

Short communication

Combined effect of IL-10 and TGF- β 1 promoter polymorphisms as a risk factor for aspirin-intolerant asthma and rhinosinusitis

Background: It has been known that interleukin (IL)-10 promoter polymorphisms at -1082A/G, -819T/C and -592A/C, may influence IL-10 expression and associate with asthma. Interleukin-10 facilitates the regulatory function of transforming growth factor (TGF)- β . The goal of this study was to investigate a gene-gene interaction between *IL-10* and *TGF- β 1* polymorphisms in Korean asthmatics with aspirin hypersensitivity.

Methods: Single-nucleotide polymorphism genotyping of *IL-10* and *TGF- β 1* genes was performed and the functional effect of the *IL-10* polymorphisms was analysed applying a luciferase reporter assay and an electrophoretic mobility shift assay.

Results: Among the patients with asthma, polymorphism at -1082A/G was significantly associated with the phenotype of aspirin-intolerant asthma, AIA ($P = 0.007$, $P_c = 0.021$). Moreover, a synergistic effect between the *TGF- β 1* -509C/T and *IL-10* -1082A/G polymorphisms on the phenotype of AIA was noted; when stratified by the presence of rhinosinusitis, the frequency of rare alleles (the CT or TT genotype of *TGF- β 1* -509C/T and AG or GG genotype of *IL-10* -1082A/G) was significantly higher in the patients with AIA (15.2%) when compared with those with ATA (6.3%, $P = 0.031$; odds ratio 4.111; 95% confidence interval 1.504–11.235). In an *in vitro* functional assay, the -1082G reporter plasmid exhibited significantly greater promoter activity when compared with the -1082A construct in Jurkat T cells ($P = 0.011$). Moreover, we found that the transcription factor Myc-associated zinc-finger protein preferentially bound the -1082G allele.

Conclusion: Our results suggest that *IL-10* promoter polymorphisms contribute to the development of AIA and that rhinosinusitis may interact genetically with *TGF- β 1*.

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Aspirin-intolerant asthma (AIA) is characterized by eosinophilic rhinosinusitis, nasal polyposis, aspirin sensitivity and asthma (1). The reported incidence of rhinosinusitis identified by radiography in patients with AIA is around 70% (2). *TGF- β 1* promoter (-509C/T) polymorphism has been recently reported to be associated with rhinosinusitis of AIA patients (3). Furthermore, the regulatory function of transforming growth factor (TGF)- β may be interrelated with interleukin (IL)-10 (4), suggesting that IL-10 is necessary to facilitate TGF- β production as a secondary factor. A gene-gene interaction between *TGF- β 1* and *IL-10* polymorphisms has been also implicated in allergies and asthma (5).

Interleukin-10 is a multifunctional cytokine produced by numerous immune cells (6). Interleukin-10 is a potent growth and differentiation factor in activated human B-lymphocytes, which may contribute to the severity of allergic disease (7). The number of *IL-10* mRNA-expressing cells in bronchoalveolar lavage fluid is

significantly elevated in subjects with atopic asthma when compared with control subjects without asthma (8). Animal models have also demonstrated that IL-10 plays an important role in the induction of airway hyper-responsiveness (9, 10).

In this study, we performed genetic association study of *IL-10* promoter polymorphisms (-1082A/G, -819T/C and -592A/C) and investigated gene-gene interaction between IL-10 and *TGF- β 1* gene polymorphisms among Korean patients with asthma.

Methods

Subjects

The subjects (498 with asthma and 448 normal controls) were enrolled at the Ajou University Medical Center in Suwon, Korea. The diagnosis of AIA was based on a positive response to lysine-aspirin broncho-provocation test (11). The presence of

