

Letter to the Editor

Claudin-1 polymorphism modifies the effect of mold exposure on the development of atopic dermatitis and production of IgE

To the Editor:

Skin barrier dysfunction, which plays a key role in the initiation of atopic dermatitis (AD), may be associated with both genetic susceptibility and environmental risk factors for AD. Importantly, the skin of patients with AD may exhibit not only loss-of-function mutation in filaggrin¹ but also defects in tight junction (TJ) function,² all of which can contribute to decreased skin barrier function.

Consistent with these observations, disruption of the epidermal barrier (comprising the stratum corneum and epithelial TJs) is now recognized as a common feature in subjects with AD. A recent study showed that the nonlesional epithelium of patients with AD exhibited marked defects in TJs, possibly due to reduced levels of claudin-1 (CLDN1), a key adhesive protein.²

Elevated levels of fungal and bacterial components in house dust,³ or diversity of the mold components in the environment,⁴ may have protective effects to the development of allergic diseases. However, exposure to indoor mold is a risk factor for childhood asthma and allergy,⁵ and exposure to fungi in early

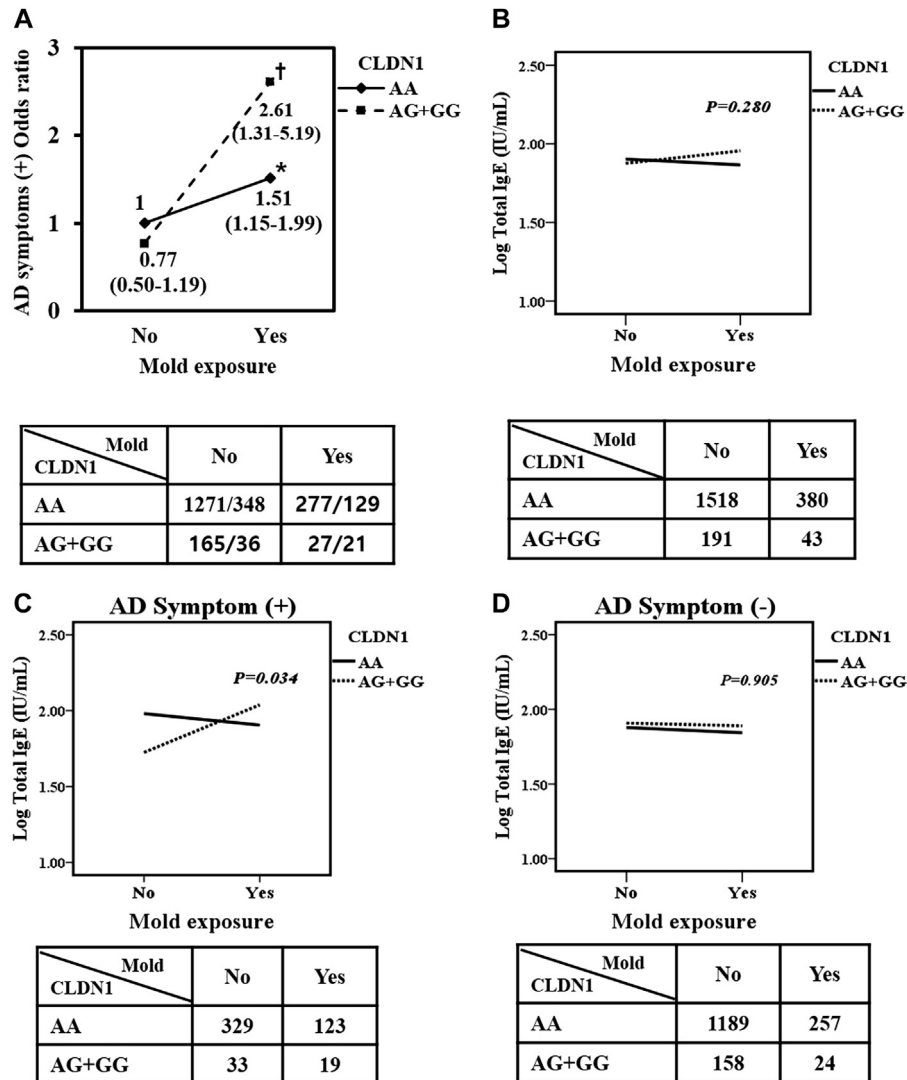


FIG 1. aOR for lifetime AD symptoms and total serum IgE levels according to mold exposure during the first year of life and CLDN1 polymorphism in the population-based study. **A**, Odds ratios for AD symptoms increased in children who had a history of mold exposure in infancy and the CLDN1 AG or GG genotype. There was significant additive interaction ($P = .008$), multiplicative interaction ($P = .149$), and trend ($P = .001$) after adjusting for age, sex, body mass index, family income, environmental tobacco smoke, and parental history of AD. * $P = .003$. † $P = .006$. **B**, The influence of the interaction between mold exposure and CLDN1 polymorphism on total IgE levels before stratification according to AD symptoms. **C** and **D**, The influence of the interaction between mold exposure during infancy and CLDN1 polymorphism on total IgE levels in patients with (+) or without (-) AD symptoms (P refers to the multiplicative interaction). Additive interaction P values were .035 for AD symptom (+) and .869 for AD symptom (-).

