and all gave a negative skin prick test result.

The total serum IgE levels in the peripheral blood were measured in all subjects using a fluorescent enzyme immunoassay (ImmunoCAP system; Phadia AB, Uppsala, Sweden). Subjects were also tested for their sensitivity to the following 16 allergens: *Dermatophagoides pteronyssinus, Dermatophagoides farinae*, dog epithelium, cat epithelium, cockroach, grass, mixed tree pollen 1 and 2, *Alternaria, Aspergillus*, ragweed, mugwort, milk, egg white, peanut and soybean. A positive skin reaction was defined as a wheal size ≥ 3 mm after subtraction of a negative control. The Korean version of the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire was used.[17] Approval to conduct the study was obtained from the human ethics committees of Asan Medical Center and from the principals of the schools attended by the children (Institutional Review Board No. 2015-1031). Written informed consent was obtained from the parents of all children who participated.

SNP genotyping

Seventeen SNPs with a previously reported AD association were selected from a panel of immune-related genes, and from the results of previous GWASs and meta-analysis. More detailed information on each SNP was shown in Supplementary Table 1. DNA was isolated from the peripheral blood of our 885 study participants (425 AD patients and 460 unaffected controls) using the WizPrepTM gDNA Mini Kit (Wizbiosolutions, Seongnam, Korea). Genotyping of these blood samples was performed using the high-throughput Fluidigm EP1 system (Fluidigm, South San Francisco, CA, USA) with a Fluidigm SNP Type™ assay platform. According to the manufacturer's instructions, a specific target amplification reaction was used to increase the copy number of targeted genomic regions using Qiagen 2X Multiplex PCR Master Mix (Qiagen, Hilden, Germany). Following the amplification reactions on the dynamic array Integrated Fluidic Circuits (IFCs; Fluidigm), fluorescence intensities were measured with the EP1 reader. Genotypes were determined using the Fluidigm SNP Genotyping Analysis program. Visual inspections were performed for all SNP determinations, and call rate over 95% was applied to further SNP analysis.

Statistical analysis

Logistic and linear regression analyses using Statistical Analysis System software (SAS v9.4; SAS Inc., Cary, NC, USA) were performed to determine associations. For the multivariate analysis, age and sex were adjusted as covariates. A P value of < 0.05 was considered statistically significant. *In silico* analyses including the Signal Scan program (http://www-bimas.cit.nih.gov/molbio/signal/) and the SNP functional predictions program (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process = home) were performed to investigate the potential functions of any SNPs found to be significantly associated with AD. The SNPSpD program (http://gump.qimr.edu.au/general/daleN/SNPSpD) was used to correct for multiple testing errors.

RESULTS

Characteristics of the study subjects

A cohort comprising 425 AD patients and 460 unaffected controls was recruited. The mean age was lower in the AD patients than in the control subjects (6.95 ± 3.58 vs 11.84 ± 2.09, $P < 0.01$).

Table 1. Clinical profiles of the study subjects

Variables	AD	Unaffected control	*P* value
No. of subjects	425	460	-
Age (yr)	6.95 ± 3.58	11.84 ± 2.09	< 0.01
Sex (male, %)	54.8	41.7	-
SCORAD	69.66 ± 18.28	NA	-
Total IgE (IU/mL)	$732.3 \pm 1,483.0$	119.8 ± 224.0	< 0.01
Severity of AD			
Severe (≥ 40)	161	NA	-
Moderate (≥ 15, < 40)	264	NA	-

AD, atopic dermatitis; SCORAD, SCORing AD; IgE, immunoglobulin E; NA, not applicable.

