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Association of the *CCR3* gene polymorphism with aspirin exacerbated respiratory disease[☆]

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Gene polymorphism

Summary

Introduction: Aspirin hypersensitivity represents two distinct clinical syndromes, such as aspirin exacerbated respiratory disease (AERD) and aspirin-intolerant chronic urticaria/angioedema (AICU) which have different clinical phenotypes resulting from different genetic backgrounds in a Korean population. Persistent eosinophilic inflammation in airway is a characteristic feature of AERD and chemokine CC motif receptor 3 (*CCR3*) plays an important role in eosinophilic infiltration into the asthmatic airway.

Objectives: The main objective of this study is to investigate the association between *CCR3* gene polymorphisms and aspirin hypersensitivity, including AERD and AICU.

Methods: *CCR3* mRNA expression was measured after an aspirin provocation test by real-time PCR. In total, 330 patients with aspirin hypersensitivity (191 AERD and 139 AICU) and 217 normal healthy controls (NC) were genotyped for two *CCR3* promoter polymorphisms (-520T/G and -174C/T), and the functional effects of the polymorphisms were analyzed applying a luciferase reporter assay and an electrophoretic mobility shift assay.

Results: *CCR3* mRNA expression was significantly increased after aspirin provocation in AERD patients ($P = 0.002$) but not in AICU patients. An *in vitro* functional study showed that the reporter construct having a -520G allele exhibited significantly higher promoter activity compared with the construct having a -520T allele in human myeloid (U937), lymphoid (Jurkat), and mast (HMC-1) cell lines ($P < 0.001$). We found -520G and -174T specific bands on EMSA.

Conclusion: This result suggests that the *CCR3* genetic polymorphisms may contribute to the development of the AERD phenotype and may be used as a genetic marker for differentiating between the two major aspirin hypersensitivity phenotypes.

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Introduction

A hypersensitivity to aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) results in two distinct clinical syndromes, such as aspirin exacerbated respiratory disease (AERD) and aspirin-intolerant chronic urticaria/angioedema (AICU).^{1–3}

In a Korean population, ingestion of aspirin or NSAIDs has been found to exacerbate asthmatic symptoms in about 20% of adult asthmatics⁴ and to worsen cutaneous symptom, such as wheal and swelling, in more than 30% of chronic urticaria patients.⁵ Aspirin hypersensitivity may result from the inhibitory action of aspirin on the formation of prostaglandin, but the pathogenic mechanisms and genetic backgrounds of two clinical syndromes, AERD and AICU, may be different. In the Korean population, the majority of AERD patients demonstrated a non-allergic and non-IgE-mediated phenotype, while AICU patients showed higher atopic rates and higher total serum IgE levels than aspirin-tolerant chronic urticaria patients.⁵ We have also reported the differing genetic backgrounds for AERD and AICU; cysteinyl leukotriene receptor type 1 (*CysLTR1*) genetic polymorphisms are associated with AERD but not with AICU,^{6,7} while high-affinity IgE receptor alpha (*FCER1A*) genetic polymorphisms are associated with AICU.⁸ These findings indicate that these two distinct syndromes of aspirin hypersensitivity have different clinical phenotypes resulting from different genetic backgrounds. Therefore, the aim of this study was to find a genetic marker that differentiates between AERD and AICU, so that it could be used as a genetic marker for diagnosing these two major aspirin hypersensitivity phenotypes.

The *CCR3* gene encodes a G protein-coupled receptor that responds to a variety of CC chemokines, including eotaxin (CCL11), eotaxin-3 (CCL26), MCP-3 (CCL7), MCP-4 (CCL13), and RANTES (CCL5).^{9,10} It expressed not only in eosinophils but also in basophils, Th2 lymphocytes, macrophages, and airway epithelial cells.^{9–12} Its importance in airway eosinophilic inflammation has been addressed in several studies.^{12–14} *CCR3* is critical for eosinophilic infiltration into airways,^{12,13} and *CCR3* antagonists are likely to be effective therapies for asthma.¹⁴

Aspirin hypersensitivity and the aspirin triad are usually accompanied by eosinophilia of the upper and lower airways.¹⁵ Eosinophilic infiltration in bronchial or nasal polyps is a characteristic feature of AERD.^{16,17} In aspirin-sensitive polyps the number of eosinophils is significantly increased and eotaxin levels are also higher than in non-allergic or allergic patients.¹⁸ Considering the pronounced eosinophilic infiltration in AERD patients, we hypothesized that airway eosinophilic inflammation via dysregulation of the *CCR3* gene may play an important role in AERD.

