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Interleukin-10 Gene –627 Allele Variants, Not Interleukin-I Beta Gene and Receptor Antagonist Gene Polymorphisms, Are Associated With Atopic Bronchial Asthma

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Asthma is an airway hyperresponsive disease characterized by the expression of multiple inflammatory genes, including cytokines. Interleukin-I and interleukin-10 (IL-1 and IL-10) are cytokines that might play a role in the process of inflammation and are therefore considered to be involved in the pathogenesis of bronchial asthma. The aim of this study was to test whether the polymorphisms of the promoter region and exon 5 of the IL-1 gene, intron 2 of the IL-1Ra gene, and –627 nucleotide (C/A) of the IL-10 gene could be genetic markers for the susceptibility of bronchial asthma. A normal control group made up of 47 healthy volunteers and 117 patients with bronchial asthma were examined in this study. We analyzed the variable number of tandem repeats at intron 2 of the IL-1Ra gene for the polymorphisms by polymerase chain reaction (PCR). PCR-based restriction analysis of the IL-1 gene polymorphisms of the promoter region and exon 5 was carried out by the endonucleases *Ava* I and *Taq* I, respectively. The IL-10 gene –627 C/A polymorphisms were investigated by PCR-based restriction analysis. The distribution of CC homozygotes in the IL-10 gene was significantly lower in asthma patients than in controls ($P = 0.013$, OR = 3.599, 95% CI = 1.240 ~ 10.441). The polymorphisms studied in the IL-1 genes did not reveal any significant association with bronchial asthma when compared with the control group (promoter region by chi-square test, $P = 0.627$; exon 5 region by Fisher's exact test, $P = 0.403$). Only two alleles of the IL-1Ra gene corresponding to one and two copies of an 86-base pair sequence repeat were identified by PCR in the control group. There were three alleles found in the asthmatic patient group. The results revealed no significant differences between normal individuals and asthma patients ($P = 0.454$, Fisher's exact test). The IL-10 gene –627 "A" allele is an associated risk factor of developing atopic asthma. J. Clin. Lab. Anal. 17:168–173, 2003.

Key words: interleukin-1 beta; interleukin-1 receptor antagonist gene; interleukin-10 gene; genetic polymorphisms; bronchial asthma

INTRODUCTION

Asthma is an airway hyperresponsive and polygenic inherited disease involving many factors (1). Genetic markers of asthma could be clinically useful both for identifying patients at risk and for preventing its occurrence. However, identifying a common genetic maker associated with the susceptibility to asthma presents an important challenge. In a collaborative study on the genetics of asthma, researchers reported that chromosomal regions 2q33, 5p15,

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11p15, l7p11.1-q11.2, 19q13, and 21q21 are linked to asthma (2). Therefore, appropriate association studies of genetic polymorphisms might be useful for searching the possible genetic markers of asthma.

Cytokines are present in serum, and several researchers have reported their association with asthma (3–5). One such cytokine, interleukin-1 (IL-1) located at chromosome 2q12, is a potent proinflammatory agent, which plays a central role in joint inflammation and destruction. Also, increased plasma concentrations of the interleukin-1 receptor antagonist (IL-1Ra) have been found to be associated with disease severity in asthmatic patients (6). The A2 allele of the IL-1Ra gene is known to be associated with non-atopic asthma and therefore could be a potential genetic maker for the prediction of asthma (7). However, a survey of the association between IL-1 gene polymorphisms and atopic asthma disease has yet to be reported. Polymorphisms of the IL-1β promoter region and exon 5, and of the receptor antagonist (IL-1Ra) gene exon 2, have been used to screen the relationship between the occurrence and severity of rheumatoid arthritis and osteoporosis (7,8).

