

# Interleukin 6 Gene Polymorphisms Are Associated with Systemic Lupus Erythematosus in Koreans

JA-YOUNG JEON, HYOUN-AH KIM, SEUNG-HYUN KIM, HAE-SIM PARK, and CHANG-HEE SUH

**ABSTRACT. Objective.** Interleukin 6 (*IL-6*) gene polymorphisms are known to play a role in chronic inflammatory disorders. We searched for polymorphisms in the *IL-6* gene and described their pathogenic role in Korean patients with systemic lupus erythematosus (SLE).

**Methods.** Genomic DNA was extracted from 151 patients with SLE and 151 controls, and about 1.4 kb-sized *IL-6* genes located between promoter region and exon 2 region were amplified by polymerase chain reaction. The promoter activity was analyzed by luciferase reporter assay in Hep3B cells and HeLa cells.

**Results.** We identified 4 single-nucleotide polymorphisms (SNP; -572 C > G, -278 A > C in the promoter, and 330 T > G, and 334 A > T in exon 2) and a -373 A<sub>n</sub>T<sub>n</sub> tract polymorphism in the *IL-6* gene. The genotype frequency, -373 A<sub>10</sub>T<sub>11</sub>, -278 C, and 334 T allele were significantly associated with SLE ( $p < 0.001$ ,  $p = 0.03$  and  $p = 0.005$ , respectively). Patients with SLE carrying the -572 G allele had anti-dsDNA more frequently ( $p = 0.007$ ). In addition, thrombocytopenia was significantly more common in patients carrying the -278 C allele ( $p = 0.006$ ). In the haplotype analysis, patients with SLE had more frequently haplotype HT3 (CA<sub>10</sub>T<sub>11</sub>ATA, dominant model,  $p = 0.012$ ) that was associated with arthritis, leukopenia, anti-dsDNA, and hypocomplementemia. Promoter reporter structures carrying the -278 C allele displayed significantly higher promoter activity than the -278 A allele in Hep3B cells ( $p < 0.001$ ) and HeLa cells ( $p < 0.001$ ).

**Conclusion.** These data suggest that *IL-6* gene polymorphisms are associated with disease susceptibility and phenotype of SLE. In addition, promoter polymorphisms may be involved in regulation of *IL-6* expression. (J Rheumatol First Release Sept 15 2010; doi:10.3899/jrheum.100170)

## Key Indexing Terms:

SYSTEMIC LUPUS ERYTHEMATOSUS  
SINGLE NUCLEOTIDE POLYMORPHISM

INTERLEUKIN 6  
PROMOTER

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by dysregulation of the immune system, involving the hyperactivity of T cell and B cell, elevated production of pathogenic autoantibodies, complement activation, and the formation of immune complexes causing multiorgan damage by deposition in host tissue<sup>1</sup>. A multifactorial interaction between genetic and environmental factors may be involved. Women of African American, Hispanic, and Asian ethnicity appear to have a higher risk of SLE, and a strong familial aggregation, with a much higher

frequency among first-degree relatives. The concordance of the disease in identical twins is approximately 25-50%, while it is around 2-5% in dizygotic twins<sup>2,3</sup>. These findings suggest that genetic factors play an important role in predisposition to the disease<sup>4,5</sup>.

Interleukin 6 (IL-6) is a multifunctional cytokine involved in regulation of the acute inflammatory response as well as modulation of specific immune responses including B cell and T cell differentiation<sup>6,7,8</sup>. B cell hyperactivity, elevated production of autoantibodies, and overexpression of T<sub>H</sub>2 cell cytokines IL-6 and IL-10 are characteristic of SLE<sup>9,10</sup>. In a previous study, we found that patients with SLE had higher serum IL-6, IL-10, IL-12, and interferon- $\gamma$  levels, but lower serum IL-2 levels than controls<sup>11</sup>. In addition, the serum IL-6 level was significantly elevated in patients with active SLE and correlated with the SLE Disease Activity Index (SLEDAI), erythrocyte sedimentation rate, and C-reactive protein (CRP). *IL-6* overexpression in SLE could result from an abundance of upregulating factors and/or polymorphisms in regions having gene regulatory implications. The *IL-6* gene is located on the short arm of chromosome 7p21 and organized in 5 exons and 4 introns. To date, several studies have been published suggesting that

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the *IL-6* gene polymorphism is associated with susceptibility and outcome of a variety of acute and chronic inflammatory diseases, including rheumatoid arthritis<sup>12</sup>, diabetes mellitus<sup>13</sup>, atherosclerosis<sup>14</sup>, Alzheimer disease<sup>15</sup>, and juvenile chronic polyarthritis<sup>16</sup>.

Although several genetic association studies of the *IL-6* gene polymorphisms with SLE have been reported, most of them have focused on 2 common polymorphisms, such as -174 G > C and variable number tandem repeat. Moreover, the possible association between *IL-6* gene polymorphisms and SLE with functional relevance has rarely been addressed. Therefore, we investigated whether genetic polymorphisms of the *IL-6* gene are associated with the pathogenesis of SLE in Koreans.