In this study, we examined *CCR3* gene expression in peripheral blood mononuclear cells from patients with aspirin hypersensitivity after an aspirin challenge test, and then investigated whether the *CCR3* gene polymorphism is associated with the differentiation of aspirin hypersensitivity by conducting a genetic case–control study among Korean subjects.

Materials and methods

Study subjects of genetic association study

Three hundred and thirty patients with aspirin hypersensitivity which consist of 191 AERD patients and 139 AICU patients, and 217 normal healthy volunteers were enrolled at Ajou University Hospital, Suwon, Korea. The AICU patients were defined as those who had had more than 6 weeks of daily itchy wheals; had a history of chronic urticaria/angioedema, which was aggravated by ingestion of more than two kinds of NSAIDs; and showed positive responses to the oral aspirin challenge test. The AERD patients were chronic asthmatic patients showing a positive response to a lysine aspirin bronchoprovocation test. The AERD patients had upper and lower respiratory symptoms without any history of drug-induced cutaneous symptoms. Patients who had both AERD and AICU were excluded for the study. All subjects gave informed consent, and the protocol was approved by the institutional review board of Ajou University Hospital, Korea.

Genomic DNA preparation and SNP genotyping

Genomic DNA was prepared from peripheral blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocol. Genotyping was performed using the SNaPSHOT ddNTP primer extension kit (Applied Biosystems, Foster City, CA, USA). The sequences of the amplifying and extension primers for *CCR3*-520T/G were as follows: forward, 5'-AGC CAGGTTCTCAAGTC-3'; reverse, 5'-TCTCTTACGGCATTTC C-3'; and extension, 5'-TTTCCCCATTAACATAATGAATG-3'. The sequences of the amplifying and extension primers for *CCR3*-174C/T were: forward, 5'-AATGCAAAATGCCGTAAG-3'; reverse, 5'-ACAAACTGGGCCATCAG-3'; and extension, 5'-GG CATATCAGAGGTGCCAGGA-3'.

Changes of mRNA expression from peripheral blood mononuclear cells during the aspirin challenge test

For this study, we enrolled 15 AERD, 23 AICU and 26 normal volunteers among the study subjects enrolled for the genetic association study. We matched age and gender distribution, and gave them an aspirin challenge test. All subjects gave informed consent, and the protocol was approved by the institutional review board of Ajou University Hospital, Korea. All medications, including theophyllines, β_2 agonists, leukotriene receptor antagonist, anti-histamines, and inhaled corticosteroids were stopped 3 days before the aspirin provocation procedure.⁷ The AERD patients were challenged with lysine aspirin inhalations and the AICU patients were challenged via oral routes as described previously.^{6–8} At first, a placebo challenge was performed for AERD patients followed by a lysine aspirin (L-ASA) bronchoprovocation test with increasing doses of aspirin (75–300 mg/ml Althargyl; Dong A Pharmaceutical Co., Ltd., Korea) with monitoring of lung function for more than 5 h. A percent change of FEV₁ within 5 h without any cutaneous symptoms was considered a positive

result for the AERD patients. The changes of FEV₁ in 1 s were monitored following the final dose of the aspirin challenge. The ASA induced change in FEV₁ was calculated as the percentage of post-challenge FEV₁ relative to pre-challenge FEV₁ and was defined as positive if the FEV₁ level declined more than 20% from the baseline.

For normal healthy controls and the AICU patients, 500 mg of aspirin (Rhonal; KunWha Pharmaceutical Co., Seoul, Korea) in tablet form was administered orally, and the subjects were observed for 4 h with monitoring for urticaria and change in lung functions every 30 min. The appearance within 4 h of urticaria without any changes in FEV₁ was considered a positive result for the AICU patients, and no change in FEV₁ within 7 h and any cutaneous symptoms were considered as normal healthy controls.

