

A synonymous variation in protease-activated receptor-2 is associated with atopy in Korean children

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Background: Atopic diseases are the most common chronic diseases of childhood, and the genetics of atopy are complex and heterogeneous. Protease-activated receptor-2 (PAR-2) is involved in various inflammatory diseases, but the association of PAR-2 with allergic diseases remains unclear.

Objective: To examine the contribution of genetic variation of PAR-2 to atopic phenotypes in the Korean childhood cohort.

Methods: We identified PAR-2 variations in a Korean population and conducted association analyses by using 316 unrelated atopic and 210 nonatopic subjects. We analyzed serum IgE and total eosinophil count levels and examined PAR-2 mRNA and protein expression levels.

Results: In the case-control association analysis, atopy was significantly associated with a single c.621C>T (p.I207I, rs631465) polymorphism of PAR-2 ($P = .001$, odds ratio = 1.95). Subjects with the c.621T risk allele had significantly higher serum IgE ($P = .004$) and total eosinophil count ($P = .03$) levels. Moreover, the positive association of c.621T was reproduced in the replication study ($P = .01$, joint P value of the replication < .001). An *in silico* analysis of RNA secondary structure prediction revealed that the C to T conversion at c.621 greatly increased predicted PAR-2 mRNA stability. This was also confirmed by an *in vitro* assay for mRNA stability. Furthermore, following an *in vivo* approach on gene expression in PBMCs showed that the expression levels of PAR-2 mRNA and protein

in subjects with the c.621CT or TT genotype were significantly higher than in those with the c.621CC genotype.

Conclusions: These results indicate that the synonymous c.621C>T polymorphism in PAR-2 might be associated with the risk of atopy, potentially by altering PAR-2 gene expression. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Protease-activated receptor-2, SNP, association study, atopy, mRNA stability

Atopy is a well-defined immune phenotype that is reported to be a risk factor for asthma and other allergic diseases.¹ The prevalence of atopy-related diseases in childhood has risen since the latter part of the 20th century.^{2,3} The pathogenesis and etiology of atopy are very complex and not completely understood. Although environmental factors may contribute to atopy,⁴ several family studies have indicated that genetic factors influence the risk of atopy.⁵⁻⁷ Little is known about the specific genes associated with susceptibility to atopy, but genes identified to be associated with atopy thus far tend to be involved in allergic responses or the immune system.⁸⁻¹⁰

Protease-activated receptors (PARs) are G protein-coupled 7-transmembrane domain receptors that can be activated by serine proteases via proteolytic cleavage.¹¹ Recent evidence suggests that the activation of PARs plays an important role in the inflammatory response and immunological reactions.^{12,13} PARs are widely expressed in immune and inflammatory cells. Allergen-derived exogenous proteases as well as endogenous proteases can react with cell surface PARs, which can then modulate the secretion of inflammatory mediators and further amplify the response. To date, 4 human PARs (PAR1-4) have been identified and cloned. PAR-1, -3, and -4 are activated by thrombin, and PAR-2 is activated by trypsin and tryptase.¹¹ PAR-2 can also be activated by a range of exogenous proteases, including some contained within important airborne allergens, such as house dust mite and the German cockroach. Thus, PAR-2 appears to play a critical role in the pathogenesis of allergic responses.¹⁴⁻¹⁶

The human PAR-2 gene is located in 5q13, tightly clustered with PAR-1 and -3 while PAR-4 is located separately at 19p12.¹⁷ PAR-2 is expressed in human lung epithelial cells, airway smooth muscle,¹⁸ and fibroblasts¹⁹ as well as in vascular smooth muscle and endothelial cells.²⁰ PAR-2 activation on airway epithelial cells causes the release of prostaglandin E₂,¹⁸ GM-CSF, eotaxin,²¹ and matrix metalloproteinase-9.²² PAR-2 expression is increased in respiratory epithelial cells of patients with asthma,²³ but the physiological role of PAR-2 in the airways remains unclear. In mice, overexpression of PAR-2 increases

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Abbreviations used

ECP: Eosinophil cationic protein
 GADPH: Glyceraldehyde-3-phosphate dehydrogenase
 PARs: Protease-activated receptors
 SNP: Single nucleotide polymorphism
 TEC: Total eosinophil count

eosinophil infiltration and airway hyperresponsiveness whereas absence of PAR-2 expression has the opposite effect.²⁴ It has recently been suggested that exogenous PAR-2 activation enhances allergen-mediated airway hyperresponsiveness and airway inflammation through distinct mechanisms.²⁵ However, there is also evidence that PAR-2 may play a protective role in the airways. PAR-2 activation induces bronchodilation^{18,26,27} and attenuates antigen-induced bronchoconstriction in animal models.²⁸

We conducted an association study of PAR-2 polymorphisms with susceptibility to atopy in a Korean childhood cohort. Functional effects of the PAR-2 variation were also examined. We provide evidence that the c.621C>T polymorphism (p.I207I, rs631465) in the coding region of *PAR-2* gene is associated with the increased risk of atopy.

METHODS**Subjects**

In the initial exploratory stage of the study, 316 atopic children were recruited from the Severance Hospital of Yonsei University, Seoul, Korea. Diagnosis of atopy was made based on either a positive skin test result to more than 1 extract of the 12 common local aeroallergens (Torii & Co, Tokyo, Japan) or serum IgE concentrations above 100 IU/mL. Two hundred ten nonatopic subjects were age-matched, healthy control children who visited the hospital for general health workups. The nonatopic children included in this group had to have a negative skin prick result test and not be taking any medication. Detailed information for atopic and nonatopic subjects is shown in Table I. Asthma was defined as recurrent wheezing or cough in the absence of a cold in the preceding 12 months with a physician's diagnosis (at any time in the patient's history), bronchial hyperresponsiveness upon methacholine challenge ($PC_{20} \leq 16$ mg/mL), or at least 12% reversibility of the FEV₁ after inhalation of a β_2 -agonist as per criteria of the American Thoracic Society.^{29,30} For detailed information on asthmatic subjects, see Table E1 in this article's Online Repository at www.jacionline.org.

