Journal of Human Genetics (2012) 57, 247–253

www.nature.com/jhg

ORIGINAL ARTICLE

Association analysis of formyl peptide receptor 2 (*FPR2*) polymorphisms and Aspirin exacerbated respiratory diseases

Hee-Jeong Kim[1,9], Sung-Hwan Cho[1,9], Jong-Sook Park[1], Tae-Hyeong Lee[1], Eun-Ju Lee[1], Yong-Hoon Kim[2], Soo-Taek Uh[3], Il Yup Chung[4], Mi-Kyeong Kim[5], Inseon S Choi[6], Byung-Lae Park[7,9], Hyoung-Doo Shin[7,8] and Choon-Sik Park[1]

Aspirin-exacerbated respiratory diseases (AERD) are associated with the metabolism of arachidonic acid. *FPR2* (formyl peptide receptor2) is a high-affinity ligand receptor for potent anti-inflammatory lipid metabolites: lipoxins. Thus, functional alterations of the *FPR2* may contribute to AERD. We investigated the relationship between single-nucleotide polymorphisms (SNPs) in the *FPR2* and AERD. Asthmatics were categorized into AERD <15% decreases in forced expiratory volume in one second (FEV_1), and/or naso-ocular reactions after oral aspirin challenge (n=170) and aspirin-tolerant asthma (ATA, n=268). In all, 11 SNPs were genotyped. *FPR2* protein expressions on CD14-positive monocytes in peripheral blood were measured using flow cytometric analysis. We performed RT-PCR of the *FPR2* mRNA expressed by peripheral blood mononuclear cells. Logistic regression analysis showed that the minor allele frequency of *FPR2 −4209T>G* (rs1769490) in intron 2 was significantly lower in the AERD group (n=170) than in the ATA group (n=268) (P=0.006, P^{corr}=0.04, recessive model). The decline of FEV_1 after aspirin challenge was significantly lower in the subjects with GG homozygotes of *FPR2 −4209T>G* than those with the other genotypes (P=0.0002). Asthmatic homozygotes for *FPR2 −4209T>G* minor allele exhibited significantly higher *FPR2* protein expression in CD14-positive monocytes than did those with the common allele of *FPR2 −4209T>G* allele (P=0.01). There was no difference in the expression of the wild form and the exon 2 deleted variant form of *FPR2* gene according to the genotypes of *FPR2 −4209T>G*. The minor allele at *FPR2 −4209T>G* may have a protective role against the development of AERD, via increase of *FPR2* protein expression in inflammatory cells.

Journal of Human Genetics (2012) **57,** 247–253; doi:10.1038/jhg.2012.12; published online 1 March 2012

Keywords: aspirin; asthma; *FPR2* (formyl peptide receptor 2); SNPs (single-nucleotide polymorphisms)

INTRODUCTION

Aspirin-exacerbated respiratory diseases (AERD) refer to the development of bronchoconstriction in asthmatic individuals following the ingestion of aspirin (acetylsalicylic acid) or other non-steroidal anti-inflammatory drugs. This syndrome is characterized by the 'aspirin triad,' which consists of aspirin hypersensitivity, bronchial asthma, nasal polyposis and chronic hyperplastic eosinophilic sinusitis.[1–3] Similar to other asthmatic individuals, the airways of patients with AERD show signs of persistent inflammation with marked eosinophilia, epithelial disruption, cytokine production and upregulation of inflammatory molecules.[4] Overproduction or underproduction of critical modulators important for the metabolism of arachidonic acids, including leukotrienes, lipoxins, thromboxane and prostaglandins, likely accounts for the observed susceptibility to aspirin. Variations within the genes of the arachidonate pathway are responsible for changes in the production and metabolism of these modulators. Polymorphisms in genes such as *LTC4S*,[5] *ALOX5*,[6] *PTGER2*,[7] *PTGER3*,[8] *CYSLTR2*,[9] *TBXA2R*[10] and *CYSLTR1*[11] have previously been shown to be associated with AERD. Among the proteins encoded by these genes, the cysteinyl leukotriene (CysLT) receptor is selectively

[1]Genome Research Center for Allergy and Respiratory Disease, Soonchunhyang University Bucheon Hospital, Wonmi-gu, Bucheon, Republic of Korea; [2]Division of Allergy and Respiratory Medicine, Soonchunhyang University Cheonan Hospital, Bongmyeong-dong, Cheonan, Korea; [3]Division of Allergy and Respiratory Medicine, Soonchunhyang University Seoul Hospital, Daesagwan-gil, Yongsan-Gu, Seoul, Republic of Korea; [4]Division of Molecular and Life Sciences, College of Science and Technology, Hanyang University, Ansan, Republic of Korea; [5]Division of Internal Medicine, Chungbuk National University, College of Medicine, Cheongju, Republic of Korea; [6]Department of Allergy, Chonnam National University Medical School and Research Institute of Medical Sciences, Gwangju, Republic of Korea; [7]Department of Genetic Epidemiology, SNP Genetics Inc., Seoul, Republic of Korea and [8]Department of Life Science, Sogang University, Seoul, Republic of Korea
[9]Equally contributed as the first authors.
Correspondence: Dr Choon-Sik Park, Division of Allergy and Respiratory Medicine, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, 1174, Jung Dong, Wonmi Ku, Bucheon, Gyeonggi Do, 420-021, Republic of Korea.
E-mail: mdcspark@unitel.co.kr
or Dr H-D Shin, Department of Life Science, Sogang University, 1 Shinsu-dong, Mapo-gu, Seoul, 121-742, Republic of Korea.
E-mail: hdshin@sogang.ac.kr
Received 3 November 2011; revised 22 December 2011; accepted 12 January 2012; published online 1 March 2012

antagonized by several currently available leukotriene modifiers, including montelukast, pranlukast and zafirlukast. However, clinical studies have demonstrated that the response to these medications is incomplete.[12,13] These results suggest that an alternative pathway that does not include CysLT or the CystLT receptor may lead to aspirin hypersensitivity in asthma.