## MATERIALS AND METHODS

**Subjects.** One hundred fifty-one patients with SLE and 151 controls were enrolled from Ajou University Hospital in Suwon, Korea. All patients satisfied at least 4 of the 1982 revised American College of Rheumatology (ACR) criteria for SLE<sup>17</sup>. The patients' medical histories were reviewed from the onset of disease until admission to the study. Clinical features of the disease as defined by ACR criteria were recorded in standardized questionnaires. The controls were chosen from the general population using a screening questionnaire, which had to indicate no history of rheumatic diseases or autoimmune disorders. All the subjects participating in this study were ethnically Korean. The study was approved by the Institutional Review Board of Ajou University Hospital and all subjects gave their informed consent.

**Identification and genotyping of single-nucleotide polymorphisms (SNP).** Fifty patients with SLE and 50 Korean volunteer controls were used for SNP identification. Genomic DNA was extracted from whole blood using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). An approximately 1.4 kb-sized *IL-6* gene located between the promoter region and exon 2 region was amplified by polymerase chain reaction (PCR) under the following conditions: hot start at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 1 min 30 s with a final extension at 72°C for 7 min. SNP genotyping was then conducted by direct sequencing (Bionics Co., Seoul, Korea). The following primers were used for amplification and sequencing: forward primer 1: 5'-GAG ACG TTC TAC GGT GT T-3', reverse primer 1: 5'-CCG TCG AGG ATG TAC CGA-3', reverse primer 2: 5'-CCG TCG AGG ATG TAC CGA-3', respectively. A minor allele frequency of greater than 5% was considered to indicate an SNP. Additionally, we amplified that and the detected SNP were genotyped using direct sequencing for patients with SLE (n = 101) and controls (n = 101).

**Preparation of promoter structures.** A 497 bp-sized fragment (from -492 to +5) of the human *IL-6* gene was amplified by PCR amplification using either -278 A homozygous or -278 C homozygous human genomic DNA as a template and the following primers: (forward primer 2: 5'-CAA TGG TAC CCG CTA CCT CAG TC TCC TTT G-3'; the bold character represents the KpnI site, reverse primer 3: 5'-CAATCT CGA GCA GAA TGA GCC TCA GAC ATC-3'; the bold character represents the XhoI site). Each PCR product was subcloned separately into the KpnI-XhoI site of the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA).

**Transfection and luciferase reporter assays.** Hep3B cells [hepatocellular carcinoma cell line #58064; Korean Cell Line Bank (KCLB), Seoul, Korea] were cultured in RPMI 1640 (Invitrogen, Grand Island, NY, USA), and HeLa cells (adenocarcinoma cell line #10002; KCLB) were cultured in high-glucose DMEM (Hyclone, Logan, UT, USA) at 37°C in a 5% CO<sub>2</sub> incubator. All media were supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml of penicillin G, and 100 mg/ml streptomycin (Invitrogen).

Hep3B and HeLa cells were transfected using FuGENE6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the day before the transfection, 1 x 10<sup>5</sup> cells per well of a 12-well plate were seeded in 1 ml of the medium with 10% FBS. Reporter plasmid DNA (0.5 µg) and 1.5 µl of FuGENE6 reagent were added to 50 µl medium without FBS and antibiotics, mixed gently and incubated at room temperature for 15 min and then added to cells that had attained 50–80% confluence in 12-well plates containing 400 µl of medium without FBS and antibiotics. The pGL3-Control and the promoter-less pGL3-Basic vectors (Promega) were used as the positive and negative controls, respectively. Transfection efficiency was determined by measuring β-galactosidase activity assay after cotransfection of both constructors, reporter construct, and pSV-β-galactosidase control vector into the cell line. After incubation for 5 h, the medium was added with 500 µl of fresh medium with 20% FBS, and the cells were incubated a further 24 h for Hep3B and HeLa cells at 37 µl in a 5% CO<sub>2</sub> incubator.

A luciferase reporter assay was performed following the protocol supplied by the manufacturer (Promega). Cells were lysed with 100 µl of reporter lysis buffer per well. From 100 µl of the cell lysate, 20 µl were assayed for luciferase activity using a luminometer, the TD-20/20 (Turner BioSystems, Sunnyvale, CA, USA). Assays were conducted in triplicate, and the experiments were repeated at least 3 times.

**Statistical analysis.** The genotype frequency was tested for significant departures from Hardy-Weinberg equilibrium at each SNP by chi-squared analysis. Differences in genotype frequency between the case and control were tested by the chi-squared test and calculation of the OR and the 95% CI. Three logistic regression models (codominant, dominant, and recessive) were used to analyze the SNP after controlling for age and sex as covariates. Differences in the mean value of the phenotypic characteristics between groups were compared by an ANOVA test and a t-test. P values of < 0.05 were considered to be significant. Haplotypes were analyzed using Arlequin version 3.1 software (CMPG, Bern, Switzerland). Statistical analyses were conducted using the SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

**Clinical characteristics of the study subjects.** The clinical characteristics of the subjects are summarized in Table 1.