For the replication stage, a total of 391 independent subjects including 148 atopic and 243 nonatopic subjects were recruited from 2 elementary schools in Jeju, Korea. The study design included administering a survey, measuring serum total IgE level, and performing skin prick tests. We used a modified International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire³¹ for determining the prevalence of symptoms and diagnosing allergic diseases. Atopy was defined as a positive skin test result on exposure to 1 or more extract of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* or serum total IgE concentrations above 100 IU/mL in the subjects with a history of asthma or allergic rhinitis. The nonatopic controls included in this group had to have a negative skin prick test result and serum total IgE concentrations below 100 IU/mL in the subjects without a history of respiratory symptoms or allergy. For detailed information on atopic and nonatopic subjects, see Table E2 in this article's Online Repository at www.jacionline.org.

All were unrelated Korean subjects and provided written informed consent to participate in the study according to the appropriate Institutional Review Board.

Variation screening of PAR-2

We carried out screening of single nucleotide polymorphisms (SNPs) with genomic DNA from 48 healthy subjects. A total of 7 overlapping

TABLE I. Baseline characteristics of the subjects

Characters	Atopy	Nonatopy	P value*
No. of patients, n (%)	316 (60.1)	210 (39.9)	
Age (y), mean (SD)	10.2 (2.79)	9.5 (3.72)	.03
Sex, male, n (%)	204 (64.6)	121 (57.6)	.18
Total serum IgE levels (IU/L), mean (SEM)	524.43 (30.58)	54.57 (3.46)	<.001
ECP (μ g/L), mean (SEM)	22.06 (1.53)	14.20 (1.42)	<.001
TEC levels (cells/L), mean (SEM)	381.55 (20.07)	210.48 (14.83)	<.001
Asthma, n (%)	96 (30.4)	62 (29.5)	.48

* χ^2 Test or *t* test was used where appropriate.

primer sets were designed by using the PAR-2 genomic sequence available from the National Center for Biotechnology Information (accession no., NM_005242.4). A promoter region including -2.0 kb from the translation initiation site, all coding exons in their entirety, and flanking intronic sequences of the *PAR-2* gene were amplified. PCR products were purified, reacted with BigDye Terminator v3.1, and applied to an ABI 3730xl automated sequencer (Applied Biosystems, Foster City, Calif). Mutation analyses were performed by using Phred, Phrap, Consed, and Polyphred 5.04 software (<http://droog.mbt.washington.edu/PolyPhred.html>). Positions of the genetic variations are given according to the recommended nomenclature for human gene mutations.³²

Selection of PAR-2 polymorphisms for genotyping

Blood samples were collected from each participant, and genomic DNA was extracted by using a purification kit (Qiagen GmbH, Hilden, Germany). For genotyping in individual subjects, we selected 3 SNPs of PAR-2 with a minor allele frequency of greater than 5% in the screening stage. Haplotype analysis was performed by using the Haploview 3.2 program based on a standard expectation-maximization algorithm to construct haplotype blocks.³³ Genotype screening of each sample was performed by using the SNaPshot or SNaPIT method (Applied Biosystems) according to protocols supplied by the manufacturer.

Measurements of blood eosinophil count, serum eosinophil cationic protein, and serum total IgE

Peripheral venous blood was collected from all subjects. An eosinophil count was performed on an aliquot of whole venous blood by using the NE-8000 system (Sysmex, Kobe, Japan). The serum fraction was then separated from whole blood, and eosinophil cationic protein (ECP) and serum total IgE levels were measured by using the CAP system FEIA (Pharmacia, Diagnostics, Uppsala, Sweden).

Skin prick test

Skin prick tests were performed with 12 common allergens, including 2 types of house dust mites, cat and dog epithelia, and mold and pollen antigens (Torii & Co). Negative and positive controls were saline and 0.5% histamine HCl, respectively.

mRNA structure modeling of PAR-2 sequences

To evaluate the effect on mRNA structure of the c.621C>T variant in the *PAR-2* gene, the mRNA secondary structure of PAR-2 (accession no., NM_005242.4) was predicted by using Vienna RNA Package version 1.8.4 software (RNAfold; <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>).^{34,35} Full-length mRNA sequences were compared with the wild-type mRNA sequence. The amount of minimum free energy of centroid secondary structure in the dot-bracket notation was calculated. Qualitative assessments of the folded structures were also made.

mRNA stability analysis

The human PAR-2 was PCR-amplified by using appropriate oligonucleotide primers and subcloned into the pEGFP-N1 vector. The c.621C>T polymorphism was generated by a PCR-based site-directed mutagenesis method using the following primers: 5'-GATTCTGCTGGTCACCATTCCTTTG TATGTCGTGAAG-3' and 5'-CTTCACGACATACAAAGGAATGGTGAC CAGCAGAATC-3'. THP-1 cells originated from human monocyte were cultured in RPMI 1640 (Invitrogen, Carlsbad, Calif) supplemented with 10% FBS and penicillin (50 U/mL)/streptomycin (50 µg/mL). Plasmids were transiently transfected into THP-1 cells by using Lipofectamine LTX and PLUS reagents (Invitrogen) according to the manufacturer's instructions. To evaluate the effect of c.621C>T polymorphism of PAR-2 on mRNA stability, pEGFP-N1-PAR2-621C and pEGFP-N1-PAR2-621T plasmids were transfected separately into THP-1 cells. At 24 hours after transfection, cells were treated with actinomycin D (Sigma, St Louis, Mo) at a final concentration of 5 µg/mL for 0, 2, 4, 6, 8, 16, or 24 hours, respectively. Total RNA was then isolated for quantitative real-time RT-PCR assay.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