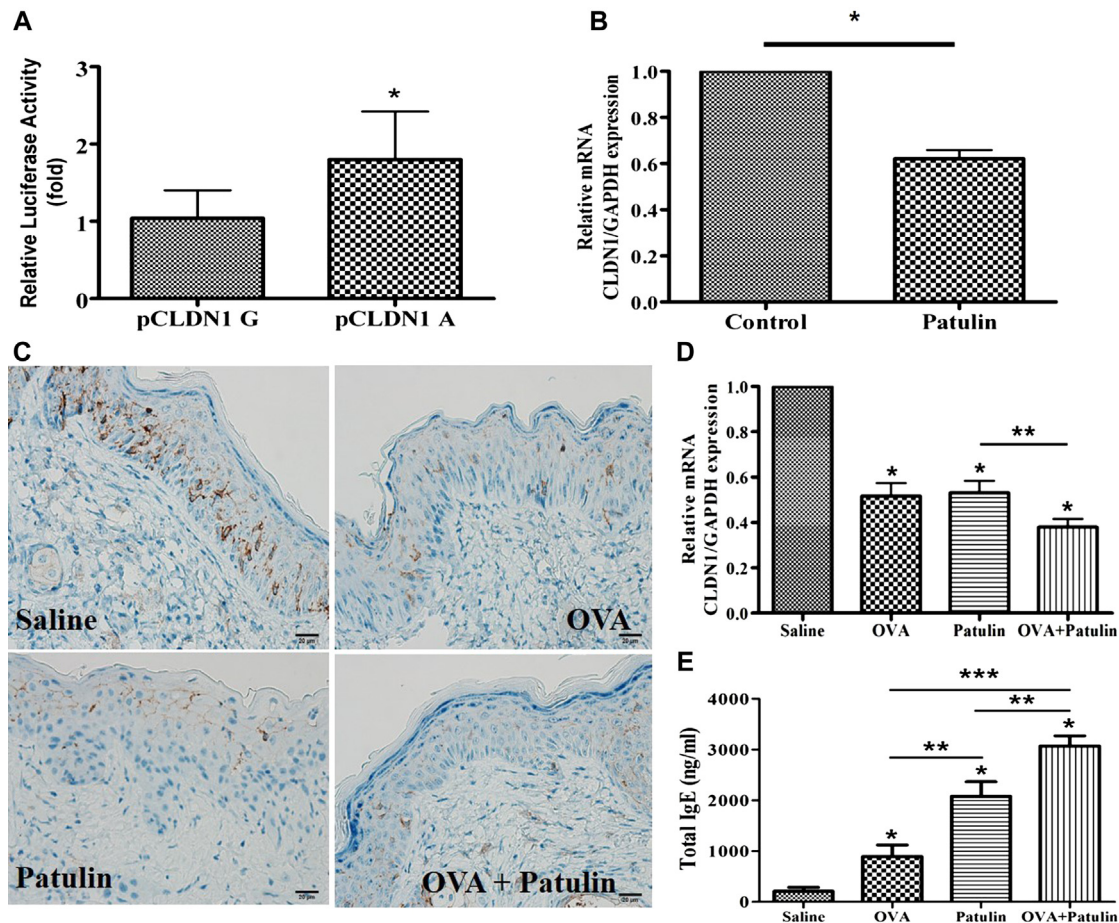


FIG 2. A, *CLDN1* promoter activities of the G and A alleles (rs9290929), determined by luciferase assay (**P* = .037). B, Decreased *CLDN1* expression after mycotoxin (patulin) treatment of human keratinocyte (**P* = .037). C, Immunohistochemical staining showed that *CLDN1* protein level was reduced in the skin of patulin-treated mice relative to the negative control (saline). D, In the mouse model, patulin and ovalbumin (OVA + patulin) decreased the cutaneous expression of *CLDN1*, as shown by real-time quantitative PCR. Data are shown as means $\pm$ SE from 2 samples in triplicate, relative to the control (**P* = .002, ***P* = .037). E, OVA + patulin treatment significantly increased serum IgE levels relative to the negative control group (saline) (*n* = 5 per group). Data are shown as means $\pm$ SE. *P* value was determined by using the nonparametric *t* test. **P* < .001, ***P* = .008, and ****P* < .001. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogen.

infancy is associated with an increased risk of AD.⁶ The reason for these discrepancies among the effects of various molds on allergy-related health outcomes remains largely unknown.

Several studies have focused on gene-environment interactions in the context of mold exposure. Our group's previous studies showed that mold exposure is a risk factor for the development of AD and allergic rhinitis in children with susceptibility.^{6,7} However, no study has yet examined the associations between *CLDN1* polymorphisms and mold exposure, or investigated whether such an association has a functional impact on the development of AD. In this study, we examined the combined effect of the rs9290929 polymorphism, located in the *CLDN1* promoter, and mold exposure during the first year of life on the development of AD symptoms and production of IgE and