Table 1. Clinical characteristics of the study subjects.

| Characteristics                          | SLE,<br>n = 151(%) | Controls,<br>n = 151(%) | SLE vs<br>Controls, p |
|--|--------------------|-------------------------|-----------------------|
| Age, yrs, mean ± SD                      | 34.21 ± 12.35      | 26.16 ± 4.27            | 0.001                 |
| Men                                      | 28 (18.5)          | 34 (22.5)               | 0.393                 |
| Women                                    | 123 (81.5)         | 117 (77.5)              |                       |
| Oral ulcer                               | 72 (47.7)          |                         |                       |
| Arthritis                                | 102 (67.5)         |                         |                       |
| Serositis                                | 24 (15.9)          |                         |                       |
| Rash                                     | 68 (45.0)          |                         |                       |
| Nephritis                                | 51 (33.8)          |                         |                       |
| Leukopenia                               | 92 (60.9)          |                         |                       |
| Lymphopenia                              | 139 (92.1)         |                         |                       |
| Thrombocytopenia                         | 37 (24.5)          |                         |                       |
| Anti-dsDNA                               | 108 (71.5)         |                         |                       |
| Hypocomplementemia                       | 114 (75.5)         |                         |                       |
| Anticardiolipin antibody                 | 63 (41.7)          |                         |                       |
| Major organ involvement                  | 69 (45.7)          |                         |                       |
| C-reactive protein (mg/dl,<br>mean ± SD) | 0.063 ± 0.121      |                         |                       |

SLE: systemic lupus erythematosus.

The mean age of the patients with SLE was  $34.2 \pm 12.4$  years and 81.5% were women. The mean age of the controls was  $26.2 \pm 4.3$  years and 77.5% were women. The patients with SLE were significantly older than the controls ( $p = 0.001$ ); therefore, the data were analyzed with logistic regression analysis to control for age. Major organ involvements, nephritis, severe thrombocytopenia, pneumonitis, myocarditis, and gastrointestinal were positive in 69 patients with SLE (45.7%).

**SNP discovery of the IL-6 gene.** Based on an allele frequency of greater than 5%, 4 SNP of the *IL-6* gene were identified: -572 C > G, -278 A > C, 330 T > G, and 334 A > T (Figure 1A). In addition, there was a -373 A<sub>n</sub>T<sub>n</sub> tract polymorphism in the promoter region of the *IL-6* gene. However, there was no polymorphism in the -174 position, which is the most commonly reported SNP in whites.

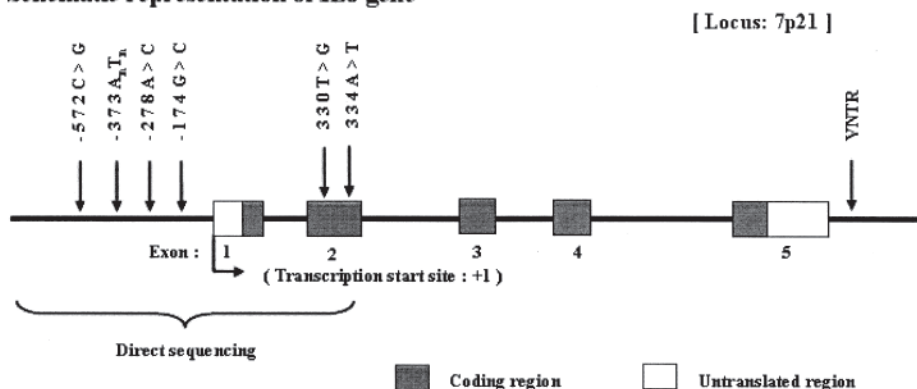
**Genotype and haplotype frequencies of the IL-6 gene.** The allele and genotype frequencies of the *IL-6* polymorphisms are presented in Tables 2 and 3. The genotype distributions of all polymorphisms were consistent with Hardy-Weinberg

equilibrium in patients with SLE and controls ( $p > 0.05$ ). In the -278 A > C polymorphism, the genotype frequency of the homozygous minor allele was significantly higher in the patients with SLE when compared to the controls ( $p = 0.03$  for the recessive model, OR 1.779, CI 1.057-2.994; Table 2). In addition, the minor allele of the 334 A > T polymorphism was frequently associated with SLE ( $p = 0.005$  for codominant model, OR 2.188, 95% CI 1.267-3.774).

In the -373 A<sub>n</sub>T<sub>n</sub> tract polymorphism, the genotype frequency of -373 A<sub>10</sub>T<sub>11</sub> was higher in patients with SLE than in the controls ( $p < 0.001$ , OR 3.535, 95% CI 1.839-6.794; Table 3). However, patients with SLE had a lower -373 A<sub>10</sub>T<sub>10</sub> genotype frequency ( $p = 0.038$ , OR 0.579, 95% CI 0.345-0.97).