Both LXA4 and aspirin- triggered 15-epi lipoxin 4 (ATL) serve as 'stop' signals for leukocyte trafficking, facilitating the resolution of inflammatory responses.[14,15] These responses are mediated by formyl peptide receptor 2 (*FPR2*, also known as *FPRL1, ALXR, HM63, FMLPX, FPR2A, FPRH1* and *FPRH2*), which is the surface membrane seven-transmembrane G-protein-coupled receptors. *FPR2* is expressed by various kinds of inflammatory cells, including macrophages, monocytes, neutrophils, T lymphocytes, bronchial epithelial cells and microvascular endothelial cells.[16,17] These inflammatory cells are involved in the pathogenesis of asthma and in asthmatic subphenotypes, including aspirin hypersensitivity. On the basis of these biological functions, genetic alterations in *FPR2* may be postulated to contribute to the pathophysiological processes of AERD. However, a comprehensive genetic analysis of *FPR2* in patients with AERD has not been performed to date. To investigate the genetic role of *FPR2* in AERD, we identified single-nucleotide polymorphisms (SNPs) of the *FPR2* and performed genetic association analyses and functional validation studies to determine the association between the alterations in *FPR2* with the risk of AERD.

MATERIALS AND METHODS

Subjects and aspirin challenge

Subjects were recruited from the Asthma Genome Research Center, which is comprised of nine university hospitals in Korea. All subjects were Korean. All patients were diagnosed by physicians based on the definition of asthma by the Global Initiative for Asthma guidelines.[18] All patients had a history of dyspnea and wheezing during the previous 12 months and also met one of the following criteria: (1) $>15\%$ increase in forced expiratory volume in one second (FEV_1) or $>12\%$ increase plus 200 ml following inhalation of a short-acting bronchodilator, (2) $<10\,mg\,ml^{-1}$ PC20 methacholine and (3) $>20\%$ increase in FEV_1 following 2 weeks of treatment with inhaled steroids and long-acting bronchodilators. A total of 24 common inhalant allergens were utilized for a skin prick test. Atopy was defined as having a wheal reaction equal to or greater than 3 mm in diameter or than that of histamine. Total IgE was measured using the CAP system (Pharmacia Diagnostics, Uppsala, Sweden).

Asthmatic patients had experienced no exacerbation of asthma or respiratory tract infection in the 6 weeks preceding oral aspirin challenge. Oral aspirin challenge was performed with increasing doses of aspirin using methods slightly modified from those described previously.[2,19] Briefly, the patient having history of aspirin hypersensitivity was given 30 mg and those having no history started 100 mg of aspirin orally. Respiratory and naso-ocular symptoms, blood pressure, external signs (urticaria and angio-oedema) and FEV_1 were documented every 30 min for a period of 1.5 h. In the absence of any symptoms or signs suggestive of adverse reaction, 60 mg or 100 mg of aspirin was administered and the same measurements were repeated every 1 h, with a final dose of 450 mg or until the patient developed a reaction. If no reaction occurred 4 h after the final dose, the test was deemed negative. Aspirin-induced bronchospasms, reflected by decline (%) of FEV_1, were calculated as the pre-challenge FEV_1 minus the post-challenge FEV_1 divided by the pre-challenge FEV_1. Oral aspirin challenge reactions were categorized into two groups as follows: (1) 15% or greater decrease in FEV_1 and/or naso-ocular reactions (AERD) or (2) less than 15% decrease in FEV_1 without naso-ocular or cutaneous reactions (aspirin tolerant asthma (ATA)). The ethics committee at each hospital approved the protocols and a written informed consent was obtained from all patients.

Genotyping of FPR2 polymorphisms

In all, 11 polymorphisms found in the *FPR2* in the National Center for Biotechnology Information (Build 36) were selected for this study, and amplification and extension primers were designed for genotyping each of the polymorphic sites by single-base extension.[20] All primer extension reactions were performed using the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA). Following the extension reaction, the reaction mixtures were incubated at 37 °C for 1 h with 1 U of shrimp alkaline phosphatase, followed by incubation for 15 min at 72 °C to inactivate the enzyme. The extension products and Geescan 120 Liz size standard solution were then mixed with Hi-Di formamide according to the manufacturer's instructions and incubated at 95 °C for 5 min, followed by 5 min on ice. The products of the reaction mixture were resolved using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Results were analyzed using GeneScan and Genotyper (Applied Biosystems). Primer sequences are shown in Supplementary Table 1. For quality control for genotyping, 16 blank and 16 duplicate were used, and the concordance rate was 100% in this study.