also investigated the response of cell and mouse skin tissue mediated by mycotoxin (patulin). The definition of AD, the characteristics of the study subjects, the total IgE levels, and the genotyping of the *CLDN1* polymorphism are described in the Methods section in this article's Online Repository at www.jacionline.org. Our results demonstrated that visible mold exposure during the first

year of life was an independent risk factor for lifetime AD symptoms (adjusted odds ratio [aOR], 1.60; 95% CI, 1.26-2.03) in the general population-based study (see Table E2 in this article's Online Repository at www.jacionline.org). *CLDN1* polymorphism was associated with susceptibility to AD in the hospital-based case-control study (aOR, 1.48; 95% CI, 1.04-2.12) (see Table E3 in this article's Online Repository at www.jacionline.org). We then analyzed the association between *CLDN1* polymorphism and mold exposure in the general population-based study. The odds of lifetime AD symptoms were higher among children with the AG or GG genotype (aOR, 2.61; 95% CI, 1.31-5.19; interaction *P* = .008) at rs9290929 who had been exposed to mold during infancy than among those with the AA genotype who had not been exposed to mold (Fig 1, A). We indicated that a regression analysis with log total IgE as the dependent variable and with mold exposure status, *CLDN1* polymorphism, and the interaction term was performed. No interaction between mold exposure during infancy and *CLDN1* polymorphism for total IgE levels was found (Fig 1, B). In the population with AD symptoms, mold exposure

during infancy was associated with elevated total IgE levels in children with the AG or GG genotype (no exposure, 1.72 ± 0.41 vs exposure, 2.04 ± 0.61 ; $P = .034$) but not in children with the AA genotype (no exposure, 1.98 ± 0.62 vs exposure, 1.90 ± 0.57 ; $P = .905$) (Fig 1, C and D).