Linkage disequilibrium was examined between SNP and locus by locus. Only 2 genetic polymorphisms of the *IL-6* gene, 330 T > G and 334 A > T, were in linkage disequilibrium ( $D' = 1$  and  $r^2 = 0.023$ ; Figure 1B), and 4 common haplotypes for 4 polymorphisms were constructed using the Arlequin software: HT1 (CA<sub>10</sub>T<sub>10</sub>ATA), HT2 (GA<sub>10</sub>T<sub>10</sub>ATA),

### A. Schematic representation of *IL6* gene



### B. Linkage disequilibrium coefficients ( $D'$ and $r^2$ ) among *IL6* polymorphisms

| Loci 1  | Loci 2  | $D'$  | $r^2$ |
|---------|---------|-------|-------|
| -572C>G | -278A>C | 0.109 | 0.006 |
| -572C>G | 330T>G  | 0.016 | 0.000 |
| -572C>G | 334A>T  | 0.116 | 0.001 |
| -278A>C | 330T>G  | 0.205 | 0.020 |
| -278A>C | 334A>T  | 0.743 | 0.026 |
| 330T>G  | 334A>T  | 1.000 | 0.023 |
| 373AnTn | -572C>G | 0.064 | 0.000 |
| 373AnTn | -278A>C | 0.086 | 0.000 |
| 373AnTn | 330T>G  | 0.151 | 0.048 |
| 373AnTn | 334A>T  | 0.209 | 0.000 |

Extension to multi-allelic marker loci is straightforward;

$$D'_{m} = \frac{\sum_{i=1}^{a_i} \sum_{k=1}^{b_k} p_i p_k |D'_{A_i B_k}|}{\sum_{i=1}^{a_i} \sum_{k=1}^{b_k} D_{A_i B_k}^2}$$

$$R = \frac{\sum_{i=1}^{a_i} \sum_{k=1}^{b_k} D_{A_i B_k}^2}{\left(1 - \sum_{i=1}^{a_i} p_{A_i}^2\right) \left(1 - \sum_{k=1}^{b_k} p_{B_k}^2\right)}$$

Figure 1. A. The *IL-6* gene consists of 5 exons separated by 4 introns. Filled boxes denote translated regions and open boxes are untranslated regions. Arrows mark single-nucleotide polymorphisms within the *IL-6* locus. Location numbers are relative to the transcription starting site. B. Linkage disequilibrium by Arlequin software with permutation test using the expectation maximization algorithm<sup>35,36</sup>.

**Table 2.** The genotype and allele frequencies of polymorphisms in the promoter region and exon2 region of the *IL-6* gene. Logistic regression analysis was applied to control for age and sex as covariables. Each p value was calculated with codominant (co), dominant (do), and recessive (re) models. p values in q rows were analyzed by chi-square test.

| Loci       | Genotype | Patients with SLE, n = 151 (%) | Controls, n = 151 (%) | SLE vs Controls |                       |
|------------|----------|--------------------------------|-----------------------|-----------------|-----------------------|
|            |          |                                |                       | p               | OR (95% CI)           |
| -572 C > G | CC       | 74 (49.0)                      | 76 (50.3)             | co: 0.470       | 1.143 (0.795 ~ 1.643) |
|            | CG       | 55 (36.4)                      | 61 (40.4)             | do: 0.715       | 1.047 (0.817 ~ 1.343) |
|            | GG       | 22 (14.6)                      | 14 (9.3)              | re: 0.334       | 1.211 (0.822 ~ 1.786) |
|            | q        | 0.328                          | 0.295                 | 0.540           | 1.062 (0.878 ~ 1.282) |
| -278 A > C | AA       | 108 (71.5)                     | 104 (68.9)            | co: 0.325       | 1.229 (0.815 ~ 1.852) |
|            | AC       | 28 (18.5)                      | 41 (27.2)             | do: 0.945       | 1.008 (0.768 ~ 1.290) |
|            | CC       | 15 (9.9)                       | 6 (4.0)               | re: 0.030       | 1.779 (1.057 ~ 2.994) |
|            | q        | 0.199                          | 0.175                 | 0.226           | 1.148 (0.553 ~ 1.437) |
| 330 T > G  | TT       | 117 (77.5)                     | 125 (82.8)            | co: 0.220       | 1.473 (0.794 ~ 2.732) |
|            | TG       | 34 (22.5)                      | 26 (17.2)             | do: 0.220       | 1.214 (0.891 ~ 1.653) |
|            | GG       | 0 (0)                          | 0 (0)                 | re: NA          | NA                    |
|            | q        | 0.113                          | 0.086                 | 0.248           | 1.188 (0.887 ~ 1.587) |
| 334 A > T  | AA       | 85 (56.3)                      | 115 (76.2)            | co: 0.005       | 2.188 (1.267 ~ 3.774) |
|            | AT       | 65 (43.0)                      | 36 (23.8)             | do: 0.005       | 1.478 (1.123 ~ 1.945) |
|            | TT       | 1 (0.7)                        | 0 (0)                 | re: 1.000       | 0                     |
|            | q        | 0.222                          | 0.119                 | 0.011           | 1.374 (0.518 ~ 1.754) |

SLE: systemic lupus erythematosus; q: minor allele frequency; NA: not applicable.