Measurement of FPR2 protein expression on CD14-positive monocytes of asthmatics

Peripheral blood mononuclear cells (PBMC) were purified from the heparinized peripheral blood of the study subjects by using a density gradient reagent (Histopaque 1077). FITC-conjugated anti-rabbit antibody (BD Pharmingen,

Table 1 Clinical profile of aspirin-exacerbated respiratory diseases (AERD) and aspirin-tolerant asthma (ATA)

Clinical profile	*AERD*	*ATA*	P-*value*
N	170	268	
Onset of age (mean (range))	36 (10–66)	40 (8–72)	0.002
Sex (male/female)	62/108	87/181	NS
Current Smoker/Ex smoker (%)	8.82/13.53	17.54/15.67	0.02
BMI ($kg\,m^{-2}$)	23.69 ± 3.29	24.56 ± 3.52	0.01
FVC (%), predicted	90.37 ± 14.59	87.83 ± 14.90	NS
FEV_1 (%), predicted	87.12 ± 17.44	89.75 ± 18.46	NS
PC20, methacholine ($mg\,ml^{-1}$)	4.86 ± 7.90	2.79 ± 3.34	NS
Atopy (%)	50.91%	56.34%	NS
Total IgE ($IU\,ml^{-1}$)	355.40 ± 615.90	353.31 ± 561.73	NS
Blood eosinophil (%)	5.84 ± 5.13	6.22 ± 5.61	NS
Decline of FEV_1 after OAC (%)	24.77 ± 16.33	3.48 ± 4.52	<0.0001
History of aspirin hypersensitivity (%)	39.41	6.06	<0.0001

Abbreviations: BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; NS, non-significant difference; OAC, oral aspirin challenge; PC20, provocative concentration of 20 total in FEV_1.
Data are presented as mean ± s.e.
P values are obtained using *t*-test or χ^2-test between group with AERD and ATA.

San Diego, CA, USA), *FPR2* antibody (Abcam, Cambridge, UK) and phycoerythrin (PE)-conjugated anti-human CD14 antibody (R&D Systems, Minneapolis, MN, USA) were incubated with 5×10^5 cell ml^{-1} PBMC and then washed twice with PBS. The stained cells were analyzed by using a flow cytometer (FACScan; Becton-Dickinson, Mountain View, CA, USA). For isotype-matched controls, FITC-conjugated Rabbit IgG (Abcam) and PE-conjugated mouse IgG1 isotype (R&D Systems) were used with the same concentration of each antibody tested. The stained cells were analyzed by using a flow cytometer (FACScan; Becton-Dickinson) and Cell Quest Software (Counter Corporation, Miami, FL, USA). Levels of *FPR2* protein expression are monocyte X-mean FPR2/Isotype. Percentage of CD14 (+) monocytes is monocyte gate% FPR2-Isotype.

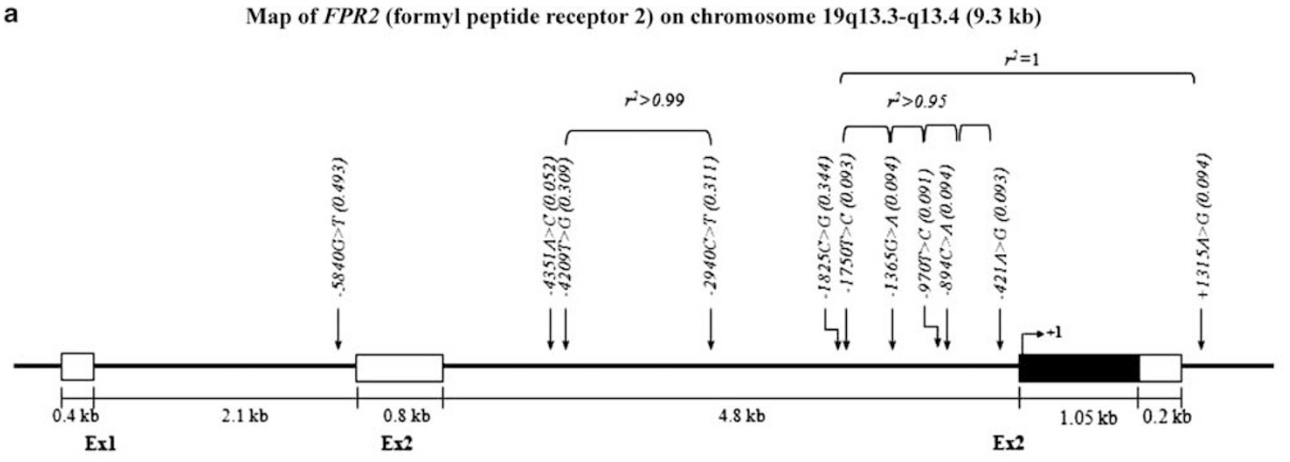
RNA extraction and RT-PCR of FPR2 mRNA

Total RNA of PBMC was isolated using the modified guanidium thiocyanate-phenol-chloroform extraction method. We quantified RNA and reverse transcribed cDNA from 3 μg of total RNA. DNase I (10 000 U ml^{-1}; Stratagene, La Jolla, CA, USA)-treated RNA was reverse-transcribed by incubating with 0.5 mM dNTP, 2.5 mM $MgCl_2$, 5 mM DTT, 1 μl of random hexamer (50 μg μl^{-1}) and SuperScript II RT (200 U μl^{-1}; Life Technologies, Grand Island, NY, USA) at 42 °C for 50 min, and heat inactivated at 70 °C for 15 min. Specific primer pairs for RT-PCR were as follows GAPDH forward; 5′-ACCCAGAAGACTGTG GATGG-3′, *GAPDH* reverse; 5′-TTCTAGACGGCAGGTCAGGT-3′ and *FPR2* forward; 5′-GCACACAGGAAAAGGAGCTT-3′, *FPR2* reverse; and 5′-ACAGAT GGTGGTGACTGTGC-3′. Amplification was performed for 35 cycles (one cycle: 30 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C) with initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 10 min. The size and amount of the PCR products generated were determined by agarose gel electrophoresis in the presence of ethidium bromide and analyzed with the Kodak EDAS 1D analysis package. The sequence of RT-PCR products was determined using the BLAST search program after direct sequencing.