Subjects were randomly selected from the nonasthma group. Total RNA was extracted from human peripheral blood by using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was assessed by gel electrophoresis and spectrophotometric measurement of OD₂₆₀/280. Purified RNA samples were reverse transcribed by using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene, La Jolla, Calif) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed by using the TaqMan Gene Expression Assay Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1 µL of cDNA was brought to a total volume of 20 µL by using RNase-free water and mixed with 10 µL of 2X TaqMan Universal Master Mix and 2 µL of the 20X TaqMan Gene Expression Assay. Target amplification was performed in 96-well plates by using ABI PRISM 7300HT (Applied Biosystems). TaqMan probes for PAR-2 (Hs00173741_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were purchased from Applied Biosystems. PCR thermal cycling conditions included an initial 10 minutes hold at 95°C to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles of denaturation (15 seconds at 95°C) and annealing/primer extension (1 minute at 60°C). The relative RNA expression levels were calculated via a comparative threshold cycle (C_t) method using GAPDH as control: $\Delta C_t = C_t(\text{GAPDH}) - C_t(\text{PAR-2})$.

Immunoblotting of PAR-2 in mononuclear cells

Mononuclear cells were isolated from fresh peripheral venous blood by using Ficoll-Paque PREMIUM (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1% (v/v) Nonidet P-40, 0.25% (v/v) sodium deoxycholate, and complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) as well as homogenized, and the debris were pelleted. For immunoblotting, protein samples were suspended in an SDS buffer and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane and blotted with appropriate primary and secondary antibodies. Protein bands were detected by enhanced chemiluminescence (GE Healthcare), and the staining intensities of immunoblots were analyzed by using imaging software (Multi Gauge ver 3.0; Fujifilm, Valhalla, NY). Anti-PAR-2 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif).

Statistical analysis

Statistical analyses were performed by using SPSS 11.5 (SPSS, Inc, Chicago, Ill) and Graphpad Prism 4.0 (Graphpad Software, Inc, San Diego, Calif) software packages. Genotype frequency comparisons between atopic and nonatopic groups were performed by using χ^2 tests. Fisher's exact test was used if expected cell frequencies were lower than 5. An adjustment for multiple tests was made by multiplying the P value with the number of SNPs being

investigated (Bonferroni correction). Genotype frequencies at each SNP were tested for the Hardy-Weinberg equilibrium.

The results of multiple measurements are presented as means $\pm$ SEM. Serum IgE and total eosinophil count (TEC) levels were not normally distributed in arithmetic scale and were thus log-transformed (normality verified by the Kolmogorov-Smirnov test) before being used for statistical comparisons. Statistical analysis was performed with Kruskal-Wallis test or analysis of variance followed by Mann-Whitney U -test, as appropriate. All P values were based on 2-sided comparisons, and P values less than .05 were considered statistically significant.

RESULTS

Demographic and clinical characteristics of the subjects

Baseline characteristics of the 316 atopic and 210 nonatopic subjects are shown in Table I. There were no differences between the 2 groups associated with gender. Subjects with atopy were slightly older ($P = .03$). Atopic subjects had significantly higher total serum IgE, ECP, and TEC levels, which are well-recognized complications of atopy. In addition, the rate of asthma did not differ significantly between the 2 groups. Baseline characteristics of subjects based on both atopy and asthma status are presented in Table E1.

PAR-2 genetic variations in the Korean population

To find PAR-2 variations in the Korean population, we performed direct DNA sequencing of the PAR-2 gene by using genomic DNA samples derived from 24 atopic and 24 nonatopic subjects. Although almost 192 SNPs have been identified to date in the human *F2RL1/*PAR-2 gene (www.genecards.org), only 8 genetic variations were discovered after comprehensive gene scanning. All PAR-2 exons and exon-intron junctions were sequenced including -2.0 kb from the translation initiation site. The linkage disequilibrium structure of the PAR-2 gene is shown in Fig 1. Of the 8 variations, 5 with an allele frequency of less than 5% were excluded from further analyses. The remaining 3 SNPs did not show linkage based on an r^2 threshold over .8, and all 3 were selected for the PAR-2 association study (Fig 1).

Association of PAR-2 polymorphisms with susceptibility to atopy

To evaluate whether PAR-2 genetic variations are associated with susceptibility to atopy, we carried out case-control association studies of the 3 SNPs in all 526 subjects (Table II). The genotype distributions of the SNPs did not deviate from the Hardy-Weinberg equilibrium ($P > .05$) in each set of samples. Of the 3 SNPs tested, one synonymous variation showed a significant association with atopy. The c.621C>T (p.I207I, rs631465) had a P value of .004 in codominant mode and .001 in the minor allele dominant mode (odds ratio [OR], 1.95, 95% confidence interval [CI], 1.30-2.94). The values remained significant after conservative Bonferroni correction for multiple testing ($\alpha/\text{number of comparison} = 0.05/3$).