**Table 3.** The genotype frequencies of -373 A<sub>n</sub>T<sub>n</sub> polymorphism in the promoter region of *IL-6* gene. Genotype identified by direct sequencing. Logistic regression analysis was applied to control for age and sex as covariables.

| Loci (-373 A <sub>n</sub> T <sub>n</sub> ) | Genotype                        | Patients with SLE, n = 151 (%) | Controls, n = 151 (%) | SLE vs Controls |                       |
|--|---------------------------------|--------------------------------|-----------------------|-----------------|-----------------------|
|  |                                 |                                |                       | p               | OR (95% CI)           |
| -373 A <sub>9</sub> T <sub>11</sub>        | A <sub>9</sub> T <sub>11</sub>  | 21 (13.9)                      | 28 (18.5)             | 0.116           | 0.577 (0.290 ~ 1.145) |
|  | Other                           | 130 (86.1)                     | 123 (81.5)            |                 |                       |
| -373 A <sub>10</sub> T <sub>10</sub>       | A <sub>10</sub> T <sub>10</sub> | 82 (54.3)                      | 104 (68.9)            | 0.038           | 0.579 (0.345 ~ 0.970) |
|  | Other                           | 69 (45.7)                      | 47 (31.1)             |                 |                       |
| -373 A <sub>10</sub> T <sub>11</sub>       | A <sub>10</sub> T <sub>11</sub> | 48 (31.8)                      | 19 (12.6)             | <0.001          | 3.535 (1.839 ~ 6.794) |
|  | Other                           | 103 (68.2)                     | 132 (87.4)            |                 |                       |

IL-6: interleukin 6; SLE: systemic lupus erythematosus.

HT3 (CA<sub>10</sub>T<sub>11</sub>ATA), and HT4 (CA<sub>10</sub>T<sub>10</sub>ATT; Table 4). There was significant difference between patients with SLE and controls in the observed haplotype HT3 (dominant model, p = 0.012).

**Associations between SLE phenotype and SNP.** The clinical characteristics according to genotype are summarized in Table 5. In the -572 C > G polymorphism, anti-dsDNA was significantly more common in the patients with SLE who had the -572 G allele (p = 0.007). In addition, the incidence of thrombocytopenia was significantly higher in patients with SLE who carried the -278 C allele (p = 0.006). No association with SLE phenotypes was observed when the other SNP were evaluated.

The clinical characteristics according to haplotype are summarized in Table 6. The frequency of arthritis was sig-

nificantly lower in patients who had haplotype HT3 (C A<sub>10</sub>T<sub>11</sub>ATA). But the frequency of leukopenia, anti-dsDNA, and complementemia was significantly higher in patients who had haplotype HT3.

**Promoter activity of the *IL-6* gene according to the -278 A > C polymorphism.** To determine if the *IL-6* -278 A > C polymorphism is associated with altered promoter activity, 2 reporter structures composed of the promoter sequence carrying either -278 A or -278 C and the luciferase reporter gene were transfected into the Hep3B cell line (Figure 2A). Luciferase activity of the structure containing -278 C was enhanced when compared to that of the structure containing -278 A (p = 0.001). Enhanced promoter activity of the -278 C structure was replicated in different cell lines of the HeLa cells (p = 0.001, Figure 2B).

**Table 4.** The haplotype frequencies of the *IL-6* gene. Haplotypes (HT) were analysed using Arlequin version 3.1 software based on the expectation maximization algorithm. Logistic regression analysis was applied to control for age and sex as covariables. Each p value was calculated with codominant (co), dominant (do), and recessive (re) models.