Interleukin-10 is a cytokine that plays a role in both immunoproliferative and inflammatory responses (9). The antiinflammatory effect of IL-10 is through the inhibition of macrophages and human polymorphonuclear leucocytes to the synthesis of proinflammatory cytokines, chemokines, and inflammatory enzymes. Low production of IL-10 was found in the alveolar macrophages and peripheral blood mononuclear cells of asthma patients. The control region of transcription of the IL-10 gene is located at 5′ flanking region and shows a polymorphism in the −627th nucleotide. Lim et al. (10) reported that haplotype of ATA among polymorphisms of −4000, –1200, and −627 of IL-10 gene was associated with severe asthma. Furthermore, Grove et al. (11) found that −627 A allele is associated with a high risk of developing chronic liver disease. Nevertheless, IL-10 −627 polymorphism might play a role in the pathogenesis of asthma.

Therefore, we used polymerase chain reaction (PCR) analysis to investigate the distribution of genotypes between a control group and patients with asthma. In order to test whether or not those polymorphisms could be markers of susceptibility to asthma disease, we compared allelic frequencies in a normal population with asthmatic patients by screening for: 1) polymorphism of the IL-1β gene at promoter region and exon 5 region, and 2) polymorphism of the IL-1 Ra gene of intron 2 region and IL-10 gene at −627 region.

PATIENTS AND METHODS

Patient Selection

A total of 117 patients (61 males and 56 females; age range: 3 to 70 yr; average: 22.7 ± 16.6) with bronchial asthma were enrolled in this study. Patients with atopy tested positive for allergen according to the radioimmunoassay test (RAIST) and pulmonary function test showed obstructive defect. Bronchial asthma (atopy) was diagnosed in patients with symptoms of chronic cough, wheezing dyspnea, and chest tightness in the night or early morning in accordance with the above test. Pulmonary function tests included FEV1 and FEV1/FVC. Results over the 80% predicted values were considered normal. A control group was drawn up of 47 healthy volunteers (17 males and 30 females; age range: l6 to 28 yr; average of 20.8 ± 2.7) who had no history of allergy and routine pulmonary function test revealed normal results. Informed consent was obtained from all individuals that participated in this study. The genomic DNA was prepared from peripheral blood using a Genomaker reagent kit (Blossom, Taiwan).

PCR

PCR was used to identify the genotypes of all of the IL-10 and IL-1 related genes. PCR of the polymorphisms were carried out to a total volume of 50 μl, containing genomic DNA (2–6 *p*mole of each primer); 1X Taq polymerase buffer (1.5 mM $MgCl_2$); and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA). The primers for the IL-1 beta promoter region, exon 5 and IL-1Ra gene polymorphisms are listed in Table 1 and were the same as those in the report by Cantagrel et al. (8). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer). The cycling conditions are also listed in Table 1.

For IL-1Ra intron 2, 10 μl of the products were loaded into 3% agarose gel containing ethidium bromide for electrophoresis and each allele was recognized according to its size. The 86-bp variable number tandem repeat (VNTR) of the IL-1Ra gene was classified as "I" for 410-bp, "II" for 240-bp, "III" for 500-bp, "IV" for 325-bp, and "V" for 595-bp allele. The IL-1 beta promoter polymorphism at position –511 was analyzed by PCR amplification followed by restriction analysis using *Ava* I digestion (New England Biolabs, Beverly, MA). The "C" allele at position 511 was categorized as "C" and showed up as 190-bp and 114-bp on agarose electrophoresis. The "T" allele was 304-bp, categorized as "T", and was encoded at position 511. The region containing the polymorphic site within exon 5 of the IL-1 beta gene was amplified and then digested

TABLE 1. Sequences of the amplification primers in the 5′ to 3′ orientation.