Because PAR-2 c.621C>T (p.I207I, rs631465) showed an association with atopy, serum IgE, TEC, and ECP levels were examined in the subjects. Higher serum IgE and peripheral blood eosinophil levels are a strong risk factor for subsequent development of atopy. Fig 2 shows log-transformed serum IgE and TEC values. The total serum IgE and TEC levels were higher for

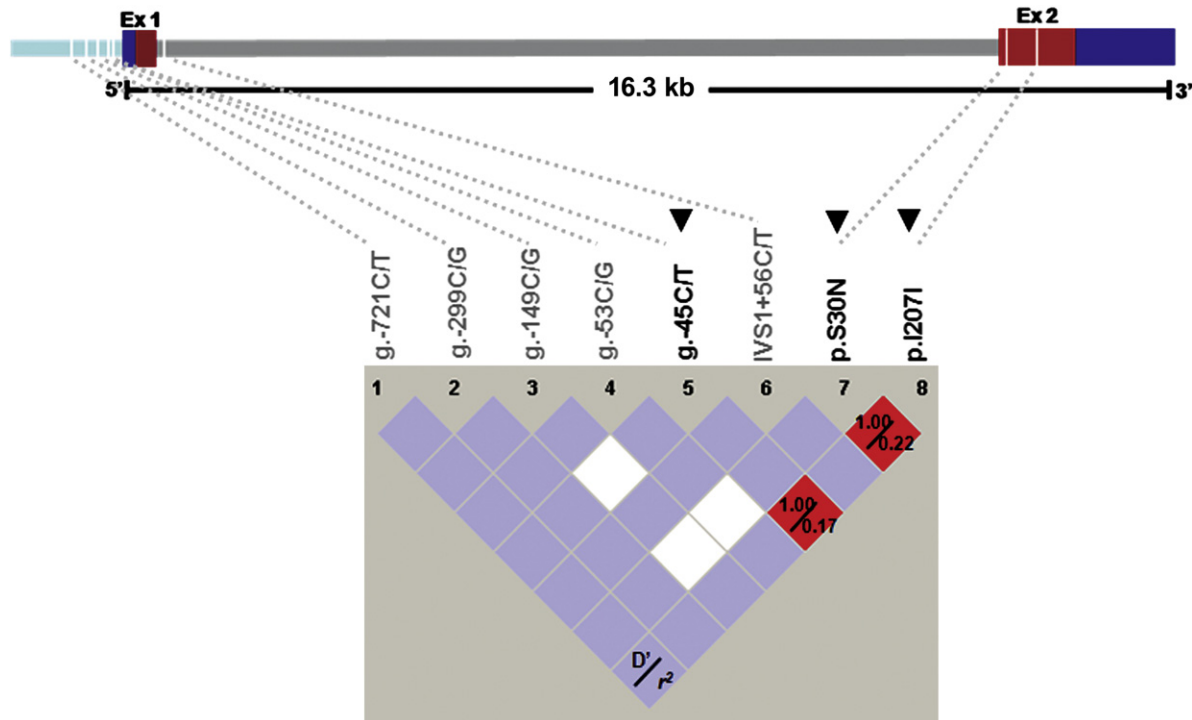


FIG 1. Linkage-disequilibrium structure of the *PAR-2* gene in Koreans. The *upper diagram* shows the genomic structure of *PAR-2*, and the *lower diagram* shows the linkage-disequilibrium (D'/r^2) among SNPs in this study. The triangles point to the SNPs selected for the association analyses. Grayed letters represent SNPs excluded from genotype analyses (with an allele frequency below 0.05).

TABLE II. Frequency of F2RL1 genetic variations in atopic and nonatopic subjects

SNP* (gene type)	Group		P value†/OR (95% CI)			
	Atopy, n (%)	Nonatopy, n (%)	Dominant	Recessive	Codominant	Allele
g.-45C/T (rs1529505)						
CC	211 (67.2)	132 (62.9)	.31	.67	.44	.48
			.83 (0.57-1.19)	1.199 (0.52-2.77)		
CT	87 (27.7)	69 (32.9)				
TT	16 (5.1)	9 (4.3)				
S30N (rs616235)						
AA	229 (72.7)	151 (72.2)	.91	.72	.84	.81
			.98 (0.66-1.45)	.66 (0.16-2.67)		
GA	82 (26.0)	54 (25.8)				
GG	4 (1.3)	4 (1.9)				
I207I (rs631465)						
CC	59 (18.8)	64 (31.2)	.001	.79	.004	.03
			1.95 (1.30-2.94)	1.06 (0.70-1.60)		
CT	176 (56.2)	92 (44.9)				
TT	78 (24.9)	49 (23.9)				

CI, Confidence interval; OR, odds ratio.

*Nucleotide location number was assigned according to the F2RL1 mRNA sequence (GeneBank accession number: NM_005242).

†P values were obtained by using the χ^2 test or Fisher exact test (expected cell value <5), and significant SNPs are shown in boldface after Bonferroni correction for the multiple testing.

individuals with the CT or TT genotype than for those with the CC genotype (IgE mean, 366.0 IU/mL vs 278.1 IU/mL, $P = .004$, Fig 2, A; TEC mean, 323.8 cells/L vs 271.7 cells/L, $P = .03$, Fig 2, B). In case of ECP, individuals with the CT or TT genotype showed a trend toward elevated levels relative to those with the CC genotype, but the difference did not have statistical significance (data not shown).

As *PAR-2* c.621C>T (p.I207I, rs631465) showed an association with atopy, this SNP was regentyped in an independent set of atopy cohort and the association was reevaluated (Table III). Importantly, results in the replication stage also showed a significant association between c.621C>T and childhood atopy (genotypic $P = .01$ in codominant mode, allelic $P = .01$). Moreover, the joint analysis of exploratory and the replication