| Haplotype                                  | Patients with SLE,<br>n = 151 (%) |            | Controls,<br>n = 151 (%) |  | SLE vs Controls |                       |
|--|-----------------------------------|------------|--------------------------|--|-----------------|-----------------------|
|  |                                   |            |                          |  | p               | OR (95% CI)           |
| HT1 [CA <sub>10</sub> T <sub>10</sub> ATA] | +/+                               | 12 (7.9)   | 19 (12.6)                |  | co: 0.241       | 0.799 (0.549 ~ 1.162) |
|  | +/-                               | 51 (33.8)  | 55 (36.4)                |  | do: 0.486       | 0.915 (0.713 ~ 1.175) |
|  | -/-                               | 88 (58.3)  | 77 (51.0)                |  | re: 0.149       | 0.727 (0.472 ~ 1.120) |
| HT2 [GA <sub>10</sub> T <sub>10</sub> ATA] | +/+                               | 1 (0.7)    | 3 (2.0)                  |  | co: 0.380       | 0.771 (0.431 ~ 1.378) |
|  | +/-                               | 23 (15.2)  | 28 (18.5)                |  | do: 0.447       | 0.883 (0.640 ~ 1.217) |
|  | -/-                               | 127 (84.1) | 120 (79.5)               |  | re: 0.480       | 0.655 (0.203 ~ 2.118) |
| HT3 [CA <sub>10</sub> T <sub>11</sub> ATA] | +/+                               | 10 (6.6)   | 1 (0.7)                  |  | co: 0.093       | 1.518 (0.933 ~ 2.472) |
|  | +/-                               | 20 (13.2)  | 0 (0)                    |  | do: 0.012       | 1.608 (1.111 ~ 2.328) |
|  | -/-                               | 121 (80.1) | 145 (96.0)               |  | re: 0.990       | 1.003 (0.594 ~ 1.695) |
| HT4 [CA <sub>10</sub> T <sub>10</sub> ATT] | +/+                               | 0 (0)      | 0 (0)                    |  | co: 0.267       | 0.614 (0.259 ~ 1.452) |
|  | +/-                               | 12 (7.9)   | 19 (12.6)                |  | do: 0.267       | 0.783 (0.509 ~ 1.205) |
|  | -/-                               | 139 (92.1) | 132 (87.4)               |  | re: NA          | NA                    |
| Other HT                                   | +/+                               | 48 (31.8)  | 40 (26.5)                |  | co: 0.450       | 1.128 (0.825 ~ 1.544) |
|  | +/-                               | 54 (35.8)  | 53 (35.1)                |  | do: 0.300       | 1.148 (0.884 ~ 1.489) |
|  | -/-                               | 49 (32.4)  | 58 (38.4)                |  | re: 0.817       | 1.033 (0.782 ~ 1.365) |

IL-6: interleukin 6; SLE: systemic lupus erythematosus; NA: not applicable.

**Table 5.** Comparison of the clinical characteristics according to the genotype of the *IL-6* gene in SLE. Logistic regression analysis was applied to control for age and sex as covariables. No association with SLE phenotypes was observed when the other SNP were evaluated. Values are number (%) unless otherwise indicated.

| Characteristics                         | -572 C > G                    |                          |       | -278 A > C                    |                         |       | 334 A > T                     |                        | p     |
|---|-------------------------------|--------------------------|-------|-------------------------------|-------------------------|-------|-------------------------------|------------------------|-------|
|   | CC, CG,<br>n = 129<br>(85.4%) | GG,<br>n = 22<br>(14.6%) | p     | AA, AC,<br>n = 136<br>(90.1%) | CC,<br>n = 15<br>(9.9%) | p     | AA, AT,<br>n = 150<br>(99.3%) | TT,<br>n = 1<br>(0.7%) |       |
| Age, yrs, mean ± SD                     | 35.25 ± 12.49                 | 35.10 ± 9.40             | 0.956 | 35.55 ± 11.85                 | 32.50 ± 13.51           | 0.371 | 35.18 ± 12.05                 | 41.00                  | 0.141 |
| Women                                   | 103 (79.8)                    | 20 (90.9)                | 0.217 | 26 (19.1)                     | 2 (13.3)                | 0.584 | 122 (81.3)                    | 1 (100.0)              | 0.632 |
| Men                                     | 26 (20.2)                     | 2 (9.1)                  |       | 110 (80.9)                    | 13 (86.7)               |       | 28 (18.7)                     | 0 (0.0)                |       |
| Oral ulcer                              | 63 (48.8)                     | 9 (40.9)                 | 0.491 | 66 (48.5)                     | 6 (40.0)                | 0.530 | 72 (48.0)                     | 0 (0.0)                | 0.338 |
| Arthritis                               | 88 (68.2)                     | 14 (63.6)                | 0.671 | 91 (66.9)                     | 11 (73.3)               | 0.614 | 101 (67.3)                    | 1 (100.0)              | 0.487 |
| Serositis                               | 21 (16.3)                     | 3 (13.6)                 | 0.754 | 20 (14.7)                     | 4 (26.7)                | 0.229 | 24 (16.0)                     | 0 (0.0)                | 0.663 |
| Rash                                    | 58 (45.0)                     | 10 (45.5)                | 0.966 | 62 (45.6)                     | 6 (40.0)                | 0.680 | 68 (45.3)                     | 0 (0.0)                | 0.364 |
| Nephritis                               | 46 (35.7)                     | 5 (22.7)                 | 0.236 | 46 (90.2)                     | 5 (9.8)                 | 0.970 | 51 (34.0)                     | 0 (0.0)                | 0.474 |
| Leukopenia                              | 79 (61.2)                     | 13 (59.1)                | 0.849 | 80 (58.8)                     | 12 (80.0)               | 0.111 | 91 (60.7)                     | 1 (100.0)              | 0.422 |
| Lymphopenia                             | 119 (92.2)                    | 20 (90.9)                | 0.830 | 124 (91.2)                    | 15 (100.0)              | 0.230 | 138 (92.0)                    | 1 (100.0)              | 0.786 |
| Thrombocytopenia                        | 33 (25.6)                     | 4 (18.2)                 | 0.456 | 29 (21.3)                     | 8 (53.3)                | 0.006 | 36 (24.0)                     | 1 (100.0)              | 0.078 |
| Anti-dsDNA                              | 87 (67.4)                     | 21 (95.5)                | 0.007 | 95 (69.9)                     | 13 (86.7)               | 0.171 | 107 (71.3)                    | 1 (100.0)              | 0.527 |
| Hypocomplementemia                      | 100 (77.5)                    | 14 (63.6)                | 0.162 | 102 (75.0)                    | 12 (80.0)               | 0.669 | 113 (75.3)                    | 1 (100.0)              | 0.568 |
| Anticardiolipin antibody                | 56 (43.3)                     | 7 (31.8)                 | 0.561 | 57 (41.9)                     | 6 (40.0)                | 0.635 | 62 (41.3)                     | 1 (100.0)              | 0.495 |
| Major organ involvement                 | 61 (47.3)                     | 8 (36.4)                 | 0.342 | 59 (43.4)                     | 10 (66.7)               | 0.086 | 69 (46.0)                     | 0 (0.0)                | 0.357 |
| C-reactive protein, mg/dl,<br>mean ± SD | 0.067 ± 0.125                 | 0.063 ± 0.097            | 0.976 | 0.063 ± 0.129                 | 0.071 ± 0.111           | 0.809 | 0.064 ± 0.122                 | 0.300                  | 0.783 |