Real-time PCR

Peripheral blood samples (10 ml) were collected from the study subjects at two points, before the provocation test and at the appearance of the disease symptoms (respiratory symptoms for AERD and cutaneous symptom for AICU patients) after the provocation using Ficoll–Paque Plus (Amersham Biosciences, Sweden) density gradient centrifugation. For normal healthy controls, samples were collected before the oral provocation test and at 6 h later. Total RNA was extracted from peripheral blood mononuclear cells using the TRI Reagent (MRC, USA). Real-time PCR was performed for examination of *CCR3* mRNA expression using the ABI Prism 7500 instrument. The PCR consisted of the cDNA product (200 ng), 125 nM of primer, and 12.5 µl of 2× SYBR Green PCR Master Mix (SYBR Green dye, AmpliTaq Gold, DNA polymerase, dNTP mix, and optimized buffer components; Applied Biosystems) in a total volume of 20 µl. The following primer pairs were used: for *CCR3*, forward 5'-CATTTCGGACCTGCTCTCC-3', and reverse, 5'-TGA CAGTCCGGGCTCGAAGG-3'; and for β -actin, forward, 5'-TCCTTCTGCATCTGTCGGC-3', and reverse, 5'-CAAGAGATGGCCACGGCTGC-3'. Amplification was performed for 40 cycles. Following an initial hot start for 10 min, each cycle consisted of 10 s of denaturation, 30 s of annealing at 60 °C, and 33 s of extension. Relative *CCR3* mRNA expression was calculated using the $2^{-\Delta\Delta C_T}$ method,¹⁹ where $\Delta\Delta C_T = (C_{T, CCR3 \text{ gene}} - C_{T, ACTB \text{ gene}})_{\text{sample}} - (C_{T, CCR3 \text{ gene}} - C_{T, ACTB \text{ gene}})_{\text{standard}}$.

In vitro promoter activity assay

A 587 bp fragment (from -558 to +29) having the human *CCR3* genetic polymorphisms was amplified by polymerase chain reaction (PCR) using the following primers: forward 5'-TC TTCAAGGTTCAATTTCCCC-3', and reverse 5'-GTCTCAACTGTAT CTAGTGAGG-3'. Following digestion with *Kpn1* and *Xho1* (Takara, Shiga, Japan), the fragment was ligated into a *Kpn1/Xho1*-digested pGL3 basic vector (Promega, Madison, WI, USA). The haplotype 1[TC], 2[GT] and 3[GC] fragments were amplified from the DNA of -520 and -174 homozygous and heterozygous subjects. The haplotype 4[TT] construct was generated by site-directed mutagenesis using the haplotype 1[TC] luciferase reporter construct as template. Site-directed mutagenesis was performed using the overlap extension PCR mutagenesis technique with the following primers: Set 1, forward 5'-TCTTC AAGGTTCAATTTCCCC-3', and reverse: 5'-GGTGCCAGGAAAGG

ATTGA-3'; Set 2, forward: 5'-TCAATCCTTTCTCTGGCACC-3', and reverse: 5'-GTCTCAACTGTATCTAGTGAGG-3'. Prior to transfection, the plasmids were sequenced by the dideoxy chain terminator method and analyzed on an ABI 310DNA sequencer (Applied Biosystems).