Table 1. Clinical characteristics of the study subjects

	Asthmatics (n = 498)	Normal controls (n = 448)	P-value	Aspirin-challenged asthmatics		
				AIA (n = 173)	ATA (n = 260)	P-value
Gender, male/total (%)	203/498(40.76)	208/448(46.4)	0.089	64/173(36.99)	112/260(43.07)	0.231
Age (year)*	42.46 ± 14.03/498	33.21 ± 14.63/448	<0.001	43.23 ± 13.55/173	42.18 ± 14.63/260	0.450
Atopy, presence/total (%)	243/419(58)	38/292(13.01)	<0.001	84/156(53.84)	117/207(56.52)	0.670
Asthma duration (years)*	5.89 ± 5.83/306	—	NA	6.59 ± 5.97/125	5.15 ± 6.10/139	0.055
FEV ₁ (% predicted)	82.15 ± 23.54/468	—	NA	79.89 ± 25.48/162	83.91 ± 21.22/252	0.096
% fall of FEV ₁ by aspirin	16.69 ± 11.74/172	—	NA	22.37 ± 10.91/100	7.08 ± 3.94/59	<0.001
PC ₂₀ methacholine (mg/ml)	8.68 ± 22.25/392	—	NA	8.90 ± 31.32/119	8.59 ± 16.71/225	0.905
Log serum total IgE (IU/ml)*	5.11 ± 1.4/458	3.64 ± 1.34/49	<0.001	5.07 ± 1.32/150	5.13 ± 1.46/254	0.679
Rhinosinusitis, presence/total (%)	272/428(63.55)	—	NA	105/137(76.64)	143/256 (55.85)	<0.001
Paranasal sinusitis, presence/total (%)	76/10(27.14)	—	NA	58/122(47.50)	9/139(6.47)	<0.001
Nasal polyp, presence/total (%)	79/283(27.92)	—	NA	65/125(52.0)	7/139(5.03)	<0.001

AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma; NC, normal controls; n, number of patients; NA, not applicable.

*This value was presented as mean ± SD. Values in bold indicate significant P value.

rhinosinusitis and nasal polyps was evaluated using a paranasal sinus X-ray and rhinoscopy. All subjects gave informed consent, and the study was approved by the institutional review board of Ajou University Medical Center. The clinical characteristics of the study subjects are summarized in Table 1.

Single-nucleotide polymorphism genotyping

Genomic DNA was extracted from 0.3 ml of whole blood using the Puregene DNA Purification Kit (Gentra, Minneapolis, MN, USA). Single nucleotide polymorphism (SNP) genotyping was performed using the SNaPshot Primer Extension Kit (Applied Biosystems, Foster City, CA, USA). The sequences of the amplification and extension primers of IL-10 genetic polymorphisms have been presented in the legend of Table 2. Single nucleotide polymorphism genotyping of *TGF-β1* -509C/T was performed according to previously reported method (3).

In vitro functional study

A 1311-bp fragment (from -1112 to +199) of the human *IL-10* promoter was amplified by polymerase chain reaction (PCR) using the following primers: forward 5'-CTTGGTAGGAAGACAA-CACTACTAAGG-3' and reverse 5'-AAAGAAAGTCTTCACTC-TGCTGAAGAGCTCAAC-3' (*KpnI* restriction site to the forward primer and *SacI* site to the reverse primer). Following digestion with *KpnI* and *SacI* (New England Biolabs, Schwalbach, Germany), the fragment was ligated into *KpnI/SacI*-digested pGL3 basic vector (Promega, Madison, WI, USA). The -1082A promoter fragments were amplified from the DNA of -1082AA homozygous subjects. The -1082G construct was generated by site-directed mutagenesis using the -1082A luciferase reporter construct as template. Site-directed mutagenesis was performed using the overlap extension PCR mutagenesis technique (12). Prior to transfection, the plasmids were sequenced by the dideoxy chain terminator method and analysed on an ABI 310 DNA sequencer (Applied Biosystems).

Human Jurkat T cells were transfected using an MP-100 micro-porator (Distal Biotechnology, Seoul, Korea) and MPK-1096 (Distal Biotechnology) according to the manufacturer's instructions.

Nuclear extracts from Jurkat cells were prepared as described previously performed (13). The oligonucleotides used for the electrophoretic mobility shift assay (EMSA) were presented in the legend of Fig. 1.

Table 2. Frequencies of genotype and haplotype in aspirin-challenged asthmatics

		AIA, (n = 173) (%)	ATA, (n = 260) (%)	P-value	OR (95% CI)
-1082A/G	AA	149 (80.34)	233 (89.61)	0.006	1.000 (reference)
	AG	32 (18.49)	26 (10.0)	0.007	2.111 (1.221, 3.648)
	GG	2 (1.15)	1 (0.38)	0.282	3.029 (0.272, 33.667)
-819T/C	TT	79 (45.66)	129 (49.61)	0.510	1.000 (reference)
	CT	74 (42.77)	104 (40.0)	0.486	1.171 (0.796, 1.722)
	CC	20 (11.56)	27 (10.38)	0.763	1.128 (0.611, 2.082)
-592A/C	AA	78 (45.08)	129 (49.61)	0.453	1.000 (reference)
	AC	75 (43.35)	104 (40.0)	0.413	1.199 (0.815, 1.763)
	CC	20 (11.56)	27 (10.38)	0.763	1.128 (0.611, 2.082)
HT1	[ATA]	228 (66.66)	360 (69.49)	0.456	1.000 (reference)
HT2	[ACC]	79 (23.09)	131 (25.28)	0.370	0.952 (0.688, 1.317)
HT3	[GCC]	35 (10.23)	27 (5.21)	0.005	2.046 (1.206, 3.473)

AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma; n, number of patients; q, minor allele frequency.