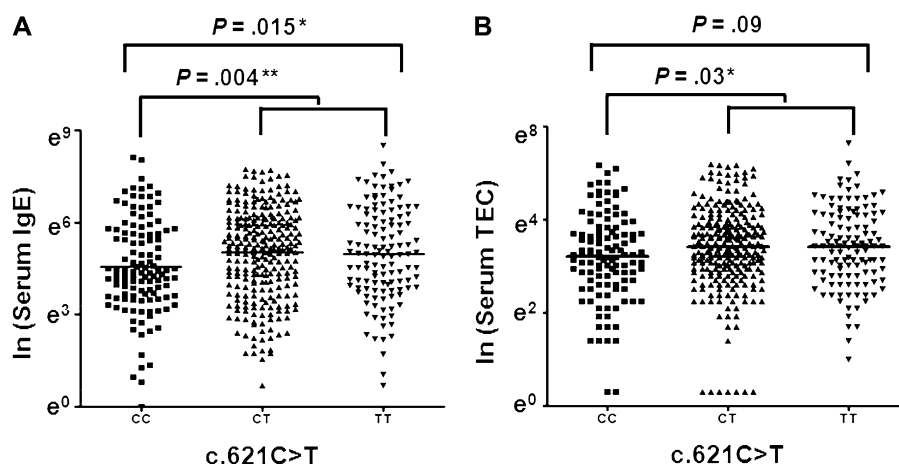


FIG 2. Serum IgE and TEC levels according to PAR-2 c.621C>T (rs631465) genotype. A scatter plot illustrating serum IgE (n = 508) (A) and TEC (n = 489) (B) levels in each genotype. The y-axis represents log-transformed serum IgE and TEC values, and horizontal bars in the scatter plots represent mean values. ** $P < .01$, * $P < .05$.

TABLE III. Association study results for c.621C>T (p.I207I, rs631465) in F2RL1 (PAR-2) and childhood atopy*

	Exploratory stage		Replication stage	
Variant	Atopy	Nonatopy	Atopy	Nonatopy
Genotypes	No. of individuals (%)			
C/C	59 (18.8)	64 (31.2)	28 (18.9)	64 (26.3)
C/T	176 (56.2)	92 (44.9)	81 (54.7)	143 (58.8)
T/T	78 (24.9)	49 (23.9)	39 (26.4)	36 (14.8)
Total	313 (100)	205 (100)	148 (100)	243 (100)
Alleles	No. of chromosomes (%)			
C	294 (47.0)	220 (53.7)	137 (46.3)	271 (55.8)
T	332 (53.0)	190 (46.3)	159 (53.7)	215 (44.2)
Total	626 (100)	410 (100)	296 (100)	486 (100)
P_{genotype} in codominant mode [$P_{\text{CC\&TT}}$ comparison , OR (95% CI)]				
	.004 [.03, 1.73 (1.04-2.86)]		.01 [.005, 2.48 (1.31-4.70)]	
Joint P_{genotype}	.001 [<.001, 2.03 (1.37-2.99)]			
P_{genotype} in minor allele dominant mode, OR (95% CI)				
	.001, 1.95 (1.30-2.94)		.093, 1.53 (0.93-2.53)	
Joint P_{genotype}	.001, 1.72 (1.26-2.35)			
P_{allele} , OR (95% CI)				
	.03, 1.34 (1.04-1.713)		.01, 1.46 (1.09-1.96)	
Joint P_{allele}	<.001, 1.38 (1.15-1.66)			

CI, Confidence interval; OR, odds ratio.

* P values were obtained by comparing atopy and nonatopy by using the χ^2 test or Fisher exact test (expected cell value <5) in each mode.

studies including 461 atopic and 448 nonatopic subjects displayed a much more significant association (joint genotypic P value = .001, joint allelic P value < .001, OR of T vs C, 1.38, 95% CI, 1.15-1.66) (Table III).

Functional evaluation of the PAR-2 c.621C >T variant

c.621C>T is a synonymous p.I207I variation that does not alter the amino acid sequence of PAR-2. However, recent studies have indicated that synonymous SNPs as well as nonsynonymous SNPs can affect mRNA stability, mRNA processing, and mRNA maturation, thereby affecting mRNA expression.^{36,37} We examined whether the risk allele of c.621C>T variation affects the stability of the PAR-2 mRNA by using an *in silico* analysis. RNA

secondary structure prediction with the RNA fold web server showed that the c.621C>T variation could greatly alter the PAR-2 mRNA secondary structure (Fig 3, A). The calculated minimum free energy level of total PAR-2 mRNA with the "T" allele at c.621 was approximately 47.16 kcal/mol lower than that with the "C" allele. In general, mRNAs that have lower minimum free energy level are expected to have more stable structure. To test this notion, we transfected the human monocyte cell line THP-1 with each PAR-2 c.621C and c.621T gene construct, treated cells with actinomycin D for a different period of time, and measured the mRNA expression level by using quantitative RT-PCR. We found that c.621T showed a significantly slower mRNA decay rate than did c.621C (Fig 3, B). These results suggest that the conversion from C to T at c.621 stabilizes PAR-2 mRNA.