Table 2. Association analysis between genetic polymorphisms and AD

SNP	(Nearby) gene, full name	Minor allele	MAF		Genetic model								
					Additive			Dominant			Recessive		
			AD cases (n = 425)	Controls (n = 460)	OR (95% CI)	*P*	$P_{cor.}$	OR (95% CI)	*P*	$P_{cor.}$	OR (95% CI)	*P*	$P_{cor.}$
rs6682925	*IL23R, Interleukin 23 receptor*	C	0.424	0.403	1.15 (0.89-1.48)	0.29	-	0.96 (0.66-1.41)	0.84	-	1.69 (1.06-2.70)	**0.03**	NS
rs7622183	*IL5RA, Interleukin 5 receptor subunit alpha*	T	0.464	0.517	0.74 (0.57-0.96)	**0.02**	NS	0.66 (0.44-0.99)	0.05	-	0.69 (0.45-1.06)	0.09	-
rs17454584	*(IL2, Interleukin 2)*	G	0.127	0.101	1.69 (1.14-2.50)	**0.009**	NS	1.78 (1.16-2.72)	**0.008**	NS	1.76 (0.34-9.06)	0.50	-
rs17389644	*(IL21, Interleukin 21)*	A	0.127	0.103	1.75 (1.17-2.61)	**0.006**	NS	1.86 (1.20-2.87)	**0.005**	NS	1.79 (0.34-9.35)	0.49	-
rs10214237	*(IL7R, Interleukin 7 receptor)*	C	0.173	0.179	1.00 (0.71-1.41)	0.99	-	1.00 (0.76-1.31)	0.99	-	1.00 (1.00-1.01)	0.97	-
rs13361382	*TMEM232, Transmembrane protein 232*	A	0.125	0.106	1.02 (0.67-1.55)	0.92	-	1.05 (0.67-1.64)	0.85	-	0.59 (0.11-3.21)	0.54	-
rs6871536	*RAD50, RAD50 double strand break repair protein/TH2LCRR, T helper type 2 locus control region associated RNA*	C	0.211	0.185	1.23 (0.89-1.71)	0.21	-	1.35 (0.92-1.98)	0.12	-	0.91 (0.34-2.40)	0.84	-
rs2569190	*CD14, CD14 molecule/TMCO6, Transmembrane and coiled-coil domains 6*	G	0.364	0.384	0.90 (0.68-1.18)	0.45	-	0.83 (0.57-1.19)	0.31	-	1.00 (0.57-1.74)	0.99	-
rs11741861	*IRGM, Immunity related GTPase M/ZNF300, Zinc finger protein 300*	G	0.376	0.365	1.26 (0.96-1.64)	0.09	-	1.19 (0.82-1.72)	0.36	-	1.72 (1.02-2.90)	**0.04**	NS
rs4713555	*(HLA-DRB1, Major histocompatibility complex class II DR beta 1/HLA-DQA1, Major histocompatibility complex class II DQ alpha 1)*	T	0.326	0.281	1.32 (1.00-1.76)	0.05	-	1.34 (0.93-1.92)	0.12	-	1.73 (0.91-3.28)	0.09	-
rs2275913	*IL17A, Interleukin 17A*	A	0.443	0.431	0.97 (0.75-1.27)	0.83	-	0.84 (0.57-1.25)	0.39	-	1.17 (0.73-1.87)	0.51	-
rs9357733	*EFHC1, EF-hand domain containing 1*	G	0.309	0.338	0.95 (0.73-1.25)	0.72	-	0.91 (0.63-1.32)	0.62	-	1.00 (0.57-1.75)	0.99	-
rs4271002	*NAT2, N-acethyltransferase 2*	C	0.221	0.223	0.98 (0.72-1.34)	0.91	-	1.03 (0.71-1.48)	0.89	-	0.75 (0.31-1.82)	0.53	-
rs6473227	*(ZBTB10, Zinc finger and BTB domain containing 10)*	A	0.358	0.427	0.72 (0.56-0.93)	**0.01**	NS	0.74 (0.51-1.07)	0.10	-	0.50 (0.30-0.83)	**0.008**	
rs4246905	*TNFSF15, TNF superfamily member 15*	T	0.329	0.330	0.94 (0.72-1.23)	0.66	-	0.96 (0.67-1.38)	0.82	-	0.84 (0.47-1.50)	0.56	-
rs2212434	*(EMSY, EMSY BRCA2 interacting transcriptional repressor)*	C	0.474	0.516	0.79 (0.61-1.01)	0.06	-	0.81 (0.53-1.22)	0.32	-	0.64 (0.42-0.98)	**0.04**	NS
rs2143950	*(PPP2R3C, Protein phosphatase 2 regulatory subunit B' gamma)*	T	0.403	0.353	1.30 (1.00-1.71)	0.05	-	1.51 (1.04-2.19)	**0.03**	NS	1.21 (0.71-2.06)	0.49	-

P value of logistic regression analysis by adjusting age and sex as covariates. Bold values indicate the statistical significance of $P < 0.05$. $P_{cor.}$ value corrected for multiple testing (effective number of correction = 15.98) using the SNPSpD program.