Set	Position	Primer and PCR conditions	PCR product (bp)	Restriction enzyme
Il-1 beta promoter	−511 C/T	U 5′-TGGCATTGATCTGGTTCATC-3′ D 5′-GTTTAGGAATCTGGACCAGA-3′ 95°C × 30″, 55°C × 30″, and 72°C × 30″	190 + 114 or 304	*Ava* I
IL-1 beta exon 5	Exon 5	U 5′-GTTGTCATCAGACTTTGACC-3′ D 5′-TTCAGTTCATATGGACCAGA-3′ 95°C × 30″, 55°C × 30″, and 72°C × 30″	135 + 114 or 249	*Taq* I
LI-1 Ra	Intron 2	U 5′-CTCAGCAACACTCCTAT-3′ D 5′-TCCTGGTCTGCAGGTAA-3′ 95°C × 30″, 58°C × 30″, and 72°C × 30″	I:410 II:240	VNTR
IL10 gene	−627	U 5′-CCTAGGTCACAGTGACGTGG-3′ D 5′-GGTGAGCACTACCTGACTAGC-3′ 95°C × 30″, 50°C × 30″, and 72°C × 30″	176 + 236 or 412	*Rsa* I

U and D respectively indicate upstream and downstream primers; VNTR, variable number tandem repeats.

by *Taq* I (New England Biolabs). Category "E1" was 135-bp + 114-bp and "E2" was 249-bp as shown on electrophoresis.

The IL-10 gene −627 A/C polymorphism was analyzed by PCR amplification followed by restriction analysis using *Rsa* I digestion (New England Biolabs). PCR products of 412-bp that were digested by restriction enzyme were categorized as "A" and showed up as 236-bp and 176-bp on agarose electrophoresis. The categorized "C" allele was undigestable and remained as 412-bp on agarose electrophoresis (Fig. 1).

For statistical analysis, the allelic frequency distributions of polymorphisms in the control and asthma patient groups were compared using the chi-square test. When the assumption of the chi-square test was violated (i.e., when one cell had an expected count of <1, or more than 20% of the cells had an expected count of <5), the Fisher's exact test was used. Odds ratios (OR) with 95% confidence intervals (CI) for disease susceptibility of specific alleles in the polymorphisms of IL-1Ra and IL-10 genes were determined. Results were considered statistically significant when the probability of findings occurring by chance was less than 5% ($P<0.05$).

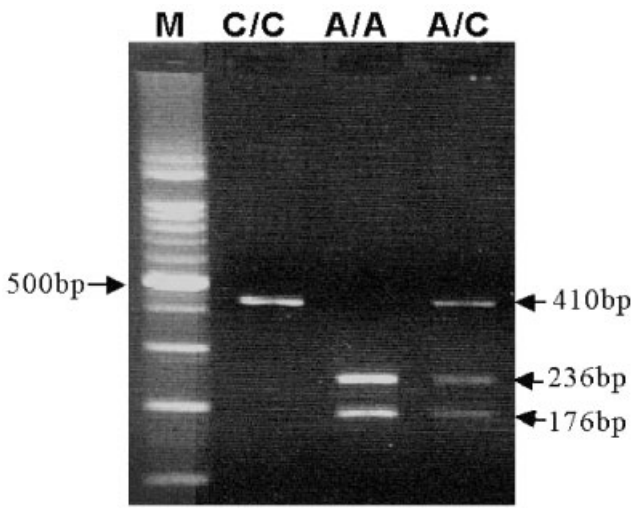

Fig. 1. Product of polymerase chain reaction based restriction analysis of the IL-10 gene −627 A/G polymorphism shown on agarose electrophoresis. Lane 1: marker, 100-bp ladder; Lane 2: CC homozygote, undigested, 412-bp; Lane 3: AA homozygote, 236-bp + 176-bp; and Lane4: C/A heterozygote.