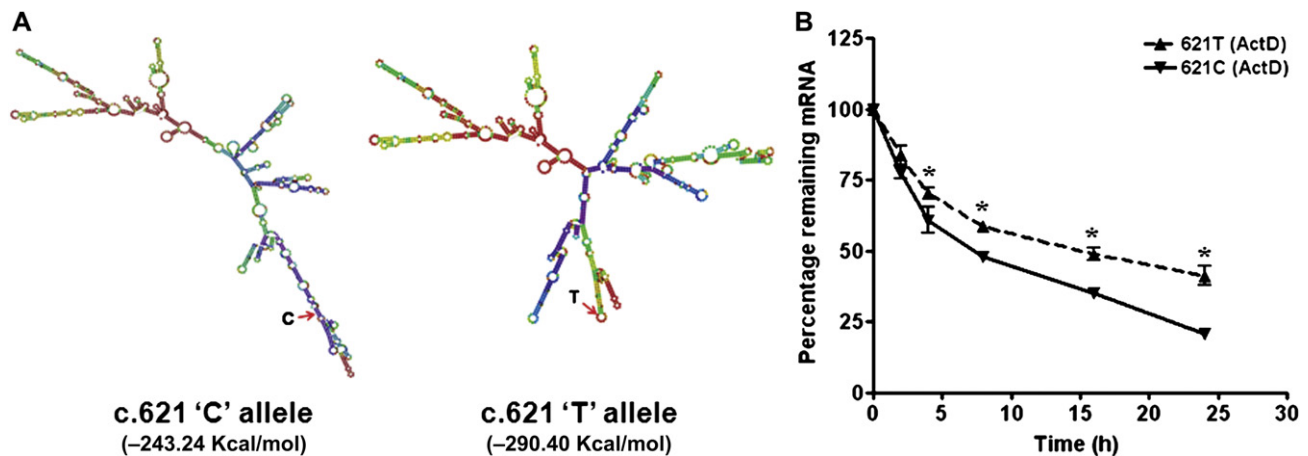


FIG 3. The mRNA structures and stability according to the c.621C>T genotype. **A**, RNA structures were predicted by using the Vienna RNA v.1.8.4 software package. The values are free energy level in the centroid secondary structure. **B**, Time course changes in the remaining amount of PAR-2 mRNA after ActD treatment. The data are presented as mean $\pm$ SD ($n = 4$). * $P < .05$.

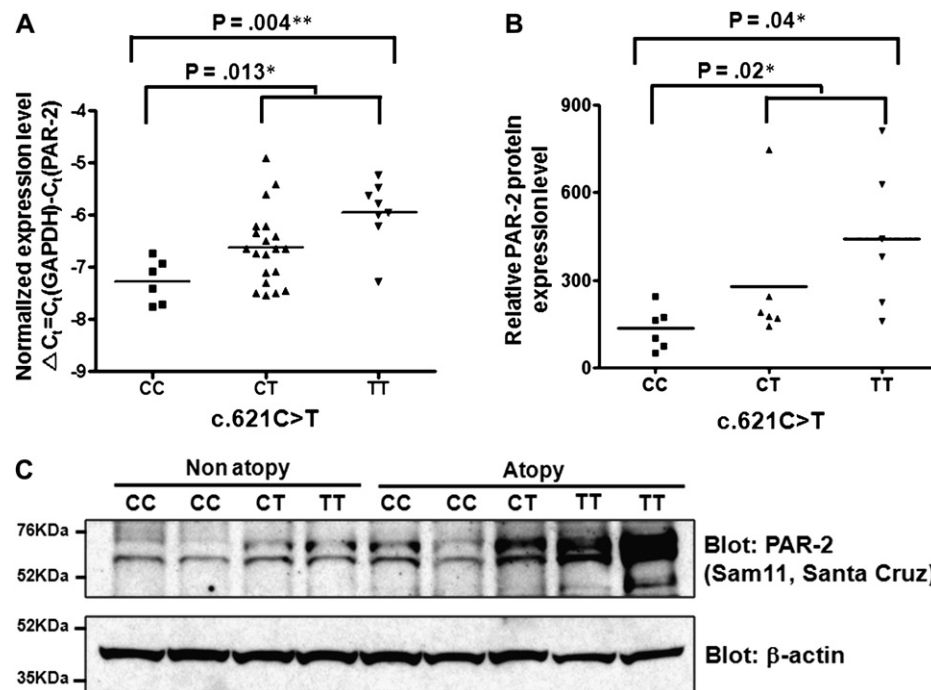


FIG 4. The expression of PAR-2 mRNA and protein in human peripheral blood cells. **A**, The mRNA expression levels in each genotype ($n = 34$). The amount of PAR-2 mRNA was calculated by using the comparative CT method. **B**, The PAR-2 protein expression levels in each genotype ($n = 18$). The data are presented as percentage of the control values (=100%) after normalizing to the β -actin internal control bands. **C**, A representative immunoblot. ** $P < .01$, * $P < .05$.

To confirm *in vitro* observed mRNA stability effect of PAR-2 c.621C>T variation, *in vivo* experiments were undertaken. First, we performed quantitative real-time RT-PCR to investigate whether c.621C>T elevated PAR-2 mRNA transcript levels in human samples (Fig 4, A). RNA samples were prepared from fresh peripheral blood cells as detailed in the Methods section. As shown in Fig 4, A, individuals with the c.621TT genotype showed 2.53 ($2^{1.33}$)-fold more PAR-2 mRNA in blood cells than did those with the c.621CC genotype (mean ΔC_t against GAPDH, -5.95 vs -7.27 , $P < .01$). Next, we performed Western blot analysis in each

genotype from atopic and nonatopic subjects to see whether the PAR-2 c.621C>T variation affects the final protein expression levels. The relative protein expression levels of PAR-2 were significantly higher in individuals with the c.621CT or TT genotype than in those with the c.621CC genotype (Fig 4, B and C). Particularly, the PAR-2 expression levels in the atopy group showed a significant difference (1.84-fold higher, $P = .03$) from that of the nonatopy group after removing outliers. Three samples were detected as outliers by a standard quartile method (outside of 1.5 times interquartile range from the first or third quartile)

because the expression levels were highly variable between individuals (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Moreover, a highly significant positive correlation was found between PAR-2 protein expression and serum IgE levels (Fig E1, B). Thus, a more stable mRNA structure may explain the functional association of the *c.621* allele with atopy.

DISCUSSION

All atopic disorders are type I hypersensitivity disorders, meaning they are associated with an exaggerated IgE-mediated immune response. In the present study, we identified significant associations between human PAR-2 polymorphisms and susceptibility to atopy. Atopic subjects had a significantly higher frequency of the CT or TT genotype at the *c.621C>T* locus compared with nonatopic subjects. The risk *c.621T* allele was also associated with higher serum IgE and TEC levels. Moreover, the positive association was reproduced in an independent replication cohort (Table III). C to T conversion at *c.621* was predicted to result in an increased mRNA stability at both *in silico* and *in vitro* assays (Fig 3) and, indeed, was associated with increased PAR-2 mRNA and protein expression in human blood cells (Fig 4). These observations implicate human PAR-2 in the hypersensitive immune reaction in atopy and possibly in type I hypersensitivity disorders in general.