The relative luciferase activity for the G promoter of rs9290929 was lower than that of the A promoter (1.04 ± 0.36 -fold vs 1.80 ± 0.62 -fold, $P = .037$) (Fig 2, A). After 24-hour stimulation with patulin in human skin keratinocytes, *CLDN1* mRNA expression was significantly reduced in patulin-treated cells relative to untreated controls ($P = .037$) (Fig 2, B). In addition, the expression of *CLDN1* by immunohistochemistry was markedly reduced in patulin-treated skin relative to the control skin of AD mice (Fig 2, C). *CLDN1* expression by real-time PCR was also significantly reduced in ovalbumin-treated skin and ovalbumin + patulin-treated skin ($P = .002$) relative to the control skin of AD mice (Fig 2, D). Epicutaneous serial treatments of patulin increased total serum IgE levels (patulin, 2080 ± 289.0 ng/mL; saline, 213.0 ± 74.96 ng/mL; $P < .001$) relative to the control group of AD mice (Fig 2, E). A detailed description is provided in the Methods section. In this study, we found that exposure to mold during the first year of life is associated with a risk of developing AD symptoms. In addition, mold exposure decreased *CLDN1* expression in both keratinocytes and a mouse model and increased total serum IgE.

The mechanisms underlying the association between exposure to mold and AD are poorly understood. Not only fungi, such as *Malassezia*, *Candida* spp, and *Trichophyton rubrum*, but also bacteria, especially *Staphylococcus aureus*, have been associated with AD.⁸ Autoreactive skin-homing T cells and IgE might play an important role in the chronic inflammation associated with AD, particularly in patients sensitized to molds such as *Malassezia sympodialis*.⁹ Also, mold exposure promotes AD development via mechanisms mediated by reactive oxygen species (ROS).¹⁰ Consistent with this, exposure to specific mold spores is associated with elevated intracellular levels of ROS in mice.¹¹ Defects of the skin barrier favor the penetration of microbes and allergens, such as mold.¹² These results suggest that lack of the *CLDN1* protein in the epidermis tends to increase the penetration of mycotoxin and production of ROS. Taken together, the present study might suggest that *CLDN1* expression could be reduced in subjects with AD on the specific polymorphism and mold exposure.

This study has some limitations, such as cross-sectional study, relatively small number of subjects, and questionnaire-based assessment of mold exposure. But the results were replicated in 2 different study groups and functional studies were investigated further through *in vivo* and *in vitro* studies.

This is the first study to identify a combined effect of the gene-environment interaction between *CLDN1* polymorphism and mold exposure on the development of AD and IgE production; furthermore, this effect was confirmed by both *in vitro* and *in vivo* experiments. In conclusion, mold exposure in early life is a risk factor for AD, and *CLDN1* polymorphism may influence the development of AD and IgE production in children.

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METHODS

Information regarding study subjects can be found in the article's main text.

Study subjects

Hospital-based case-control study. The study subjects ($n = 1686$) were recruited from a single tertiary referral hospital (Table E1). The study population comprised 1239 patients with AD and 447 normal control subjects with no history of AD or other allergic diseases, including food allergies, allergic rhinitis, and asthma.^{E1} Patients with AD were examined at the Childhood Asthma Atopy Center of Asan Medical Center in Seoul and diagnosed according to the criteria of Hanifin and Rajka.^{E2}

General population-based study. Study subjects ($n = 3361$) were recruited from 9 primary schools in Seoul and Jeongseup (Republic of Korea) between July and October 2008,^{E3} and 16 kindergartens in Seoul and the province of Gyeonggi-do between July and August 2010.^{E4} Approval to conduct the study was obtained from the human ethics committees of Hallym University and Asan Medical Center and from the principals of the schools attended by the children. Written informed consent was obtained from the parents of all children who participated in these studies.