Table 2 Logistic analysis for the risk of aspirin intolerance associated with *FPR2* polymorphisms controlled for age, sex, atopy, smoking status and BMI in Korean asthmatics

				N(%)		*Codominant*			*Dominant*			*Recessive*		
Loci	*rs number*	*Position*	*Genotype*	*AIA*	*ATA*	*OR (95% CI)*	P	P^{corr}	*OR (95% CI)*	P	P^{corr}	*OR (95% CI)*	P	P^{corr}
−5840G>T	rs4801893	Intron1	GG	48 (29.6%)	60 (22.4%)									
			GT	82 (50.6%)	144 (53.7%)	0.83 (0.62–1.11)	0.21	1	0.75 (0.48–1.20)	0.23	1	0.81 (0.50–1.33)	0.40	1
			TT	32 (19.8%)	64 (23.9%)									
−4351A>C	rs10853843	Intron1	AA	145 (90.1%)	238 (89.5%)									
			AC	16 (9.9%)	28 (10.5%)	0.98 (0.50–1.91)	0.94	1	0.98 (0.50–1.91)	0.94	1		.	.
			CC	0 (0%)	0 (0%)									
−4209T>G	rs17694990	Intron1	TT	86 (53.4%)	122 (45.7%)									
			TG	68 (42.2%)	109 (40.8%)	**0.67 (0.49–0.93)**	**0.02**	0.11	0.73 (0.49–1.10)	0.13	0.91	**0.30 (0.13–0.71)**	**0.006**	**0.04**
			GG	7 (4.4%)	36 (13.5%)									
−2940C>T	rs4802863	Intron1	CC	86 (53.1%)	122 (45.7%)									
			CT	68 (42.0%)	109 (40.8%)	**0.69 (0.50–0.95)**	**0.02**	0.16	0.75 (0.50–1.12)	0.16	1	**0.34 (0.15–0.78)**	**0.01**	0.07
			TT	8 (4.9%)	36 (13.5%)									
−1825C>G	rs17834679	Intron1	CC	62 (38.3%)	123 (45.9%)									
			CG	72 (44.4%)	114 (42.5%)	1.28 (0.95–1.71)	0.10	0.69	1.34 (0.89–2.02)	0.17	1	1.47 (0.83–2.61)	0.19	1
			GG	28 (17.3%)	31 (11.6%)									
−1750T>C	rs17695020	Intron1	TT	135 (83.3%)	218 (82.0%)									
			TC	27 (16.7%)	43 (16.2%)	0.78 (0.47–1.30)	0.34	1	0.84 (0.49–1.45)	0.53	1			
			CC	0 (0.0%)	5 (1.9%)									
−1365G>A	rs17756793	Intron1	GG	135 (83.3%)	218 (81.3%)									
			GA	27 (16.7%)	45 (16.8%)	0.77 (0.46–1.27)	0.30	1	0.82 (0.48–1.41)	0.47	1			
			AA	0 (0.0%)	5 (1.9%)									
−970T>C	rs17695032	Intron1	TT	135 (83.3%)	221 (82.5%)									
			TC	27 (16.7%)	42 (15.7%)	0.80 (0.48–1.33)	0.39	1	0.86 (0.50–1.49)	0.60	1			
			CC	0 (0.0%)	5 (1.9%)									
−894C>A	rs17756805	Intron1	CC	135 (83.3%)	218 (81.3%)									
			CA	27 (16.7%)	45 (16.8%)	0.76 (0.46–1.25)	0.28	1	0.81 (0.47–1.39)	0.44	1			
			AA	0 (0.0%)	5 (1.9%)									
−421A>G	rs17756817	Intron1	AA	135 (83.3%)	219 (82.0%)									
			AG	27 (16.7%)	42 (15.7%)	0.77 (0.47–1.28)	0.32	1	0.84 (0.49–1.45)	0.54	1		.	
			GG	0 (0.0%)	6 (2.3%)									
+1315A>G	rs17695052	Exon2	AA	135 (83.3%)	217 (81.6%)									
			AG	27 (16.7%)	44 (16.5%)	0.77 (0.46–1.27)	0.31	1	0.82 (0.48–1.42)	0.48	1			
			GG	0 (0.0%)	5 (1.9%)									
ht2			−/−	70 (43.2%)	119 (44.4%)									
			ht2/−	72 (44.4%)	123 (45.9%)	1.11 (0.82–1.51)	0.49	1	1.10 (0.73–1.65)	0.66	1	1.29 (0.68–2.44)	0.44	1
			ht2/ht2	20 (12.4%)	26 (9.7%)									
ht3			−/−	109 (67.3%)	163 (60.8%)									
			ht3/−	49 (30.3%)	85 (31.7%)	0.74 (0.52–1.05)	0.09	0.64	0.78 (0.51–1.20)	0.26	1	**0.33 (0.11–1.02)**	**0.05**	0.37
			ht3/ht3	4(2.5%)	20(7.5%)									