PAR-2 belongs to a subfamily of G-protein-coupled 7-transmembrane receptors. PAR-2 is present on all the cells involved in allergic diseases and can be activated by serine proteases such as trypsin and mast cell tryptase. Recently, the potential role of PAR-2 in the pathogenesis of inflammation and immune diseases via interaction with serine protease has attracted attention.^{14,15,19,23-25} Most studies show that PAR-2 has a proinflammatory effect via PAR-2 agonist experiments, but some reports suggest that PAR-2 can have a protective effect against inflammation.^{18,24-28} Clearly, the role(s) of PAR-2 in inflammation is still not fully understood. Other recent results include the finding of increased PAR-2 expression in respiratory epithelial cells from patients with asthma.²³ PAR-2 expression and activation can be modulated by differential glycosylation,³⁸ and activation can also be altered by polymorphic variation. For example, variations in the human PAR-2 in extracellular loop 2 (Phe240→Ser, PAR2F204S) result in significant differential receptor activation by trypsin and PAR agonists.³⁹

We hypothesized based on these and other data that SNPs in PAR-2 would be associated with atopy and asthma and set out to analyze the association in a Korean childhood cohort. However, we found a strong association of one SNP in PAR-2 with atopy rather than asthma. These association results are difficult to explain because asthma and atopy are both complex diseases, with a somewhat unclear relationship with each other. Although atopy is the most significant risk factor for asthma development in children,⁴⁰ atopy alone does not account for asthma.⁴⁰ Furthermore, many persons are atopic but not asthmatic.⁴¹ Thus, untangling the genetics of these disorders will be challenging, and candidate genes for asthma and for atopy might be relevant to the other disease. In keeping with this, immune activation through PAR-2 might be a candidate mechanism in the development of atopy, and asthma as well, although this particular SNP did not show an association with asthma.

Recent studies show that PARs, especially PAR-2, play an important role in allergic reactions through regulation of protein expression. In skin inflammatory disease patients, PAR-2 expression was found to be increased in mast cells,⁴² and the expression levels of PAR-2 mRNA were also significantly upregulated in patients with allergic rhinitis.⁴³ Reduced expression of PARs by RNA interference can modulate PAR-related cytokine production in mast cells, which are a key effector cell in allergic inflammation.⁴⁴ Given these results, genetic variation that influences PAR protein expression or results in significant differential receptor activation may represent a genetic basis for individual differences in disease susceptibility. However, to date there have been relatively few studies of PAR-2 genetic variation with association to allergic disease.

The *PAR-2* gene spans 16.3 kb on the long arm on human chromosome 5 in position 5q13.3. It is composed of an upstream regulatory region and 2 exons separated by a large intron. In this Korean population, a total of 8 genetic variations were discovered and 3 had an allele frequency greater than 5%. According to International HapMap Project data (<http://www.hapmap.org/>), allelic frequencies for these 3 SNPs differed significantly among ethnic groups. Minor allele frequencies in Caucasians are much lower than in Asians. For example, the “T” allele of *c.621C>T* (rs631465) was found less frequently in the Utah residents with ancestry from northern and western Europe population (0.05) than in the Asian populations (Korean [50.3], Japanese [43.2], Chinese [46.7]). The “T” allele of *c.621C>T* associated with atopy in this study might be a susceptible SNP in other Asian populations, but this would need further study.

With regard to the molecular mechanism by which PAR-2 might influence atopy, the *c.621C>T* variation is a synonymous (not amino acid changing) SNP at position 207. Therefore, it is formally possible that the positive associations seen could potentially originate from other functional polymorphisms located in the *PAR-2* gene or nearby. To examine this possibility, we searched for other functional variations linked with *c.621C>T* in the HapMap project database. However, we found no linked causal polymorphisms and focused on the *c.621C>T* SNP itself to test the biological relevance of sequence variation. Growing evidence indicates that synonymous variation in coding regions can alter mRNA levels by affecting mRNA stability.^{35,36} An *in silico* analysis performed by using RNA structure software package (version 4.5) revealed that *c.621C>T* greatly alters the predicted PAR-2 mRNA secondary structure (Fig 3, A). The calculated minimum free energy level of the PAR-2 mRNA with the “T” allele at *c.621* was approximately 47.16 kcal/mol lower than that of the mRNA with the “C” allele, leading to the expectation that PAR-2 mRNA with the “T” allele is more stable than the mRNA with the “C” allele. This was confirmed by an *in vitro* assay for mRNA stability (Fig 3, B). Besides *in vitro* assays, we confirmed increased mRNA and protein expression in an *in vivo* assay using human blood cells (Fig 4). Furthermore, subjects with the *c.621T* risk allele had significantly higher serum IgE and TEC levels, which are highly correlated with atopy risk. Thus, it appears that the more stable mRNA structure associated with the *c.621T* allele causes an increase in PAR-2 mRNA and leads to make more final protein products. Increased PAR-2 proteins in immune and inflammatory cells might be responsible for increased cellular responsiveness to allergen stimuli in relevant tissues and therefore to increased risk of atopy.

Despite strong molecular evidence for associations between PAR-2 c.621C>T polymorphism and atopy, the replication study in independent samples is strongly needed for the reliability of results in genetic association studies. To do this, we endeavored to find a replication cohort having similar phenotype and diagnostic criteria for the atopy and we found an independent atopy cohort. In the second case-control cohort, we also found a significant association of PAR-2 c.621C>T SNP with atopy and the individuals with the TT genotype showed a significantly higher level of serum IgE than those with the CC genotype (data not shown). Considering the difficulty of replication, it is quite interesting to show the same results in the relationship between PAR-2 c.621C>T polymorphism and atopy.