Questionnaire data

Demographic factors, potential explanatory and confounding factors, and the prevalence of diseases and symptoms were assessed by a questionnaire issued to the parents. The Korean versions of the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire and an additional environmental questionnaire were used.^{E5}

The primary question related to the diagnosis of AD was, "Has your child ever been diagnosed as AD by a doctor?" In the general population-based study, lifetime AD symptoms and mold exposure were estimated using a questionnaire, on the basis of answers to the following questions: "Has your child ever had an itchy rash that came and went for at least 6 months?" and "Did you ever see mold in your home during your child's first year of life?" The questionnaire was completed by the parents or guardians of the subjects.

Total serum IgE and genotyping

Total serum IgE levels from peripheral blood in all subjects were measured using a fluorescent enzyme immunoassay (ImmunoCAP system; Phadia AB, Uppsala, Sweden). Genotyping for the rs9290929 single nucleotide polymorphism in *CLDN1* was performed in 384-well plates using TaqMan assay C_2057740_10 (ABI, Foster City, Calif).

Luciferase assay

To create the *CLDN1* promoter-luciferase reporter constructs, the upstream region (rs9290929) of *CLDN1* was amplified and cloned into the pGL3-Basic luciferase vector (Promega, Madison, Wis). Briefly, to compare the activities of *CLDN1* promoters containing the A and G alleles of SNP, 20-bp flanking of rs9290929 was synthesized as 2 complementary oligonucleotides (sense primer, A allele: CAACACAGTCAGTGTGTCGTTACATCAAAATCATTG GACTTC, G allele: CAACACAGTCAGTGTGTCGTTACGTCAAAAT CATTGGACTTC and antisense primer, A allele: TCGAGAAGTCCAA TGATTTTGATGTAACGACACACTGACTGTGTTGGTAC, G allele: TCGAG AAGTCCAATGATTTTGACGTAACGACACACTGACTGTGTTGGTAC). Restriction enzyme sites *KpnI* and *XhoI* were incorporated at the ends of the forward and reverse antisense primers, respectively. Each PCR product was gel-purified using an agarose gel purification kit (iNtRON, Daejeon, Korea) and ligated into the TA vector (Invitrogen, Carlsbad, Calif). The plasmid was then digested with *KpnI* and *XhoI* (Takara, Shuzo, Japan), and the *CLDN1* promoter fragment was ligated into similarly digested pGL3-Basic luc using T4 DNA ligase (iNtRON). All constructs were verified by direct sequencing. Plasmid DNA was prepared for subsequent transfection using a Plasmid Midi kit (Qiagen, Hilden, Germany).

Human skin keratinocytes (HaCaT cells) were cultured using Dulbecco modified Eagle medium containing 10% (v/v) FBS (Invitrogen, Carlsbad, Calif) and antibiotic-antimycotic (10×) in 5% CO₂ at 37°C.

HaCaT cells were cotransfected with a mixture of pGL3-Basic-luciferase (Promega) and pRL-TK-Renilla-luciferase (Promega) using the Lipofectamine 2000 reagent (Invitrogen). Plasmid DNA was incubated in Dulbecco modified Eagle medium without antibiotics. Standard 6-well tissue culture plates were used for all experiments. After 24 hours, cells were harvested after a medium change, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Both firefly and *Renilla* luciferase activities were measured using a luminometer (Victor3, PerkinElmer, Waltham, Mass); *Renilla* luciferase activity was used as a control for transfection efficiency. *Promoter activity* was defined as the ratio of firefly luciferase activity to *Renilla* luciferase activity. Each experiment was run at least in triplicate.

Real-time quantitative PCR

HaCaT cells (3×10^5 cells/well) were seeded in 6-well plates and cultured overnight, followed by stimulation with patulin (500 ng/mL, Sigma-Aldrich, St Louis) for 24 hours. Total RNA was isolated using the TRIzol Reagent (Invitrogen, Grand Island, NY). Extracted RNA was stored at -80°C . Concentration and integrity were monitored using a NanoDrop (NanoDrop Technologies) spectrophotometer. cDNA was synthesized from 4 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the kit protocol.