AD, atopic dermatitis; SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; NS, not significant.

The total IgE levels were significantly higher in the AD group (732.3 ± 1,483.0 IU/mL vs 119.8 ± 224.0 IU/mL, $P < 0.01$). The detailed characteristics of these subjects are summarized in Table 1.

Association analysis with AD development and severity

To conduct a replication study of previously described susceptible loci in AD, we selected a total of 17 SNPs (10 located at gene regions and 7 at nearby genes) that had been identified by previous GWASs and meta-analyses of AD and also from a panel of immune-related genes (Supplementary Table 2). These SNPs were then successfully genotyped in our AD and control populations and were found to be common variants in these subjects with a minor allele frequency (MAF) above 0.05.

A case-control analysis adjusted for age and sex as covariates was performed. The results replicated significant associations between 8 SNPs (rs6682925, rs7622183, rs17454584, rs17389644, rs11741861, rs6473227, rs2212434 and rs2143950) and AD development (minimum $P = 0.005$ at rs17389644 under a dominant model, Table 2). In addition, most of these significant SNPs found in the case-control analysis and an additional polymorphism, rs11741861, that is co-located at *IRGM* and *ZNF300* showed significant signals in the association analysis with AD severity (minimum $P = 0.004$ at rs6473227 under a dominant model, Table 3).

Association analysis with SCORAD and total IgE

Additional linear regression analysis using SCORAD and total IgE, as important indices of AD assessment, was performed. In SCORAD association analysis in AD patients (n = 189), 4 SNPs showed significant signals (rs13361382 on *TMEM232*, rs6871536 co-located at *RAD50* and *TH2LCRR*, rs11741861 co-located at *IRGM* and *ZNF300*, rs2275913 on *IL17A*; minimum $P = 0.003$ at rs13361382 under additive model, Table 4) even after correction for multiple testing ($P_{cor} = 0.04$). Analysis using the total IgE level revealed that rs17454584 near *IL2*, rs17389644 near *IL21* and rs4713555 near *HLA-DRB1* and *HLA-DQA1* had a potential association in AD patients (minimum $P < 0.0001$ at rs4713555 under a recessive model, $P_{cor} < 0.001$, Table 4).

Meta-analysis and *in silico* analyses of significant SNPs

Based on the SNPs identified from previous studies of AD, a meta-analysis was performed. Among the variants that were replicated with significance in our analysis, 4 SNPs (rs17389644, rs6473227, rs2212434 and rs2143950) appeared to have positive associations with AD (minimum overall $P = 8.85 \times 10^{-10}$ for