Four luciferase reporter constructs for the common and rare genotypes were transiently transfected into a myelomonocytic cell line (U937) with lipofectamin (Invitrogen, CA, USA). Other cell lines, consisting of human monocyte cells (THP-1) and human mast cells (HMC-1), were transfected by an electroporator. Briefly, 2×10^5 cells were seeded in 12-well plates and transfected with 1 µg of the reporter construct and 5 ng of PRL SV40 renilla control vector using lipofectamin (ratio, 1:5). The THP-1 and HMC-1 cells were seeded in 24-well plates and transfected using a micro-porator (Incyto, Suwon, Korea). At 24–48 h post-transfection, the cells were lysed and assayed for firefly luciferase activity according to the manufacturer's instructions (Promega, Madison, WI, USA). The transfections and luciferase assays were repeated four times in triplicate ($n = 12$).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed according to the method described previously using nuclear extracts from U937 cells.⁷ The following double-stranded oligonucleotides were used as probes for the electrophoretic mobility shift assay (EMSA): for *CCR3* -520T/G, 5'-AT AATGAATG [T/G] CTCATCATTA-3'; for *CCR3* -174A/C, 5'-CT CAATCCTT [C/T] TCCTGGCAC-3'; and for OCT1 as a nonspecific competitor, 5'-TGTCGAATGCAAATCACTAGA-3'. The double-stranded oligonucleotides were annealed by incubation at 95 °C for 5 min, followed by gradual cooling to room temperature. The double-stranded DNA probes were radiolabeled at their 5'-ends with DNA polymerase I (Klenow fragment) (Promega, Madison, WI, USA) and [α -³²P]CTP. The reaction mixtures (10 µl), which contained 5 µg of nuclear extract, 10 mM HEPES (pH 7.9), 200 ng of poly(dI:dC), 0.005 mM MgCl₂, 70 mM NaCl, 5 mM DTT, 1 mM NaVO₃, 10 mM NaF, 1 mM EDTA, and 5% glycerol, were preincubated on ice for 20 min and then incubated for 20 min on ice in the presence of 0.2 pmol of radiolabeled probe. The reaction mixtures were then subjected to electrophoresis on a native 6% polyacrylamide gel. For competition experiments, unlabeled blunt-ended competitor oligonucleotides were added to the binding reaction mixtures before the addition of the radiolabeled oligonucleotide probes. The gels were dried, and radioactivity was detected using a FLA7000 scanner (Fuji Photo Film, Tokyo, Japan).

Data analysis

We employed a widely used measure of linkage disequilibrium between all pairs of biallelic loci, Lewontin's D' ($|D'|$) and r^2 . The haplotypes of the individuals were inferred using the Expectation-Maximization Algorithm. The genetic effects of the inferred haplotypes were analyzed in the same way as the SNPs. We assessed the differences in genotype frequency between the case and control groups with a chi-square test and by calculating odds ratio (OR) with 95% confidence intervals (CI). The distributions of

CCR3 SNP genotypes and haplotypes were analyzed with logistic regression models that controlled for sex and age as covariables with three alternative models (co-dominant, dominant, and recessive models). We studied the differences in clinical characteristics between the groups with the Student's *t* test (for continuous variables) or a chi-square test (for categorical variables). Differences in the mRNA levels of the target genes were assessed with the Mann–Whitney test. A *P* value of less than 0.05 denoted a statistically significant difference. The statistical analyses were completed using SPSS version 12 software (SPSS Inc., Chicago, IL, USA).

Results

Case–control association study of *CCR3* gene polymorphisms with aspirin hypersensitivity

To identify whether *CCR3* gene polymorphisms may offer a differential genetic marker between AERD and AICU, we performed a genetic association study of the two polymorphisms of the *CCR3* gene (-520T/G and -174C/T) in 330 patients with aspirin hypersensitivity and 217 normal healthy controls. The clinical characteristics of the study subjects are summarized in Table 1. AERD was more commonly found in middle-aged females. The AICU patients showed a higher rate of atopy than did the AERD patients ($P < 0.001$).

In the genetic association analysis among patients with aspirin hypersensitivity, two polymorphisms, -520T/G and -174C/T, were found to be significantly associated with the risk of AERD (Table 2); the frequency of the rare genotype was significantly higher in AERD group than in the AICU group ($P = 0.015$, OR = 2.003 for -520T/G, $P = 0.011$, OR = 2.378 for -174C/T in a recessive analysis model). The significance of each polymorphism remained after Bonferroni's correction ($P = 0.030$ for -520T/G, $P = 0.022$ for -174C/T). The genotype frequency of the synonymous SNP, 51T/C, did not differ between the AERD and AICU groups. In the genetic association analysis between group of patients with aspirin hypersensitivity and the normal healthy control group, no significant association was observed (data not shown). There were no significant differences in mean age, gender distribution, prevalence of atopy, or total IgE level for the *CCR3* genetic polymorphism (data not shown).

The two polymorphisms of the *CCR3* gene, -520T/G and -174C/T, were strongly linked with each other ($ID'1 = 1$, $r^2 = 0.778$), so the haplotype analysis in association with AERD was consistent with the genotype association result (data not shown).