IL-6: interleukin 6; SLE: systemic lupus erythematosus; SNP: single-nucleotide polymorphism.

## DISCUSSION

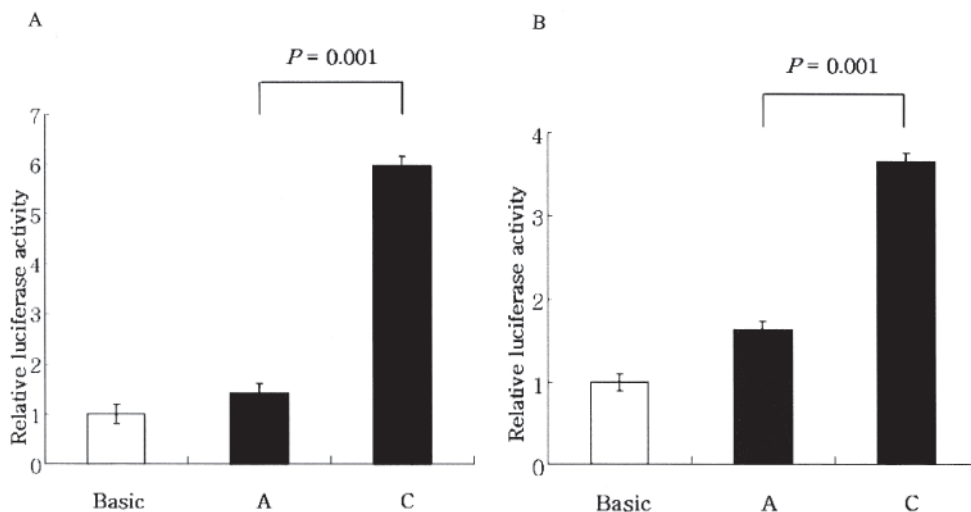
SLE is a complex multigenic disease in which the contributing genetic systems are being rapidly identified. In human SLE, genes of early components of complements as well as many polymorphic genes (including the MHC, Fc-gamma receptors, mannose-binding protein, PCDC-1, CRP, Bcl-2, IL-1 receptor antagonist, IL-10, tumor necrosis factor- $\alpha$  genes, etc.) were found to be associated with SLE by

population-based case-control studies<sup>18,19,20</sup>. Although the initiating immunological event in SLE remains unknown, it has been shown that an imbalance is involved between depressed T<sub>H</sub>1 cell cytokines, which promote cell-mediated immunity, and enhanced T<sub>H</sub>2 cell cytokines, which support physiological cascade<sup>21</sup>. Cytokines are also known to be involved in the pathogenesis of SLE. In particular, IL-6 has an important role in the regulation of immune responses, by

**Table 6.** Comparison of the clinical characteristics according to the haplotype (HT) of the *IL-6* gene in systemic lupus erythematosus (SLE). Logistic regression analysis was applied to control for age and sex as covariables. No association with SLE phenotypes was observed when the other haplotypes were evaluated. Values are number (%) unless otherwise indicated.