Logistic regression analysis was used to control for age and gender as covariables. The sequences of the amplification and extension primers were as follows: for -1082A/G, forward 5'-acacacacacaaatcaag-3', reverse 5'-ataggaggctcctacttcttc-3', and extension 5'-tcctctacatccctacttccc-3'; for -819T/C, forward 5'-gaaaccaaattctcagttggc-3', reverse 5'-atgaccctaccgtctctattt-3', and extension 5'-tggtgtaccctgtacaggtgatgtaa-3'; and for -592A/C, forward 5'-aaacatrgcctga-gaatcctaa-3', reverse 5'-aagtaaaaatgaggggtgg-3', and extension 5'-ctttccagagactggcttctcag-3'.

*Each P-value was calculated with co-dominant, dominant and recessive models. Values in bold indicate significant P value.

Statistical analyses

The chi-squared tests were used to detect a significant departure in genotype frequency from Hardy-Weinberg equilibrium at each SNP and assess differences between the *IL-10* polymorphisms. Logistic regression analysis was used to analyse the SNPs and haplotypes controlling for age and gender as covariates with three alternative models (codominant, dominant and recessive). All statistical analyses were performed using spss v.11 (SPSS Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$. The Bonferroni correction is used for a multiple-comparison correction.

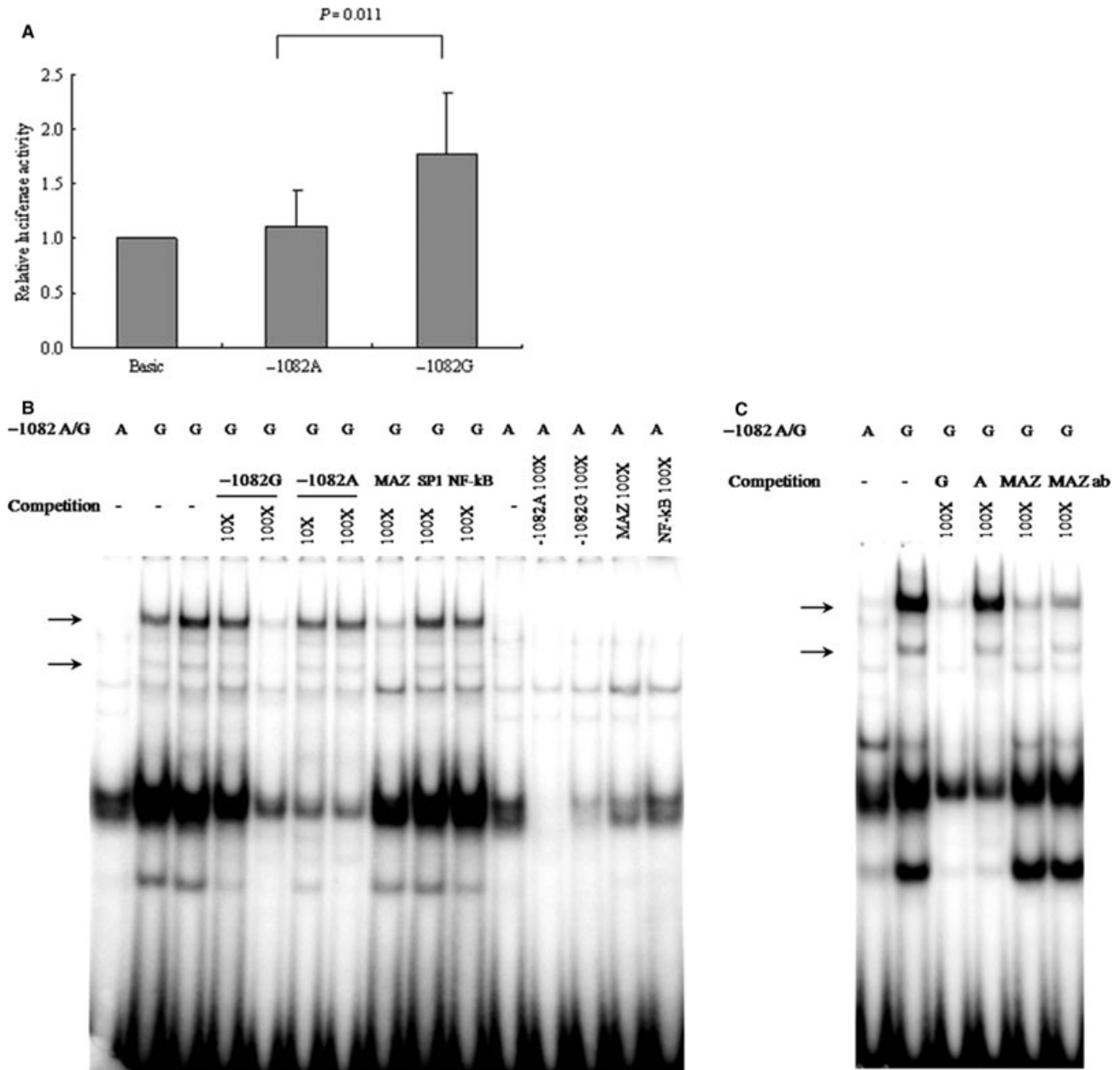


Figure 1. Functional effect of the -1082A/G polymorphism on transcriptional activity. (A) Relative luciferase production (ratio of luciferase production driven by the reporter construct to that by the promoter-less control vector, pGL3-Basic) in Jurkat T cells. The P -value was determined by a Mann-Whitney test. (B) EMSAs for the -1082A/G polymorphism using Jurkat cell nuclear extracts. (C) Identification of transcription factor binding to the -1082A/G probes using a competitive binding assay. Sequences of the oligonucleotides used as probes and competitors as follows; -1082A: gctcttttgggaagggaagtaggg; -1082G: gctcttttgggagggaagtaggg; MAZ: ctgcgcctcccccgc; SP-1: attcgatcggggcgggcgagc; NF-kb: agttgaggggactttccagg.

Results

Case and control association study of IL-10 polymorphisms

We performed a genetic association study of polymorphisms in *IL-10* (-1082A/G, -819T/C and -592A/C) using 498 subjects with asthma who were divided into two groups, AIA and aspirin-tolerant asthma (ATA), based on the presence of aspirin hypersensitivity.