Table 3. Associations between genetic polymorphisms and AD severity

SNP	(Nearby) gene	Minor allele	MAF			AD severity					
			Control (n = 460)	AD cases		Control vs Moderate		Control vs Severe		Moderate vs Severe	
				Moderate (n = 264)	Severe (n = 161)	*P*	$P_{cor.}$	*P*	$P_{cor.}$	*P*	$P_{cor.}$
rs6682925	*IL23R*	C	0.403	0.407	0.453	0.33	-	0.19	-	0.21	-
rs7622183	*IL5RA*	T	0.517	0.471	0.451	0.05	-	0.15	-	0.66	-
rs17454584	*(IL2)*	G	0.101	0.128	0.124	**0.02**	NS	0.07	-	0.58	-
rs17389644	*(IL21)*	A	0.103	0.126	0.129	**0.02**	NS	**0.03**	NS	0.81	-
rs10214237	*(IL7R)*	C	0.179	0.183	0.156	0.98	-	0.99	-	0.26	-
rs13361382	*TMEM232*	A	0.106	0.118	0.137	0.97	-	0.48	-	0.23	-
rs6871536	*RAD50/TH2LCRR*	C	0.185	0.198	0.232	0.39	-	0.26	-	0.34	-
rs2569190	*CD14/TMCO6*	G	0.384	0.374	0.346	0.90	-	0.38	-	0.52	-
rs11741861	*IRGM/ZNF300*	G	0.365	0.362	0.399	0.60	-	**0.03**		0.45	-
rs4713555	*(HLA-DRB1/HLA-DQA1)*	T	0.281	0.322	0.331	0.12	-	0.18	-	0.90	-
rs2275913	*IL17A*	A	0.431	0.447	0.436	0.98	-	0.83	-	0.87	-
rs9357733	*EFHC1*	G	0.338	0.322	0.287	0.50	-	0.52	-	0.32	-
rs4271002	*NAT2*	C	0.223	0.232	0.203	0.68	-	0.41	-	0.28	-
rs6473227	*(ZBTB10)*	A	0.427	0.367	0.343	0.14	-	**0.004**	NS	0.58	-
rs4246905	*TNFSF15*	T	0.330	0.324	0.338	0.60	-	0.77	-	0.54	-
rs2212434	*(EMSY)*	C	0.516	0.500	0.430	0.29	-	**0.03**	NS	0.06	-
rs2143950	*(PPP2R3C)*	T	0.353	0.405	0.399	**0.01**	NS	0.47	-	0.86	-

P value of logistic analysis under additive model by adjusting age and sex as covariates. Bold values indicate the statistical significance of $P < 0.05$. $P_{cor.}$ value corrected for multiple testing (effective number of correction = 15.98) using the SNPSpD program.
AD, atopic dermatitis; SNP, single nucleotide polymorphism; MAF, minor allele frequency; NS, not significant.