Differential expression of *CCR3* gene after aspirin challenge test in patients with AERD

To investigate how the *CCR3* gene responds when asthmatic symptoms are exacerbated by aspirin, we used quantitative real-time PCR to measure mRNA expression in peripheral blood mononuclear cells from patients with aspirin hypersensitivity (AERD and AICU) and normal healthy controls. We matched age and gender distribution among all volunteers who were given the aspirin challenge test. In AERD patients the *CCR3* mRNA expression significantly increased following the aspirin challenge, together with an exacerbation of asthmatic symptoms ($P = 0.002$), while no significant changes were noted in normal healthy controls and AICU patients ($P > 0.05$) (Fig. 1).

The functional effect of the *CCR3* gene polymorphism on transcriptional activity

To evaluate whether the *CCR3* gene polymorphism was associated with altered transcriptional activity, we used luciferase reporter constructs having four different genotypes (TC, GT, GC, TT) for the two polymorphisms, -520T/G and -174C/T, of the *CCR3* gene.

The constructs with the GC genotype exhibited significantly greater luciferase activity compared with the other constructs in the three different types of cells, human myelomonocytic cells (U937, Fig. 2A), human mast cells (HMC-1, Fig. 2B), and human T lymphocyte cells (Jurkat, Fig. 2C) (all $P < 0.001$).

To investigate whether the *CCR3* gene polymorphism created a transcription factor binding site, we performed an electrophoretic mobility shift assay (EMSA) with nuclear extracts from U937 and THP-1 cells using double-stranded oligonucleotide probes that corresponded to -520T/G and -174C/T (Fig. 3). Specific bands were observed with the -520T/G probe and the -174C/T probe (Fig. 3B and C). The shifted band produced by the -520G probe completely disappeared in the presence of the unlabeled -520G probe,

Table 1 Clinical characteristics of the study subjects for case–control association study.

	ASA (N = 330)	NC (N = 217)	P value	Underlying disease		
				AERD (N = 191)	AICU (N = 139)	P value AIA vs. AICU
Sex (male/total)	138/330(41.8%)	96/217(44.2%)	0.597	69/191(36.13%)	69/139(49.64%)	0.018
Age (year) ^a	39.89 ± 13.30/330	33.75 ± 13.85/213	<0.001	43.28 ± 13.47	35.24 ± 11.59	<0.001
Atopy (presence/total)	194/316(61.4%)	21/182(11.5%)	<0.001	93/178(52.25%)	101/138(73.19%)	<0.001
Log serum total IgE (IU/ml) ^a	5.11 ± 1.21/282	3.63 ± 1.34/49	<0.001	5.06 ± 1.3	5.19 ± 1.08	0.371

ASA; patients with aspirin hypersensitivity, NC; normal healthy control. AERD; aspirin exacerbated respiratory disease, AICU; aspirin-intolerant chronic urticaria, N; number of patients.

^a This value was presented as mean ± SD.

Table 2 Allele and genotype frequencies of *CCR3* gene polymorphisms.

		ASA (N = 330)	NC (N = 217)	P value	Underlying disease		
					AERD (N = 191)	AICU (N = 139)	P value AIA vs. AICU
-520T/G	TT	131(39.7%)	79(36.4%)	0.391	72 (37.7%)	59(42.4%)	0.093
	GT	150(45.5%)	103(47.5%)	0.647	84(44%)	66(47.5%)	0.547
	GG	49(14.8%)	35(16.1%)	0.372	35(18.3%)	14(10.1%)	0.015
	q	0.376	0.399	0.384	0.403	0.338	0.088
-174C/T	CC	152(46.1%)	99(45.6%)	0.612	88(46.10%)	64(46.00%)	0.321
	CT	142(43.0%)	92(42.4%)	0.605	76(39.80%)	66(47.50%)	0.765
	TT	36(10.9%)	26(12.0%)	0.720	27(14.10%)	9(6.50%)	0.011
	q	0.324	0.332	0.606	0.34	0.302	0.317

ASA; patients with aspirin hypersensitivity, AERD; aspirin exacerbated respiratory disease, AICU; aspirin-intolerant chronic urticaria, N; number of patients, q; minor allele frequency. Each P value was calculated with co-dominant, dominant, and recessive models. Logistic regression analysis was applied to control for age and sex as covariables. Bold character indicates significance.

but it remained visible in the presence of the unlabeled -520T probe (Fig. 3B). The shifted band produced by the -174T probe was not visible in the presence of the unlabeled -174T probe, but it remained visible in the presence of the unlabeled -174G probe (Fig. 3C).