Abbreviations: AIA, aspirin induced asthma; ATA, aspirin-tolerant asthma; BMI, body mass index; CI, confidence interval; *FPR2,* formyl peptide receptor2; OR, odds ratio.
Logistic regression analyses were used to calculate the odds ratio and *p*-values of the codominant model, controlling for age (continuous value), sex (male=0, female=1), atopy status (non-atopy=0, atopy=1), BMI (continuous value) and smoking status (non-smoker=0, ex-smoker=1, smoker=2) as co-variables.



b Haplotypes in *FPR2*

Hap.	-4209T>G	-2940C>T	-1825C>G	-1750T>C	-1365G>A	-970T>C	-894C>A	-421A>G	+1315A>G	Freq.
ht1	T	C	G	T	G	T	C	A	A	0.352
ht2	T	C	C	T	G	T	C	A	A	0.336
ht3	G	T	C	T	G	T	C	A	A	0.211
ht4	G	T	C	C	A	C	A	G	G	0.091
Others	.	.	.	.	.	.	.	.	.	0.010

c LDs among *FPR2* polymorphisms

		|D'|									
		-5840G>T	-4351A>C	-4209T>G	-2940C>T	-1825C>G	-1750T>C	-1365G>A	-970T>C	-894C>A	-421A>G	+1315A>G
r^2	-5840G>T		1	0.013	0.03	0.794	1	1	1	1	0.957	1
	-4351A>C	0.051		1	1	1	0.083	0.1	0.059	0.1	0.104	0.07
	-4209T>G	0.000	0.122		1	0.985	1	1	1	1	0.977	1
	-2940C>T	0.000	0.123	0.995		0.985	1	1	1	1	0.978	1
	-1825C>G	0.330	0.030	0.235	0.234		1	1	1	1	1	1
	-1750T>C	0.098	0.000	0.228	0.231	0.056		1	1	0.986	0.986	1
	-1365G>A	0.100	0.000	0.232	0.236	0.057	0.987		1	0.987	0.973	1
	-970T>C	0.096	0.000	0.226	0.227	0.055	0.986	0.960		1	1	1
	-894C>A	0.100	0.000	0.235	0.236	0.057	0.960	0.974	0.960		0.973	0.987
	-421A>G	0.091	0.000	0.223	0.224	0.057	0.960	0.947	0.973	0.947		0.986
	+1315A>G	0.099	0.000	0.231	0.235	0.057	1	0.987	0.973	0.973	0.960	

Figure 1 Gene map indicating haplotypes of the formyl peptide receptor2 (FPR2). (**a**) Gene map and single-nucleotide polymorphisms (SNPs) of FPR2, located on chromosome 19q13.3–q13.4. Coding exons are indicated by black blocks, and the 5′ and 3′-untranslated regions (UTRs) by white blocks. The base at which translation begins was denoted as nucleotide +1. (**b**) FPR2 haplotypes. (**c**) Linkage disequilibrium coefficients (ID'I and r^2) among FPR2 SNPs.

Statistical analysis

We applied two widely used measures of linkage disequilibrium to all pairs of biallelic loci: Lewontin's D' ($|D'|$)[21] and r^2. Haplotypes for each individual were inferred using the PHASE algorithm (ver. 2.0), developed by Stephens *et al.*[22] Genotype and haplotype distributions were analyzed using logistic models with age (continuous value), gender (male=0, female=1), smoking status (non-smoker=0, ex-smoker=1, smoker=2), atopy (absence=0, presence=1) and BMI as covariates. Differences in the decline of FEV_1 following aspirin challenge among the genotypes were examined using independent *t*-test. Statistical data were managed and analyzed using SAS version 9.1 (SAS, Inc., Cary, NC, USA) and SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA). To correct *P*-values, the effective number of independent markers in *FPR2* was calculated using SNPSpD software (http://genepi.qimr.edu.au/general/daleN/SNPSpD).[23] This program is based on the spectral decomposition of matrices of pairwise linkage disequilibrium between SNPs (*P*-value X 6.7767). Corrected *P*-values less than 0.05 were regarded as being statistically significant. Intergroup comparisons of *FPR2* expression were assessed by the non-parametric Kruskal–Wallis *H*-test for continuous data. If differences were found to be statistically significant, Mann–Whitney *U*-test was applied to analyze differences between the two groups. The *FPR2* protein expressions are expressed as the mean ± s.e. of the mean (s.e.m.).

P-values less than 0.05 were regarded as being statistically significant.

RESULTS

Characteristics of the study subjects

A total of 438 subjects were recruited from the asthma cohort. The clinical characteristics of the study subjects are summarized in Table 1. Significant differences in onset age of asthma, prevalence of smoking and BMI were found between the AERD and the ATA groups ($P<0.05$, Table 2). Aspirin-induced declines in FEV_1 of −15 to 68% were observed.