Table 4. Associations of genetic polymorphisms with SCORAD and total IgE

SNP	SCORAD									Total IgE								
	Score			Genetic model						Score			Genetic model					
				Additive		Dominant		Recessive					Additive		Dominant		Recessive	
	M/M (No.)	M/m (No.)	m/m (No.)	*P*	$P_{cor.}$	*P*	P_{cor}	*P*	$P_{cor.}$	M/M (No.)	M/m (No.)	m/m (No.)	*P*	P_{cor}	*P*	P_{cor}	*P*	$P_{cor.}$
rs6682925	71.03 ± 9.02 (62)	69.77 ± 7.66 (83)	67.00 ± 10.34 (42)	0.21	-	0.34	-	0.26	-	715.1 ± 60.7 (139)	612.9 ± 45.1 (185)	624.7 ± 69.8 (80)	0.21	-	0.16	-	0.55	-
rs7622183	67.78 ± 9.14 (55)	68.55 ± 7.39 (86)	73.79 ± 10.88 (46)	0.14	-	0.42	-	0.10	-	707.9 ± 64.6 (120)	620.8 ± 44.3 (196)	665.4 ± 70.5 (89)	0.52	-	0.87	-	0.19	-
rs17454584	68.77 ± 5.88 (137)	71.99 ± 9.98 (52)	- (0)	0.21	-	0.21	-	-	-	610.6 ± 34.6 (312)	702.5 ± 71.7 (96)	2,439.5 ± 1,219.8 (4)	0.28	-	0.55	-	**0.01**	NS
rs17389644	68.77 ± 5.88 (137)	71.67 ± 10.04 (51)	- (0)	0.26	-	0.26	-	-	-	608.1 ± 34.7 (308)	695.0 ± 70.9 (96)	2,439.5 ± 1,219.8 (4)	0.30	-	0.58	-	**0.01**	NS
rs10214237	70.36 ± 6.17 (130)	67.66 ± 9.29 (53)	87.00 ± 50.23 (3)	0.91	-	0.56	-	0.09	-	610.9 ± 37.0 (273)	759.7 ± 69.4 (120)	332.6 ± 105.2 (10)	0.94	-	0.69	-	0.34	-
rs13361382	67.81 ± 5.71 (141)	76.52 ± 12.10 (40)	90.40 (1)	**0.003**	**0.04**	**0.004**	NS	0.22	-	565.3 ± 32.9 (295)	769.8 ± 81.1 (90)	821.5 ± 410.8 (4)	0.13	-	0.13	-	0.64	-
rs6871536	69.20 ± 6.45 (115)	68.77 ± 8.88 (60)	82.81 ± 26.19 (10)	0.21	-	0.62	-	**0.03**	NS	601.2 ± 38.3 (247)	718.6 ± 63.0 (130)	532.0 ± 125.4 (18)	0.39	-	0.22	-	0.63	-
rs2569190	69.85 ± 7.96 (77)	66.86 ± 7.43 (81)	77.01 ± 15.10 (26)	0.35	-	0.79	-	0.02	-	568.0 ± 44.5 (163)	792.4 ± 58.1 (186)	427.6 ± 58.2 (54)	0.75	-	0.57	-	0.14	-
rs11741861	73.50 ± 8.07 (83)	65.63 ± 7.29 (81)	69.97 ± 13.99 (25)	0.06	-	**0.009**	NS	0.96	-	552.7 ± 43.2 (164)	736.8 ± 54.3 (184)	654.9 ± 82.5 (63)	0.98	-	0.68	-	0.53	-
rs4713555	70.49 ± 8.03 (77)	69.66 ± 7.51 (86)	68.51 ± 14.29 (23)	0.61	-	0.66	-	0.70	-	679.7 ± 50.4 (182)	532.6 ± 39.6 (181)	1,081.6 ± 168.9 (41)	0.08	-	0.95	-	**< 0.0001**	**< 0.001**
rs2275913	64.62 ± 8.79 (54)	72.71 ± 7.71 (89)	71.47 ± 10.90 (43)	0.06	-	**0.01**	NS	0.66	-	569.9 ± 51.0 (125)	700.6 ± 50.4 (193)	600.9 ± 67.2 (80)	0.81	-	0.91	-	0.59	-
rs9357733	69.87 ± 6.99 (100)	71.53 ± 8.67 (68)	63.57 ± 14.58 (19)	0.47	-	0.99	-	0.11	-	615.4 ± 43.6 (199)	742.2 ± 59.4 (156)	447.1 ± 65.9 (46)	0.50	-	0.91	-	0.20	-
rs4271002	68.84 ± 6.62 (108)	70.78 ± 8.28 (73)	70.70 ± 26.72 (7)	0.43	-	0.40	-	0.87	-	683.7 ± 44.1 (240)	604.1 ± 48.8 (153)	482.8 ± 133.9 (13)	0.22	-	0.23	-	0.59	-
rs6473227	70.60 ± 8.38 (71)	68.74 ± 7.02 (96)	70.59 ± 15.40 (21)	0.74	-	0.59	-	0.89	-	643.2 ± 49.2 (171)	701.5 ± 51.3 (187)	503.5 ± 69.8 (52)	0.98	-	0.49	-	0.33	-
rs4246905	69.24 ± 7.51 (85)	69.53 ± 7.59 (84)	74.53 ± 17.57 (18)	0.35	-	0.59	-	0.24	-	704.1 ± 52.2 (182)	620.3 ± 46.4 (179)	420.9 ± 64.2 (43)	0.40	-	0.78	-	0.18	-
rs2212434	74.00 ± 11.03 (45)	67.88 ± 6.93 (96)	69.18 ± 10.55 (43)	0.19	-	0.07	-	0.77	-	742.2 ± 72.8 (104)	617.4 ± 42.8 (208)	590.8 ± 63.7 (86)	0.47	-	0.64	-	0.48	-
rs2143950	70.24 ± 8.71 (65)	68.87 ± 7.22 (91)	70.21 ± 12.41 (30)	0.87	-	0.73	-	0.89	-	599.2 ± 50.1 (143)	647.1 ± 45.9 (199)	812.5 ± 102.4 (63)	0.22	-	0.38	-	0.24	-

P value of linear regression analysis under additive model by adjusting age and sex as covariates. $P_{cor.}$ value corrected for multiple testing (effective number of correction = 15.98) using the SNPSpD program. M/M, M/m, and m/m indicate the homozygote of the major allele, heterozygote, and homozygote of the minor allele, respectively. Bold values indicate the statistical significance of $P < 0.05$.
AD, atopic dermatitis; SCORAD, SCORing AD; IgE, immunoglobulin E; NS, not significant; n, number of subjects.