TaqMan primers and probes for *CLDN1* were derived from the commercially available TaqMan Gene Expression Assays Products (Hs00221623_m1 for *CLDN1*). Real-time RT-PCR was performed in 384-well plates on an ABI PRISM 7900 HT Sequence Detection System (ABI). Amplification reactions (20 μL) containing 1 μL of template cDNA were carried out in duplicate according to the manufacturer's protocol (Applied Biosystems). Quantitation was performed by using the standard-curve method: mean C_T values were calculated for duplicates, and the concentration of test samples was interpolated on the basis of standard curves. All samples were normalized by dividing the level of the test mRNA by the level of Glyceraldehyde 3-phosphate dehydrogenase mRNA in the same cDNA sample.

Development of the AD mouse model

We developed an AD model using hairless mice, as described previously.^{E6} Briefly, to sensitize the skin, each mouse was subjected to three 1-week exposures to a mycotoxin (patulin, 100 $\mu\text{g}/100 \mu\text{L}$) patch, separated by 2-week intervals. Patulin (100 μg , Sigma-Aldrich) was applied using a gauze ($1 \times 1 \text{ cm}$) attached to the dorsum skin with Tegaderm (3M Health Care, St Paul, Minn). The patulin patch was replaced once per week. The mice were euthanized on the final day of the schedule after the third sensitization. The negative control group ($n = 5$) received sterile saline (100 μL), applied using the same procedure.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded tissue blocks of skins were cut (3 μm thickness) and mounted on coated glass slides. Immunohistochemistry was carried out using a Benchmark XT automated slide preparation system (Ventana Medical systems, Inc, Tucson, Ariz). Deparaffinization, epitope retrieval, and immunostaining were performed using cell conditioning solution (CC1) and the BMK ultraVIEW diaminobenzidine (DAB) detection system (Ventana Medical Systems). Skin sections were stained with antibody against CLDN1 (1:50; rabbit polyclonal, ab15098-500, Abcam, Mass), followed by incubation with horseradish peroxidase-conjugated polymer-labeled anti-rabbit antibody (K4003, Dako, Carpinteria, Calif). Positive signals were amplified using ultra-VIEW copper, and sections were counterstained with hematoxylin and blueing reagent.

Measurement of serum IgE

Sera were obtained from blood taken during exsanguination of mice after airway measurements on the final day of the schedule. For the detection of total IgE, 96-well plates were first coated overnight with purified anti-mouse IgE (0.5 µg in 100 µL carbonate-bicarbonate buffer; PharMingen, San Diego, Calif). The remaining binding sites were blocked, and plates were incubated with 100 µL of diluted serum (1:10 dilution in carbonate/bicarbonate buffer) per well. After washing, 100 µL of TMB solution (Sigma Chemical Co.) was added per well, followed by incubation and washing. Finally, optical density was measured at 450 nm.

Statistical analysis

Statistical analyses were performed using SPSS (version 21.0). Logistic regression analyses were performed to identify independent risk factors for AD. In the multivariate analyses, age, sex, and body mass index were adjusted as personal factors; parental AD was adjusted as a familial factor; and family income and exposure to environmental tobacco smoke were adjusted as socioeconomic factors. Continuous variables are expressed as means $\pm$ SD. Prevalence rates are presented along with 95% CIs. We evaluated the interaction for odds ratio and total IgE using a generalized linear model, a general term that applies to a broad range of statistical models. We chose the dominant model for the gene-environment interaction because the dominant model (AA vs Aa + aa) explained the risk of AD more accurately than did

either the additive model or the recessive model without environmental factors (data not shown). Data from the *in vivo* model are represented as means $\pm$ SEM. Each group was compared using the Mann-Whitney test. For all analyses, a *P* value of less than .05 was considered statistically significant.