Frequencies and haplotype construction of FPR2 polymorphisms

In all, 11 polymorphic SNPs in the *FPR2* gene were selected in the National Center for Biotechnology Information SNP database (NCBI SNP DB; build 36). In build 36 of NCBI SNP DB, there were no SNPs in exon 1 and 2 (Figure 1a). The minor allele frequencies of the 11 SNPs in the study subjects are summarized in Supplementary Table 2. The distributions of all loci were in Hardy–Weinberg equilibrium ($P>0.05$). The linkage disequilibrium coefficients ($|D'|$) and r^2 for the *FPR2* polymorphisms were calculated for all study subjects (Figure 1c). Absolute linkage disequilibrium ($|D'|=1$ and $r^2=1$) was present between *−1750T>C* and *+1315A>G*. Tight linkages were also observed between *−4209T>G* and *−2940C>*T ($r^2>0.99$), and among *−1750T>C*, *−1365G>A*, *−970T>C*, *−894C>A* and *−421A>G* ($r^2>0.95$). Ten haplotypes were constructed from the 11 SNPs. Among these haplotypes, *FPR2*-ht2 and *FPR2*-ht3 (frequency > 0.05) were used for analysis, because *FPR2*-ht1 and *FPR2*-ht4 were almost tagged with *−1825C>G* and *−1750T>C*, respectively.

Association of FPR2 polymorphisms with the risk of aspirin intolerance in asthmatics

All of the single SNPs and two haplotypes (ht2 and ht3) were analyzed for association with the risk of AERD using logistic models (Table 2). Association analysis revealed that two SNPs, *FPR2 −4209T>G* and *−2940C>T*, were significantly associated with AERD in the recessive model (P=0.006 and 0.01). The other SNPs and haplotypes and haplotypes were not associated with the risk of AERD. After correction for multiple comparisons, the significant difference remained only for *FPR2 −4209T>G* (P^{corr}=0.04). The frequency of the homozygous GG genotype for *FPR2 −4209T>G* was three times lower in AERD patients, a significant difference from ATA patients (4.4% and 13.5%, respectively).

Differences in the rates of FEV1 decline following aspirin challenge among the genotypes and haplotypes were examined using multiple regression models (Figure 2). Asthmatics homozygous for the minor allele of *FPR2 −4209T>G* exhibited a lower decline in FEV_1 induced by aspirin provocation than did heterozygotes and homozygotes carrying the common allele (11.66 ± 0.76 % vs 6.24 ± 1.19%, P=0.0002, Figure 2).

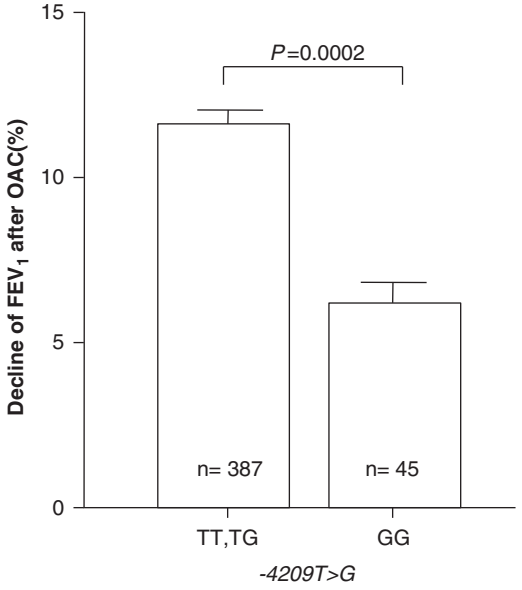


Figure 2 Comparison of the decline of forced expiratory volume in one second (FEV1) (%) induced by aspirin provocation between subjects possessing different alleles of FPR2: -4209T>G. *P*-values were obtained by linear regression analysis, controlled for age (continuous value), sex (male=0, female=1), atopy status (non-atopy=0, atopy=1), smoking status (non-smoker=0, ex-smoker=1, smoker=2) and body mass index (BMI) as co-variables.

Comparison of FPR2 protein expression between FPR2 −4209T>G genotypes using flow cytometry

To determine whether the sequence variants of *FPR2 −4209T>G* affected expression of the *FPR2* protein, we measured *FPR2* expression in CD14-positive (CD14 (+)) monocytes by flow cytometry (Figure 3a and b). *FPR2* protein expression was measured in those obtained from asthmatics with the following genotypes: *FPR2 −4209TT* (n=26), *FPR2 −4209GG* (n=6) and *FPR2 −4209TG* (n=27). *FPR2* expression in CD14 (+) monocytes was significantly different among the three groups (P=0.021). The CD14 (+) monocytes from asthmatic patients homozygous for the minor allele of *−4209T>G (GG)* exhibited a twofolds higher intensity of fluorescence stain for *FPR2* protein than did those from patients having the common allele, *FPR2 −4209 T* (69.41 ± 22.16 vs 27.91 ± 2.24, respectively; P=0.01, Figure 3c). More than 80% CD14 (+) monocytes expressed the *FPR2* protein on their surface regardless of the genotypes of *FPR2 −4209T>G* (Figure 3d).

Comparison of FPR2 mRNA expression between FPR2 −4209T>G genotypes

To investigate the genetic effect of *−4209T>G* polymorphism on the alternative splicing of *FPR2* on PBMC, RT-PCR for the *FPR2* mRNA was performed. As *FPR2 −4209T>G* is located in the intron 2, we amplified mRNA containing *FPR2* using primers to detect alternative splicing as described in methods and Figure 4a. We identified a wild-form (upper band, 360 bp) and an alternative form of *FPR2* (lower band, 231 bp) (Figure 4b). The wild and the variant forms were similarly found in the RT-PCR products on mRNA from PBMC of the subjects having *−4209GG* or those having *−4209TT*. The sequence of RT-PCR products was determined using the BLAST search program after direct sequencing. The alternative form was a variant deleted of exon 2 of 129 bp. (Figure 4c).