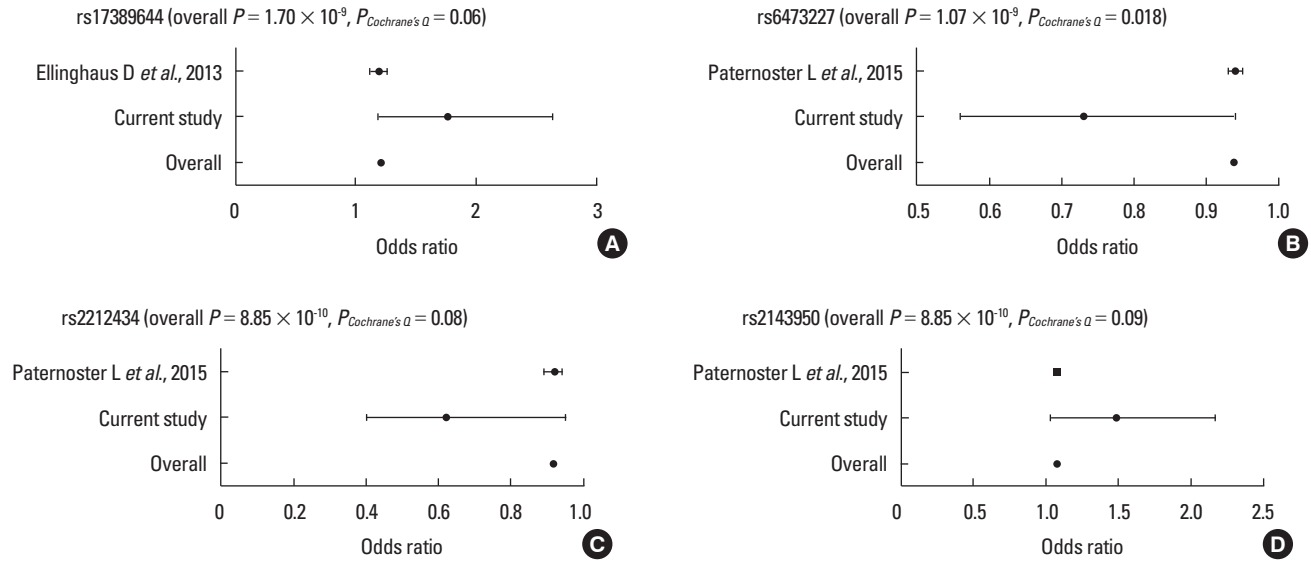

Figure. Result of meta-analysis. Plots of the replicated SNPs of (A) rs17389644, (B) rs6473227, (C) rs2212434 and (D) rs2143950 with significance in this study in relation to the published studies on AD are calculated using the software package PLINK. The association of each SNP with AD is evaluated through the fixed-effect meta-analysis *P* value. Additional significance of statistical heterogeneity measured using the χ^2-based Cochran's Q test is considered ($P_{Cochrane's\ Q} < 0.05$). Bold values indicate the statistical significance of $P < 0.05$. AD, atopic dermatitis; SNP, single nucleotide polymorphism.

rs2212434 and rs2143950, Figure). However, among the positive SNPs, only rs6473227 still retained positive significance in the Cochran's Q test established by the presence of heterogeneity ($P_{Cochrane's\ Q} = 0.018$).

We then investigated the potential functions of the significant SNPs using *in silico* analyses. Intriguingly, the minor C allele of rs6682925T>C in the regulatory upstream region was predicted to be a putative binding site for the GATA-1 and NF-E transcription factors, but not the sequence including the major T allele of this SNP (Supplementary Table 3). In the analysis to predict the functions of these SNPs, rs6682925, rs7622183 and rs6473227 were estimated to be potential exonic splicing enhancer (ESE) sites that were dependent on the major and minor alleles of each SNP (Supplementary Table 3).