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TABLE E1. Subject characteristics

Characteristic	Hospital-based study		Population-based study
	Case	Control	
Number	1239	447	3361
Responders to questionnaire, n (%)	NA	NA	3299 (98.2)
Age (y)	5.18 ± 3.86	9.32 ± 2.47	8.75 ± 2.90
Sex (M/F)	717/522	231/216	1719/1588
BMI*	NA	NA	17.89 ± 3.17
Lifetime AD symptoms	NA	NA	709 of 2349 (23.2%)
Parental history of allergic diseases	NA	NA	1068 of 3056 (34.9%)
Parental history of asthma	NA	NA	117 of 2911 (4.0%)
Parental history of AR	NA	NA	953 of 3033 (31.4%)
Parental history of AD	NA	NA	237 of 2933 (8.1%)
Environmental tobacco smoking	NA	NA	1342 of 3095 (43.4%)
Educational level of mother			
≤High school graduate	NA	NA	1242 of 3039 (40.9%)
≥University graduate	NA	NA	1797 of 3039 (59.1%)
Economic state (monthly income)			
Low (<3 million won)	NA	NA	1282 of 2977 (43.1%)
Middle (3-5 million won)	NA	NA	1280 of 2977 (43.0%)
High (≥5 million won)	NA	NA	415 of 2977 (13.9%)
Mold exposure (visible mold)			
During infancy	NA	NA	564 of 2932 (19.2%)
During the previous year	NA	NA	635 of 2930 (21.7%)
Biomarker			
Total IgE (IU/mL)	512.62 ± 1095.99	57.70 ± 124.34	204.08 ± 394.16
Claudin-1			
Frequency of rs9290929			
A	0.933	0.953	0.946
G	0.067	0.047	0.054

Values are mean ± SD or proportion.

AR, Allergic rhinitis; F, female; M, male; NA, not available.

*BMI (body mass index): weight (kg)/height² (m).

TABLE E2. Risk factors for AD in the population-based study

Risk factors	AD Sx ever*	
	aOR (95% CI)†	P value
Demographic factors		
Sex: male	1.11 (0.91-1.34)	.299
BMI	1.03 (0.99-1.06)	.142
Age	0.92 (0.89-0.96)	<.001
Higher economic state (monthly income ≥5 million won)	1.05 (0.78-1.41)	.747
Maternal education (<university graduate)	0.90 (0.73-1.12)	.338
Genetic factors		
Parental history of allergic disease	1.70 (1.41-2.06)‡	<.001
Parental history of AD	2.82 (2.09-3.79)‡	<.001
Parental history of AR	1.31 (1.06-1.61)	.013
Parental history of asthma	1.36 (0.87-2.13)	.171
Environmental factors		
Environmental tobacco smoking	1.06 (0.87-1.28)	.570
Pet ownership in the past 12 mo	0.89 (0.63-1.25)	.490
Pet ownership during infancy	1.42 (0.94-2.14)	.098
Mold exposure during infancy (visible mold)	1.60 (1.26-2.03)	<.001
Biomarkers		
Total IgE (>200.0 IU/mL)	1.20 (0.94-1.53)	.138

Data were calculated by logistic regression multivariate analysis. Risk factor analysis was not available for the subjects of the hospital-based study.

AR, Allergic rhinitis; BMI, body mass index; OR, odds ratio.

*AD Sx ever: AD symptoms in the lifetime.

†OR adjusted by age, sex, BMI, family income, environmental tobacco smoke, and parental history of atopic dermatitis.

‡OR adjusted by age, sex, BMI, family income, and environmental tobacco smoke.

TABLE E3. aORs for AD according to *CLDN1* polymorphism in subjects with AD and controls, investigated as part of the hospital-based case-control and population-based studies

<i>CLDN1</i>	Hospital-based study		Population-based study	
	N (–/+)	AD	N (–/+)	AD Sx ever
		aOR (95% CI)		aOR (95% CI)
AA	405/1074	1 (reference)	1649/520	1 (reference)
AG + GG	42/165	1.48 (1.04-2.12)*	211/62	0.93 (0.69-1.26)

Data were calculated by logistic regression multivariate analysis.

Sx, Symptoms.

**P* = .031.