DISCUSSION

In the present study, we are the first, to the best of our knowledge, to identify a SNP located in the intron of *FPR2* that is related to aspirin hypersensitivity in asthmatics. This association was characterized using a case–control association study, followed by functional validation of protein expression. The minor allele frequency of *FPR2 −4209T>G* significantly differed between the AERD and ATA groups, even after correction for multiple comparisons. Few studies have explored the association of *FPR2* polymorphisms with human disease. In a previous association study of *FPR2* genetic variants, two SNPs (F110S and C126W), located in the open reading frame of *FPR2,* were found to be associated with juvenile periodontitis.[24] These SNPs were not analyzed in our study, because the design of this study was based on the NCBI DB and these two SNPs were not in NCBI DB. These SNPs were not analyzed in the present study. We found that asthmatics homozygous for the *FPR2 −4209T>G* minor allele exhibited a lower FEV_1 decline after aspirin challenge than patients who were heterozygous or homozygous for the common allele. These data indicate that the minor allele of *FPR2 −4209T>G* may protect against the development of aspirin-induced bronchospasms in asthmatics.

As the *FPR2* was initially cloned based on homology to the *FPR* cDNA,[25,26] the importance of FPR in the host defense against bacterial infections has been demonstrated by the increased susceptibility of *Fpr2* knockout mice to Listeria monocytogenes.[27] Additionally, the *FPR2* has been demonstrated to have novel roles for lipoxin and aspirin-triggered lipoxin in the regulation of T-cell-mediated responses. The lipoxin and aspirin-triggered lipoxin inhibit TNF-alpha secretion from activated T cells via the *FPR2*.[28] In previous functional studies, transcription of *FPR2* was shown to be dramatically upregulated by IL-13, IFN-gamma, IL-4 and IL-6.[29,30] All of these modulators are involved in the development of allergic inflammation in asthmatics.[28] In the present study, asthmatic patients homozygous for the minor *−4209T>G* allele exhibited higher *FPR2* protein expression and less decrease of FEV1 fall rate after aspirin challenge. This data suggest that FPR2, a high-affinity ligand receptor for lipoxins and lipoxinA4 (LXA4) analogue, may exert protective effect against aspirin induced bronchospasm. Lipoxin A4 generation and lipoxin A4 receptor expression were found to be decreased in airway tissues of patients with severe asthma.[31] Thus, downregulation of lipoxin and aspirin-triggered lipoxin may have additive effects on inflammatory reactions in the presence of low-level expression of the *FPR2*, as seen in AERD.

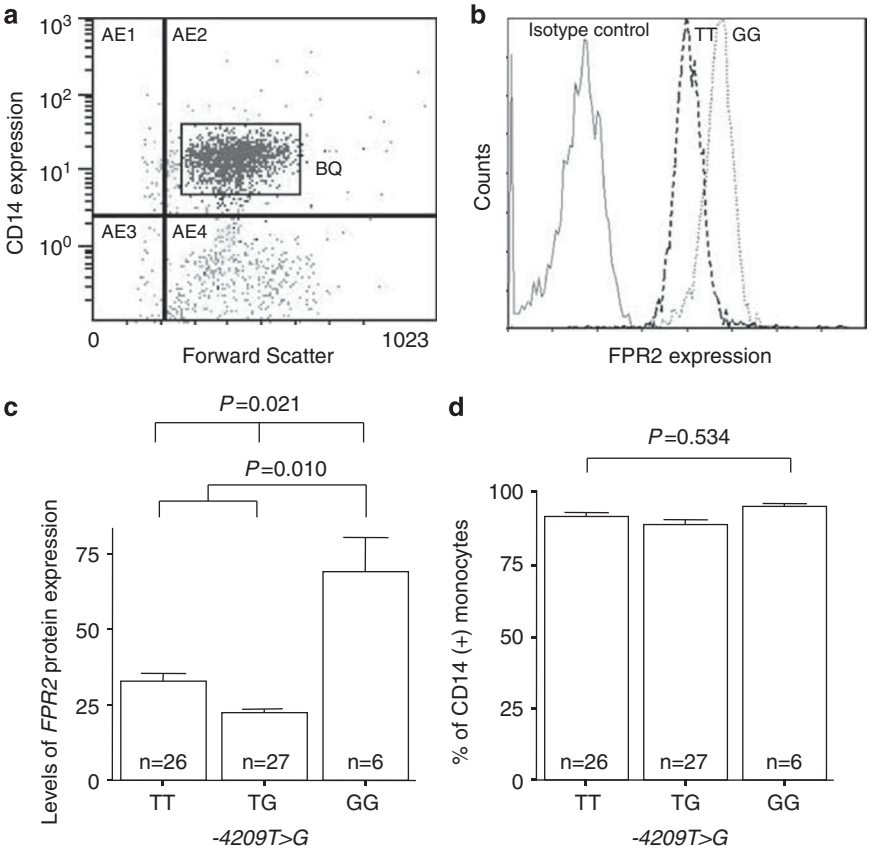


Figure 3 Flow cytometry analysis of formyl peptide receptor2 (FPR2) expression in peripheral blood cells in asthmatics. (**a**) To identify monocytes among peripheral blood cells, cells were gated by forward scatter and CD14 staining. Points inside the box represent CD14 (+) monocytes. (**b**) Histogram of FPR2 protein expression in CD14 (+) monocytes. Plot 1, isotype control; Plot2, -4209TT; and Plot3, -4209GG. (**c**) Mean levels of FPR2 protein expression per CD14 (+) monocyte from asthmatics with the -4209TT, TG and GG genotypes. (**d**) Percentage of CD14 (+) monocytes of each genotype exhibiting expression of FPR2. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