In view of the potential mechanisms for the development of allergy attributable to PAR-2, the details are still matters of speculation. One theoretical possibility is that PAR-2 directly controls T cell function(s) that cause an increased inflammatory cytokines production. PAR-2 also plays a role in the development of dendritic cells, which are critical orchestrators of the immune system and provide a link between adaptive and innate immunity.⁴⁵ Altogether, PAR-2 signaling may enhance subsequent allergic immune responses and explain the contribution of PAR-2 to the allergy development.

In summary, this study is a comprehensive assessment of polymorphisms at the PAR-2 gene and suggests that a synonymous polymorphism c.621C>T (p.I207I, rs631465) contributes to the risk of atopy.

Key message

- The atopic diseases are the most common chronic diseases of childhood, and PAR-2 genetic variation may play an important role in the development of atopy in Korean children.

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TABLE E1. Baseline characteristics of the subjects (4 group comparisons)

Characteristic	Atopy		Nonatopy		P value*
	Asthma	Nonasthma	Asthma	Nonasthma	
No. of patients	96	197	62	146	
Age (y), mean (SD)	9.9 (2.59)	10.3 (2.87)	9.81 (4.02)	9.38 (3.61)	.09
Sex, male, n (%)	68 (70.8)	123 (62.8)	40 (64.5)	80 (54.8)	.09
Lung function, mean (SD)					
FEV ₁ (L)	1.87 (0.71)	2.15 (0.74)	1.59 (0.44)	1.84 (0.68)	.001
FEV ₁ (% predicted)	89.43 (12.24)	91.56 (13.30)	84.28 (16.64)	93.19 (13.24)	<.001
FVC (L)	2.19 (0.86)	2.42 (0.86)	1.86 (0.48)	2.02 (0.83)	.003
FVC (% predicted)	91.16 (16.44)	91.33 (12.15)	86.65 (16.99)	90.50 (11.49)	.15
FEV ₁ /FVC (% predicted)	88.42 (10.16)	89.38 (6.18)	98.29 (11.50)	97.65 (8.74)	<.001
FEF ₂₅₋₇₅ (% predicted)	79.73 (18.88)	94.64 (22.52)	80.05 (24.57)	96.48 (24.49)	<.001
Methacholine PC ₂₀ (mg/mL), n (%)					
<1	21 (22.1)	0 (0.0)	15 (25.4)	0 (0.0)	<.001
1-4	37 (38.9)	0 (0.0)	17 (28.8)	0 (0.0)	
4-16	37 (38.9)	0 (0.0)	27 (45.8)	0 (0.0)	
>16	0 (0.0)	194 (100.0)	0 (0.0)	143 (100.0)	
Total serum IgE levels (IU/L), mean (SEM)	609.48 (53.76)	461.46 (35.29)	66.72 (7.13)	49.71 (3.90)	<.001
ECP (μg/L), mean (SEM)	502.56 (38.04)	334.26 (25.21)	255.59 (27.17)	192.77 (17.75)	<.001
TEC levels (cells/L), mean (SEM)	26.53 (3.25)	20.15 (1.68)	17.68 (3.06)	12.67 (1.52)	<.001

* χ^2 Test or *t*-test was used where appropriate.

TABLE E2. Baseline characteristics of the subjects in the replication cohort

Characteristic	Atopy	Nonatopy	<i>P</i> value*
No. of patients, n (%)	148 (37.9)	243 (62.1)	
Age (y), mean (SD)	10.1 (1.33)	10.2 (1.16)	.42
Sex, male, n (%)	76 (51.4)	105 (43.2)	.14
Total serum IgE levels (IU/L), mean (SEM)	364.24 (27.14)	36.36 (1.64)	<.001

* χ^2 Test or *t*-test was used where appropriate.

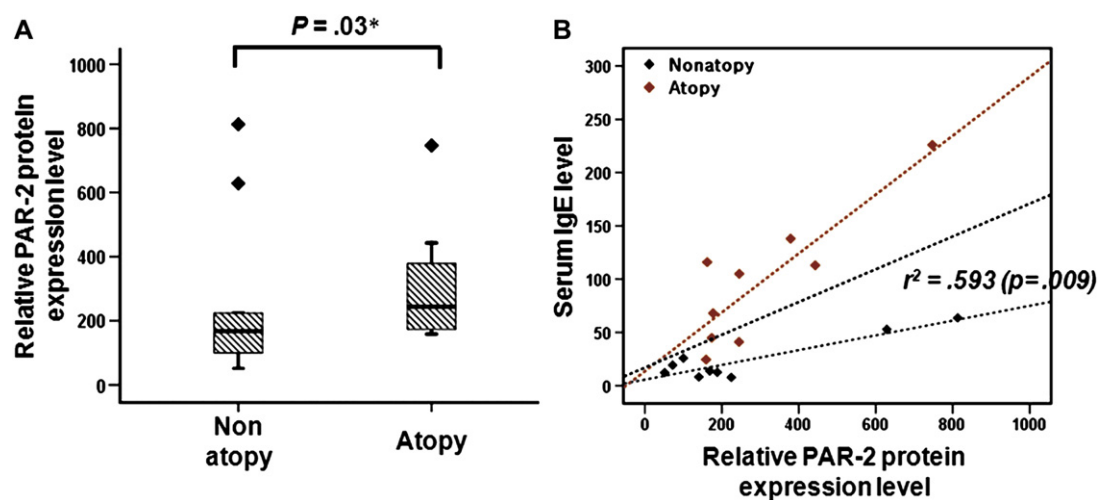


FIG E1. The relationship between relative PAR-2 protein expression levels and atopy. **A**, The relative PAR-2 expression in atopic ($n = 9$) and nonatopic ($n = 9$) subjects. The horizontal bar represented median value. **B**, The correlation between relative PAR-2 protein expression and serum IgE levels (atopy, red; nonatopy, blue; all subjects, black). r^2 , Coefficient of determination. $*P < .05$.