DISCUSSION

AD is a complex disease caused by a combination of multiple genetic and interacting environmental factors. The identification of the genetic factors that contribute to AD is therefore important developing of new therapeutic and prevention strategies for this condition. Although GWASs and meta-analyses have been performed in a number of studies and identified many susceptibility loci for AD,[6-11] replication studies for these findings are still lacking and reliable markers for AD thus remain to be identified. In our current replication and additional analysis, several previously described genetic variants again showed significant associations with AD, with the top signals found at rs13361382 in *TMEM232*, rs17389644 near *IL21*, and rs4713555 near *HLA-DRB1/HLA-DQA1* for SCORAD, AD development and the total IgE level, respectively. These results suggest that these genetic variants may contribute to a predisposition for AD.

A recent multi-ancestry meta-analysis of the largest number of samples yet analyzed (21,399 AD cases and 95,464 controls) identified 31 susceptibility loci including 10 novel loci related to innate immune signaling and T cell function.[11] Variants of immune system genes (such as *IL23R, IL5RA,* and *IL2*) have also been reported as candidates for immune-mediated disorders.[12-14] However, many previous studies have not fully replicated the associations of SNPs with AD described by previous reports, including variants identified by GWASs or meta-analyses for reasons such as small sample sizes, differences in study designs and an inappropriate reliance on standard significance thresholds.[18,19] Hence, replication studies are needed to provide more accurate estimates of the association of genetic variations with the development of diseases and related phenotypes. In our current study, the rs6473227 and rs2143950 SNPs, which were previously highlighted in a meta-analysis,[11] were found to have a significant association with AD and its severity, respectively, suggesting that they could serve as genetic markers for AD.

In our present replication study in an AD and control population, 8 previously identified SNPs again showed significant associations with AD in our case-control analysis (minimum $P = 0.005$ at rs17389644 under dominant model, Table 2). However, several known SNPs (rs6682925, rs7622183, rs11741861, rs2212434, and rs2143950) showed only nominal association with AD. On the other hand, despite higher MAFs of a few SNPs (rs17454584, rs17389644, and rs2143950) in both moderate and severe AD

cases than those of controls, the significance of these SNPs was not increased depending on AD severity. This may be due to the insufficient sample size (in particular, the low number of severe AD cases) and/or involvement of other regulators; therefore, further replication and studies are needed.

The rs17389644 variant near the *IL21* locus showing the most significant signal among our AD subjects and notably was previously identified as a novel susceptibility locus for AD using genome-wide immune chip analysis and meta-analysis.[20] Several previous studies have reported that *IL21* contributes to the pathogenesis of allergic diseases. The serum *IL21* levels were found to increase during the acute exacerbation of asthma and to fall again after treatment.[21] In other recent studies, it was found that both *IL21* and *IL21R* expression was higher in acute skin lesions of AD patients,[22] and that *IL21* levels were are higher in adult AD patients than in unaffected controls.[23] Although little is known about the biological mechanism of *IL21* on IgE production in allergic diseases, additional recent studies have suggested that *IL21* may suppress serum IgE production, with possible involvement of Th2 cells that produce IL*4* and/or *IL13*.[24-26] Our current results have indicated that the minor A allele of rs17389644 near *IL21* is associated with higher levels of IgE in AD patients ($P = 0.01$ under recessive model, Table 4), again suggesting that this variant plays a role in altering the serum IgE level in AD with hope of further functional studies elsewhere. Additional functional studies that investigate the effects of the minor A allele of rs17389644 on *IL21* expression or on its trans-acting activity in relation to AD are needed to elucidate this mechanism.

There has been some conflicting evidence as to whether previously reported susceptibility markers on candidate genes are in fact associated with AD. For instance, some previous studies have reported that SNPs (rs2040704 on *RAD50*, rs3091307 co-located within *TH2LCRR* and near *RAD50*) at chromosome 5q31.1 are significantly associated with AD.[8,27] However, another study reported only a nominal association between this rs3091307 locus and an AD patient group and no association at all with the allergic type of AD.[28] In our result, the rs6871536 SNP co-located at the *TH2LCRR* and *RAD50* was found to have no association signal, with the exception of a nominal signal only in the association with SCORAD These conflicting results may be due to insufficient sample sizes (in particular, the low number of AD cases with SCORAD) and/or different genetic backgrounds among populations that have been used thus far. Further replication studies of large cohorts comprising different populations are warranted.