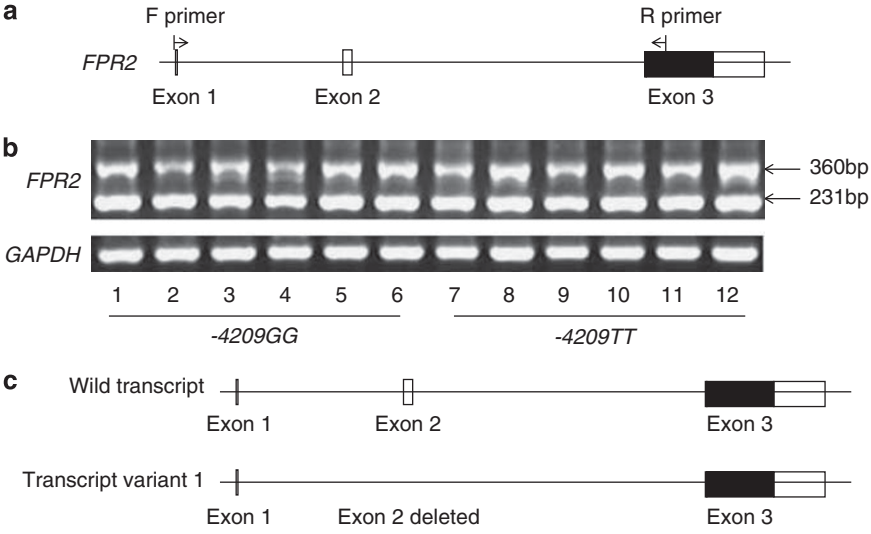


Figure 4 Comparison of formyl peptide receptor2 (FPR2) mRNA levels from its single-nucleotide polymorphisms (SNP) subtypes. (**a**) Organization of human FPR2 and diagram of RT-PCR primer. (**b**) RT-PCR Product size from Exon1 to Exon3 is 231 bp and alternative form of PCR Product size is 360 bp. PBMC containing T allele or G allele was harvested and FPR2 mRNA was measured using by RT-PCR. (**c**) The alternative form has a new transcript variant.

In the flow cytometry analysis, we found that asthmatic patients homozygous for the rare $-4209T>G$ allele exhibited higher *FPR2* protein expression than patients with the common allele. These findings demonstrate that *FPR2* protein expression may be dependent on the genetic function of the $-4209T>G$ allele. It is well known that intronic SNPs can affect transcript processing through alternative splicing or producing RNA secondary structure.[32–34] The *FPR2* $-4209T>G$ located in the second intron. Therefore, we have checked the presence of alternatively spliced forms of *FPR2* by RT-PCR over exon 1 to exon 3. A 231-bp wild form and a new alternative form of 360 bp were clearly visualized. The expression of the two forms of *FPR2* are obvious in the both genotypes of *FPR2* $-4209T>G$. This data indicate that the new alternative spliced variants may not be related with the genotypes of *FPR2* $-4209T>G$. However, we did not measure the m-RNA synthesis of the two forms of *FPR2* using real time PCR. Further investigation is needed to better understand the genetic mechanism of the intronic SNP of *FPR2* on the generation of the wild and the new-spiced variants of this gene.

Another explanation for the differential protein expression of *FPR2* may be different rate of mRNA production, which depends on the secondary structure of mRNA. The secondary structure can be predicted with using *mfold* web server version 3.1.[35] The secondary structure containing the T allele of $-4209T>G$ has a ΔG of $-150.59\,kcal\,mol^{-1}$ and that with G allele has a ΔG of $-150.00\,kcal\,mol^{-1}$. These data indicate that synthesis rate of *FPR2* mRNA may be not different according to the genotypes of *FPR2* $-4209T>G$. Further investigation is needed to better understand the genetic mechanism of the intronic SNP of *FPR2* in aspirin induced asthma (AIA) and to clarify the molecular function of the new-spiced variants of this gene.

In conclusion, we genotyped 11 SNPs located within *FPR2* and examined their associations with AERD. Logistic analysis showed that *FPR2* $-4209T>C$ is associated with AERD. This association was further validated by showing that patients homozygous for the minor allele of *FPR2* $-4209T>C$ exhibit higher levels of *FPR2* protein expression in CD14 positive peripheral blood monocytes compared with those having the common allele. These data indicate that the minor allele of *FPR2* $-4209T>G$ may have a protective role in the development of aspirin hypersensitivity in asthma through an increase in *FPR2* protein expression in inflammatory cells. These results may be useful for the development of new methods to diagnose AERD and new therapeutic strategies for the control of aspirin hypersensitivity in asthma.

ACKNOWLEDGEMENTS

DNA samples were generously provided by the Soonchunhyang University Bucheon Hospital Biobank, a member of the National Biobank of Korea, supported by the Ministry of Health, Welfare and Family Affairs, Republic of Korea.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)