Discussion

Although it is generally accepted that an altered eicosanoid metabolism is a factor in both asthma and urticaria,²⁰ the differences in clinical demographics between AERD and AICU patients in the Korean population^{5,6} suggested that different pathogenic mechanisms and genetic backgrounds may be involved. Persistent upper and lower eosinophilic inflammation with chronic rhinosinusitis and nasal polyposis may be a prominent feature of AERD, while mast cell activation through unknown mechanism may be important in the pathogenesis of AICU.⁵⁻⁸

Considering the biological role of CCR3 in eosinophil infiltration to both the skin and the lung,^{12,13} we first investigated the role of CCR3 in aspirin hypersensitivity using an aspirin challenge test and a case-control association study in Korean patients with aspirin hypersensitivity.

This study demonstrates a significant contribution of *CCR3* gene polymorphisms in AERD patients. In this study, mRNA expression of *CCR3* gene was detectable in peripheral blood mononuclear cells from each subject, and there

was a significant increase in the expression after exacerbation of asthmatic symptoms by an aspirin challenge in AERD patients, but not in normal healthy controls and AICU patients. However, we could not compare the protein expression of CCR3 during aspirin challenges in two study groups, AERD and AICU. This finding suggests that CCR3 may play a role in the exaggeration of asthmatic symptoms induced by aspirin exposure in AERD patients. However, an exacerbation of cutaneous symptoms by aspirin exposure in AICU patients may not be related to increased expression of CCR3, because no significant changes were noted after aspirin challenges.

In this study, we noted a significant association between the *CCR3* gene polymorphisms (-520T/G and -174C/T) and AERD, with supporting evidence from *in vitro* functional studies. Previously, genetic association studies of *CCR3* gene polymorphisms have been investigated on asthmatics in different ethnic groups.²¹⁻²⁴ Most of the genetic studies were focused on two synonymous SNPs (51T/C and 240C/T) and two non-synonymous SNPs (824 G/A and 1052T/C). Genetic association results might be controversial because of the ethnic differences. A silent mutation T51C in the *CCR3* gene was only associated with asthma in a British population²³ and not in Japanese,²³ Taiwanese,²² and Korean populations.²¹ In addition, three SNPs (240C/T, 824 G/A, and 1052T/C) were not polymorphic, and the synonymous SNP (51T/C) showed very

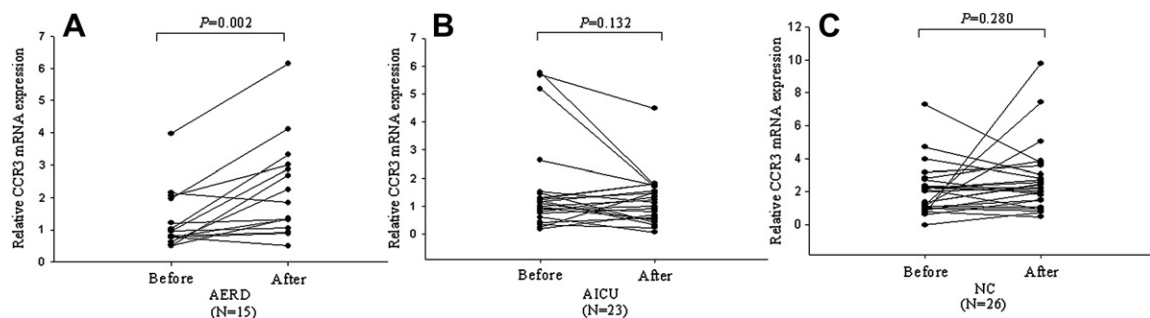


Figure 1 Changes of mRNA expression level of *CCR3* gene after aspirin challenges in three study groups, (A) aspirin exacerbated respiratory disease (AERD), (B) aspirin-intolerant urticaria/angioedema (AICU) and (C) normal healthy control (NC). Statistical analysis was done by the Wilcoxon's signed-ranks test.