Genetic association analysis of the asthma and normal control groups revealed no significant association with asthma (data not shown). However, the -1082A/G polymorphism in *IL-10* was significantly associated with the risk of aspirin hypersensitivity among aspirin-challenged subjects with asthma ($P = 0.007$, Table 2). The significance of the -1082A/G polymorphism in *IL-10* remained after a Bonferroni correction ($P_c = 0.021$). The frequency

of haplotype 3 [GCC] containing the rare allele of the *IL-10* -1082A/G polymorphism was higher in the AIA group than in the ATA group ($P = 0.005$, $P_c = 0.015$).

Combined effect of polymorphisms in *IL-10* and *TGF-β1* on aspirin hypersensitivity in subjects with asthma

We also genotyped the -509C/T polymorphism in *TGF-β1*, as the regulatory function of *TGF-β* may be interrelated with *IL-10*, and a synergistic effect on allergies and asthma has been reported between polymorphisms in *TGF-β1* and *IL-10*. A gene-gene interaction between the *IL-10* -1082A/G and *TGF-β1* -509C/T polymorphisms was observed in asthmatics [$P = 0.024$; odds ratio (OR) 2.664; 95% confidence interval (CI) 1.230–5.468; Table 3]. When stratified by the presence of rhinosinusitis, the frequency of rare allele carriers (CT or TT genotype of *TGF-β1* -509C/T and the AG or GG genotype of *IL-10* -1082A/G) was significantly higher in the AIA group (15.2%) than in the ATA group (6.3%, $P = 0.031$; OR 4.111; 95% CI 1.504–11.235).

Functional effect of the *IL-10* -1082A/G polymorphism on transcriptional activity

The construct containing the -1082G allele exhibited significantly greater luciferase activity when compared with the constructs containing the -1082A allele in human Jurkat T cells ($P = 0.011$, Fig. 1A).

In EMSA using nuclear extracts prepared from Jurkat T cells, a specific band was observed with the -1082G probe, but not with the -1082A probe (Fig. 1B). Moreover, the shifted band corresponding to the -1082G probe was successfully competed by the unlabeled MAZ probe, but not by the unlabeled SP1 and nuclear factor κB probes (Fig. 1B). The -1082G-specific band was also

competed by anti-MAZ antibodies (MAZ L-20; Biotechnology, Inc., Santa Cruz, CA, USA) but not supershifted (Fig. 1C).