SCORAD is an important AD assessment index, and the IgE level is one of the central players in allergic diseases including AD. The serum total IgE level, as a useful endophenotype, is generally increased in patients with AD.[29] Different associations of genetic variants with the SCORAD and IgE parameters have been reported in AD cohorts: for instance, no associations have previously been reported between the *FLG* R501X mutation and the SCORAD or IgE levels in AD, whereas significant associations have been described between the -1112 C/T SNP of *IL13* with both SCORAD and IgE.[30] Our current findings also identified significant association signals for several SNPs with SCORAD (at rs13361382 on *TMEM232*) and IgE (at rs4713555 near *HLA-DRB1* and *HLA-DQA1*). Another recent study has also reported a potential association between genetic variations of *TMEM232* and AD in a Chinese population.[31] In addition, considering the association between SNPs located near to the *HLA-DRB1* locus and total IgE in asthma and the involvement between human leucocyte HLA class II and IgE responses,[32,33] rs4713555 may also play a role in regulating the IgE levels in AD. Hence, although further validation is needed, it is possible that SCORAD or IgE-associated SNPs could be predictive markers for AD.

We performed a meta-analysis of 4 SNPs (rs17389644, rs6473227, rs2212434, and rs2143950) that have been identified as risk loci for AD in previous genome-wide studies.[11,20] As shown in Figure, rs2212434 near to *EMSA* and rs2143950 near to *PPP2R3C* showed the most significant signal. However, when Cochran's Q test was used to assess heterogeneity of the estimated effect-sizes from the individual studies,[34] only rs6473227 near to *ZBTB10* still retained positive significance ($P_{Cochrane's\ Q} = 0.018$). There are few clues in the current literature that might explain the direct relationship between the potential SNP and allergic/immune responses. Therefore, further evaluation will be needed to identify whether these 2 SNPs could indeed be useful markers for AD.

To estimate the potential functions of the SNPs we evaluated in AD, we employed *in silico* analysis of the variants found to be significantly associated with this disorder. Using the Signal Scan program to identify the putative transcription factor's binding sites, the CTAT**C**A and CTAT**C** sequences including the 'C' allele of rs6682925 in the promoter region of *IL23R* were estimated to be putative binding elements for the GATA-1 and NF-E regulators. However, the major 'T' allele of this SNP did not show any results in this regard, suggesting that that the minor 'C' allele of rs6682925 may affect gene expression (Supplementary Table 3). In our additional search for potential ESE sites for splicing machinery using the SNP Function Prediction program, 3 SNPs (rs6682925, rs7622183, and rs6473227) were observed to have different binding scores for splicing factors depending on the major and minor alleles of each (Supplementary Table 3). In additional search of the expression quantitative trait loci (eQTL) based on the conditional eQTL analysis (https://eqtl.onderzoek.io/index.php?page = info), rs7622183 was also found to act as *cis*-acting eQTL, suggesting that this variant might act as a potential cis-regulator for the gene. In the case of functional relevance for rs2275913, this variant is positioned in the promoter region of *IL17A* and within a binding motif for the critical regulator of nuclear factor-activated T cells (NFAT), leading to a higher promoter activity and production of *IL17A* in the minor A allele than the major G allele of rs2275913.[35,36] In addition, *IL17A*

rs2275913 has been reported to be associated with the risk of several diseases including inflammatory diseases (for instance, rheumatoid arthritis), allergic asthma and rhinitis.[37,38] Therefore, further functional studies are required.

In conclusion, we have replicated the associations between previously identified SNPs and AD in our current study and performed extended analyses of these variants to better understand the clinical phenotypes of this condition. Although this study has limitations of small number of samples and lack of functional evaluation, our results suggest that several genetic variants may indeed be associated with AD and its clinical phenotypes (SCORAD and total IgE). However, further studies that include functional evaluations of the significantly associated SNPs identified herein will be needed.

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