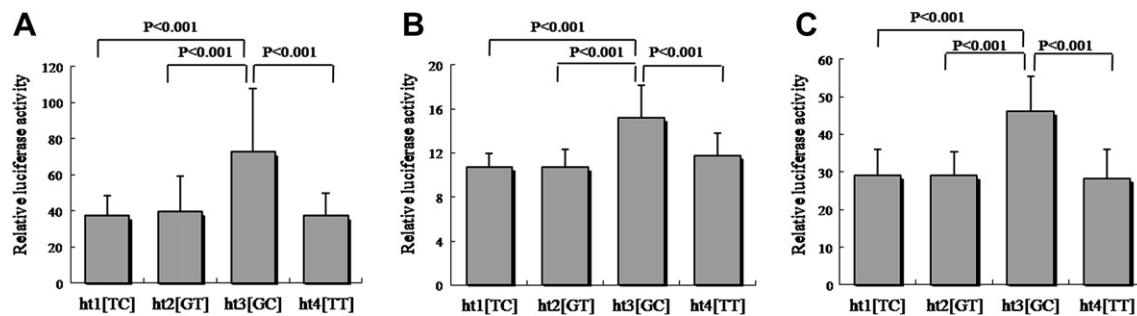


Figure 2 Effect of two promoter polymorphisms of -520T/G, and -174C/T on the transcription activity of human *CCR3* gene in various cell lines, (A) human myeloid (U937), (B) lymphoid (Jurkat), and (C) mast (HMC-1) cell lines. The reporter gene constructs contained the *CCR3* polymorphisms, ht1[TC], ht2[GT], ht3[GC], and ht4[TT]. The relative luciferase activity (RLU) is represented as the ratio of the activity to the luciferase activity in the cells transfected with the promoter-less basic vector, pGL3B. Data are the mean values of four independent experiments (in total, triplicated for each sample, $n = 12$), plus or minus the SD. Statistical analysis was done by the Mann–Whitney *U*-test.

low minor allele frequency in a Korean population.²¹ Instead, three intronic SNPs (-22557G/A, -520T/G, -174C/T) were identified in a Korean population.²¹ In this study, we targeted two intronic SNPs (-520T/G and -174C/T) because these two SNPs are located 520 and 174 bp upstream of the translation start site. Modulation of gene expression by intronic sequences has been reported. We previously reported that intronic sequences might constitute a proximal promoter element located upstream of the translation start site.⁸ According to our preliminary study, the other intronic SNP (-22557G/A) of the *CCR3* gene did not show an association with aspirin hypersensitivity, so it was excluded from further study.

The two intronic SNPs, -520T/G and -174C/T, of the *CCR3* gene were significantly associated with AERD among patients with aspirin hypersensitivity. The percentage of patients having -520 G or -174T homozygous genotypes was significantly higher in the AERD group than in the AICU group. An *in vitro* functional study using a luciferase reporter assay found that the -520G genotype exhibited significantly higher promoter activity than the -520T genotype in three different human cell lines. However, luciferase reporter constructs carrying -174C/T polymorphism did not show any functional influence on the transcriptional activity. We also found -520G specific DNA-binding protein in EMSA, although we will need further investigation to