Discussion

This is the first study to demonstrate *IL-10* as a risk factor for AIA and a combined effect with *TGF-β1* on rhinosinusitis in AIA patients.

Genetic associations have been reported between polymorphisms in *IL-10* and asthma (14–17), the exacerbation of asthma by dust mite allergen (18), and COPD (19, 20), although the direction of these associations is unclear. The *IL-10* ATA haplotype (-1082A, -819 T and -592 A) is considered to be a risk haplotype in people with atopic asthma in North India (15), and is negatively associated with PC₂₀ methacholine and the per cent predicted forced expiratory volume in 1 s (FEV₁) in children with asthma (16), as well as the eosinophil count and circulating IgE level in adults (17). An *IL-10* ATA haplotype (-1082A, -819T and -592A) associated with reduced *IL-10* production has been associated with severe asthma (14). However, the -1082GG homozygous genotype was associated with the exacerbation of asthma among Costa Rican children exposed to dust mite allergen (18). Seifart et al. (19) reported that the *IL-10* -1082G allele was more common in patients with COPD and was associated with an altered inflammatory response in COPD.

In this study, based on genotype frequency data, we could identify the *IL-10* -1082G allele as a risk factor for AIA. In addition, we identified *IL-10* -1082G as a high-producer allele in human Jurkat T cells using a luciferase reporter assay. The *IL-10* -1082G allele promoted the binding of MAZ (21), suggesting MAZ as a positive regulator of *IL-10*.

Rhinosinusitis, a key feature of AIA, was found to be associated with gene-gene interaction between the *IL-10* -1082A/G and *TGF-β1* -509C/T polymorphisms in AIA. Previously, we reported that the *TGF-β1* -509C/T polymorphism contributed to the development of AIA with rhinosinusitis by increasing *TGF-β* production in the nasal mucosa and/or polyp tissues of patients with AIA (3). In addition, *IL-10* was reported to facilitate the regulatory function of *TGF-β* via the expansion of induced *TGF-β*-producing cells and the support of *TGF-β* release through the regulation of Th1 cytokine production (4). The synergistic influence of polymorphisms in *IL-10* and *TGF-β1* was also illustrated by the altered severity and long-term complications of asthma in response to IgE synthesis (5). Taken together, our results indicate that alterations in *IL-10* production caused by the -1082A/G polymorphism in *IL-10* may contribute to the development of AIA with rhinosinusitis, and that this effect is strengthened by a genetic interaction between the *IL-10* -1082A/G and *TGF-β1* -509C/T polymorphisms.

Table 3. Frequencies of combined genotype of the *IL-10* -1082 A > G and *TGF-β1* -509C>T polymorphisms

	AIA (%)	ATA (%)	<i>P</i> -value	OR (95% CI)
Aspirin-challenged asthmatics				
Group I	32 (18.5)	62 (23.8)	0.193	1.000
Group II	107 (61.8)	171 (65.8)	0.415	1.212 (0.742, 1.979)
Group III	12 (6.9)	11 (4.2)	0.274	2.113 (0.840, 5.318)
Group IV	22 (12.7)	16 (6.2)	0.024	2.664 (1.230, 5.468)
Aspirin-challenged asthmatics with rhinosinusitis				
Group I	16 (15.2)	37 (25.9)	0.059	1.000
Group II	65 (61.9)	88 (61.5)	1.000	1.708 (0.875, 3.332)
Group III	8 (7.6)	9 (6.3)	0.800	2.055 (0.671, 6.291)
Group IV	16 (15.2)	9 (6.3)	0.031	4.111 (1.504, 11.235)

OR, odds ratio; CI, confidence interval. Values in bold indicate significant *P* value. The groups were defined by the combination of *IL-10* and *TGF-β1* polymorphisms. Patients in Group I had *IL-10* -1082 AA and *TGF-β1* -509 CC (used as the reference value). Patients in Group II had *IL-10* -1082 AA and *TGF-β1* -509 CT or TT. Patients in Group III had *IL-10* -1082 AG or GG and *TGF-β1* -509 CC. Patients in Group IV had *IL-10* -1082 AG or GG and *TGF-β1* -509 CT or TT.

The interrelated functions of IL-10 and TGF- β may lead to ongoing inflammation in the nasal mucosa and/or polyp tissues in people with asthma, ultimately contributing to AIA with rhinosinusitis.

In conclusion, polymorphisms in *IL-10* and *TGF- β 1* may positively contribute to the development of AIA with rhinosinusitis through a gene-gene interaction.

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