TABLE 2. The allelic frequencies of Il-10 gene polymorphisms among the healthy control subjects and the asthma patients

	Control	Patient	OR	95% CI
CC	11 (18.3%)	6 (5.1%)	1	
AC	22 (36.7)	58 (49.6%)	4.833	1.675 ~ 13.940
AA	27 (45%)	53 (45.3%)	3.599	1.240 ~ 10.441

Chi-square test, $\chi^2 = 8.66$; $P = 0.013$; OR = odds ration; CI = confidence interval.
Allelic frequency of C is 57.9% and 29.9% in control and patient group, respectively. Frequency of A allele is 42.1% and 70.1% in control and patient groups, respectively.

RESULTS

Table 2 shows the frequency distribution of the −627 IL-10 polymorphism genotypes in both patient and control groups. A significantly lower frequency of CC homozygotes was observed in asthma patients compared with controls (CC: 5.1% asthma vs. 18.3% controls, $P = 0.013$, OR for AA homozygote compared with CC homozygote = 3.599 [95% CI = 1.240 ~ 10.441]). These genotypes gave corresponding "A" allele frequencies of 0.421 in controls and 0.701 in asthma patients. "C" allele frequencies were 0.579 in controls and 0.299 in asthma patients.

According to the Fisher's exact test, no significant differences in allelic frequencies of the IL-1 beta gene exon 5 polymorphism between asthma patients and controls were found (Table 3, $P = 0.627$). Furthermore, no differences in the IL-1 beta gene promoter region polymorphism between patients and normal controls were found (Table 3, $P = 0.799$).

No "III" allele was found in either group and no "IV" allele was present in the control group. However, the presence of "IV" was detected in the asthma group and control group (Table 3). The distribution of IL-1Ra gene polymorphism was compared using the Fisher's exact test and no significant differences between the healthy control group and the asthma patient group were revealed ($P = 0.454$). The distribution of "I" homozygotes in the control and asthma groups was 83.0% and 86.3%, respectively.

DISCUSSION

The "A" allele of the IL-10 gene –627 polymorphism was over represented in asthma patients, indicating an association with atopic asthma. This polymorphism, therefore, is a good genetic marker for further studies of the causes of atopic asthma. Neither the IL-1 β gene nor the IL-1Ra gene polymorphisms correlated with asthma in our study. A great amount of the genetic background of asthma is still unidentified, and only a few gene polymorphisms have been studied (10,12,13). We chose to focus on cytokines as candidate genes because they have several proteins that are key components in the pathogenesis of many diseases. The results encourage us to search other cytokine network genes for association with asthma disease because they may provide further information.

IL-10 inhibits cytokine production (14), down-regulates the production of IL-4 and IL-5, and has a wide variety of effects on other immune cells, including stimulation of B cell differentiation and immunoglobulin secretion (9,15). However, the biological effects of IL-10 are difficult to delineate because the activities of this molecule on immune responsiveness vary considerably (16). The roles of IL-10 in allergic inflammation and asthma are contradictory. Makela et al. (17) identified that IL-10 expression is important to the downstream of the inflammatory cascade and it regulates the tone of the airways after allergic sensitization and challenge. Therefore, low levels of IL-10 expression might have a role on the pathogenesis of atopic asthma.

Lim et al. (10) reported that the −627 A allele/ATA haplotype is associated with low IL-10 expression. Therefore, it was evident that the "A" allele was associated with low level expression of IL-10. Low level expression of the IL-10 gene will favor inflammatory, immune mediated, and profibrotic mechanisms of bronchial cells reaction. This might explain the association of the "A" allele with asthma. However, Lim et al. (10) did not find a significant difference between mild asthma and normal controls. They concluded that the IL-10 gene −627 polymorphism has a role in determining disease severity but does not seem to be important in susceptibility. They studied this polymorphism with

TABLE 3. Distribution of interleukin-1 receptor beta gene promoter polymorphism, exon 5 polymorphism, and distribution of interleukin-1 receptor antagonist (IL-1Ra) gene polymorphism between healthy control subjects and asthma patients