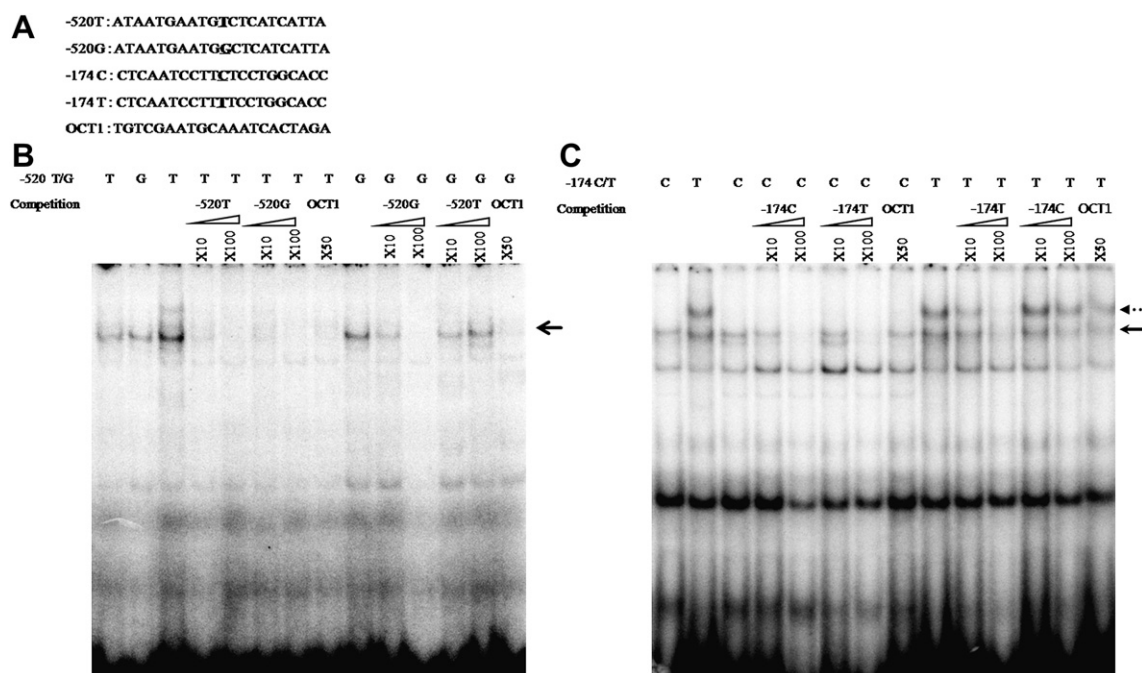


Figure 3 Electrophoretic mobility shift assay for the *CCR3* gene polymorphism using nuclear extracts from U937 cells. (A) Nucleotide sequences of oligonucleotides used as probes and competitors. (B) Differential binding of a nuclear protein to -520T/G alleles. Arrow represents -520G specific band. (C) Differential binding of a nuclear protein to -174C/T alleles. Line arrow and dot arrow represent -174T specific band. Differential binding of a nuclear protein was represented by lane 1–2 according to SNPs. The competition test was shown in lanes 3–15 using self, non-self, and nonspecific probes.

identify the transcription factor. *In vitro* functional study demonstrated that the intronic SNP, -520T/G, of the *CCR3* gene was capable of causing an increased transcription level of the *CCR3* gene.

Previously, it had been reported that eotaxin mRNA and protein expressions were significantly increased in AERD¹⁶ and the eosinophilic nasal polyps might be a source of eotaxin.¹⁸ Additionally, *CCR3* mRNA and protein expressions were increased in the bronchial mucosa of asthmatic patients and related to airway hyperresponsiveness.¹⁴ In this study, although the basal level of *CCR3* mRNA expression were not significantly different between AICU and AERD patients, it increased significantly after aspirin challenges with asthma symptom exacerbations, in the AERD patients, not in AICU patients. Activation of eosinophils is a characteristic finding of nasal and bronchial mucosa of AERD.^{1,16} Taken together, we suggest that the increased expression of *CCR3* may be responsible for eosinophilic inflammation in AERD patients. Furthermore, genetic polymorphism of *CCR3* severely increased eosinophilic inflammation.

In conclusion, these findings suggest that the *CCR3* gene polymorphisms (-520T/G and -174C/T) could increase the genetic susceptibility for AERD, which distinguishes between lung and skin manifestation of aspirin hypersensitivity, AERD and AICU. The *CCR3*-520T/G polymorphism may exaggerate asthmatic symptoms after aspirin/NSAID exposure by increasing the expression level of *CCR3* in the asthmatic airways of AERD patients, which suggests that *CCR3* antagonism might be useful in the management of AERD. However, the exacerbation of cutaneous symptoms by aspirin exposure in AICU patients may not be related to increased expression of *CCR3*.

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Conflict of interest

The authors in this article don't have any significant conflicts of interest with any companies/organizations.

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