Promoter	C/C	C/T	T/T	Total	*P* value	χ^2
Asthma patients	22 (19.0%)	54 (46.6%)	40 (34.4)	116 (00.0%)		
Control	7 (14.9%)	22 (46.8%)	18 (38.3%)	47 (100.0%)	0.799	0.449
Exon 5	E1/E1	E1/E2	E2/E2	Total	*P* value	
Asthma patients	45 (95.7%)	2 (4.3%)	0 (0.0%)	47 (100.0%)		
Control	113 (97.4%)	3 (2.6%)	0 (0.0%)	116 (100.0%)	0.627[a]	
	I	I&II	II	I/IV	Total	*P* value
Asthma patient	101 (86.3%)	12 (10.3%)	2 (1.7%)	2 (1.7%)	117 (100.00%)	
Control	39 (83.0%)	8 (17.0%)	0 (0.0%)	0 (0%)	47 (100.00%)	0.454[a]

[a]Fisher's exact test.

other polymorphisms at –1117 (C/T) and −4000 (G/A) that we did not study. Further studies should be done to clarify this polymorphism with disease severity.

IL-1Ra is an important regulator of inflammation. IL-1Ra is IL-1's natural competitive inhibitor, occupying IL-1 cell surface receptors without triggering signal transduction (18). The type II IL-1Ra allele has been previously found in association with a variety of autoimmune diseases, such as alopecia areata, systemic lupus erythematosus, and ulcerative colitis (19–21). However, instead of type II, the type I allele of IL-1Ra intron 2 influenced the susceptibility of asthma disease in our study. The allelic distribution of intron 2 of the IL-1Ra gene differed between Taiwanese and Caucasian French patients with rheumatoid arthritis (8). In our group, there was no genotype of alleles III, IV, and V in the normal control group. However, there was presence of alleles III and IV in the asthma patient group. This difference might be attributable to the small sample size of the patient and control groups in our study.

IL-1 is involved in a wide spectrum of biological activities such as elevating body temperature and protein and energy mobilization in the acute phase response (22). Although it has been proposed that the IL-1 gene contributes to regulating the level of IL-1β production, we did not find this to be the case with asthma disease. This might have been due to an inappropriate choice of the polymorphic region in IL-1 in our study, rendering the statistical difference insignificant. However, because the relationship between IL-1Ra and asthma disease is not only involved in an inflammatory pathway, the relationship between IL-1 and asthma should be further studied. The genes for IL-1 are located on chromosome 2 and are closely linked with the IL-1Ra gene (23). Linkage dysequilibrium of the genes during recombination may be one of the causes of nonassociation.

Pillay et al. (6) studied the allelic frequency of the IL-1Ra gene and noted that the I allele of IL-1Ra was significantly over represented in most black subjects (90%) as compared to white subjects in their study (74%, $P<0.0001$). The II allele was significantly under represented in black subjects (11%) as compared to white subjects in their study (27%, $P<0.0001$). However, there was no difference in the frequency of VNTR of the IL-1Ra between black asthmatics and black controls and between white asthmatics and white controls in Pillay's study. Our data are compatible with Pillay's result although we did not compare racial differences. Mao et al. (7) showed that the II allele of the IL-1Ra gene is associated with nonatopic asthma (OR = 5.71). Although we observed a relatively higher ratio of the I allele of the IL-1Ra gene in atopic asthma patients than in the control group (86% vs. 83%), the difference was not significant (OR = 0.55). Therefore, it indicates a different pathogenesis between atopic and nonatopic asthma.

In most of our patients, asthma was diagnosed after symptoms developed because there are currently no predictive markers for the disease. Therefore, a reliable marker for asthma could lead to earlier diagnosis and treatment which would have a significant impact on patient care, as well as on health care costs. Our data indicate that the IL-10 gene polymorphism is a candidate genetic marker to screen for asthma. However, the IL-1Ra gene intron 2 and IL-1 β gene polymorphisms are not associated with atopic asthma disease and therefore are not appropriate genetic markers of asthma.

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