| Characteristics                      | HT1 [CA <sub>10</sub> T <sub>10</sub> ATA] |                   |       | HT2 [GA <sub>10</sub> T <sub>10</sub> ATA] |                    |       | HT3 [CA <sub>10</sub> T <sub>11</sub> ATA] |                    |       |
|--------------------------------------|--|-------------------|-------|--|--------------------|-------|--|--------------------|-------|
|                                      | +/+, +/-                                   | -/-               | p     | +/+, +/-                                   | -/-                | p     | +/+, +/-                                   | -/-                | p     |
|                                      | n = 63<br>(41.7%)                          | n = 88<br>(58.3%) |       | n = 24<br>(15.9%)                          | n = 127<br>(84.1%) |       | n = 30<br>(19.8%)                          | n = 121<br>(80.1%) |       |
| Age, yrs, mean ± SD                  | 33.5 ± 11.8                                | 34.7 ± 12.8       | 0.568 | 32.7 ± 10.2                                | 34.5 ± 12.7        | 0.508 | 31.5 ± 11.7                                | 34.9 ± 12.5        | 0.176 |
| Women                                | 53 (84.1)                                  | 70 (79.5)         | 0.475 | 23 (95.8)                                  | 100 (78.7)         | 0.048 | 24 (80.0)                                  | 99 (81.8)          | 0.819 |
| Men                                  | 10 (15.9)                                  | 18 (20.5)         |       | 1 (4.2)                                    | 27 (21.3)          |       | 6 (20.0)                                   | 22 (18.2)          |       |
| Oral ulcer                           | 32 (50.8)                                  | 40 (45.5)         | 0.517 | 13 (54.2)                                  | 59 (46.5)          | 0.488 | 12 (40.0)                                  | 60 (49.6)          | 0.347 |
| Arthritis                            | 42 (66.7)                                  | 60 (68.2)         | 0.845 | 14 (58.3)                                  | 88 (69.3)          | 0.293 | 15 (50.0)                                  | 87 (71.9)          | 0.022 |
| Serositis                            | 12 (19.0)                                  | 12 (13.6)         | 0.370 | 1 (4.2)                                    | 23 (18.1)          | 0.087 | 3 (10.0)                                   | 21 (17.4)          | 0.324 |
| Rash                                 | 31 (49.2)                                  | 37 (42)           | 0.383 | 10 (41.7)                                  | 58 (45.7)          | 0.718 | 11 (36.7)                                  | 57 (47.1)          | 0.304 |
| Nephritis                            | 24 (38.1)                                  | 27 (30.7)         | 0.342 | 6 (25.0)                                   | 45 (35.4)          | 0.322 | 9 (30.0)                                   | 42 (34.7)          | 0.625 |
| Leukopenia                           | 37 (58.7)                                  | 55 (62.5)         | 0.640 | 14 (58.3)                                  | 78 (61.4)          | 0.776 | 23 (76.7)                                  | 69 (57.0)          | 0.048 |
| Lymphopenia                          | 60 (95.2)                                  | 79 (89.8)         | 0.221 | 21 (87.5)                                  | 118 (92.9)         | 0.369 | 29 (96.7)                                  | 110 (90.9)         | 0.297 |
| Thrombocytopenia                     | 16 (25.4)                                  | 21 (23.9)         | 0.829 | 8 (33.3)                                   | 29 (22.8)          | 0.273 | 10 (33.3)                                  | 27 (22.3)          | 0.209 |
| Anti-dsDNA                           | 42 (66.7)                                  | 66 (75.0)         | 0.263 | 18 (75.0)                                  | 90 (70.9)          | 0.681 | 26 (86.7)                                  | 82 (67.8)          | 0.040 |
| Hypocomplementemia                   | 46 (73.0)                                  | 68 (77.3)         | 0.549 | 15 (62.5)                                  | 99 (78.0)          | 0.106 | 28 (93.3)                                  | 86 (71.1)          | 0.011 |
| Anticardiolipin antibody             | 28 (44.4)                                  | 35 (39.8)         | 0.566 | 9 (37.5)                                   | 54 (42.5)          | 0.647 | 13 (43.3)                                  | 50 (41.3)          | 0.842 |
| Major organ involvement              | 32 (50.8)                                  | 37 (42.0)         | 0.287 | 9 (37.5)                                   | 60 (47.2)          | 0.379 | 14 (46.7)                                  | 55 (45.5)          | 0.905 |
| C-reactive protein, mg/dl, mean ± SD | 0.073 ± 0.158                              | 0.057 ± 0.087     | 0.422 | 0.047 ± 0.057                              | 0.067 ± 0.130      | 0.462 | 0.072 ± 0.120                              | 0.061 ± 0.122      | 0.668 |

SLE: systemic lupus erythematosus.



**Figure 2.** Effect of the  $-278 A > C$  polymorphism on the transcription activity of the human *IL-6* gene promoter in Hep3B cells (A) and HeLa cells (B). Relative luciferase activity is represented as the ratio of the activity to the luciferase activity in the cells transfected with the empty control vector, pGL3-Basic. Each experiment was conducted in triplicate for each sample, and the results are expressed as the mean ± SEM for 3 independent experiments. The p value was determined by a paired t-test.

supplying positive and negative signals to activated T and B cells<sup>22</sup>.

Therefore, we conducted this case-control study of Korean patients with SLE under the assumption that genetic polymorphisms of the *IL-6* gene may be related to the susceptibility of SLE and to its clinical manifestations. We identified 5 genetic polymorphisms ( $-572 C > G$ ,  $-373$

$A_nT_n$ ,  $-278 A > C$  in the promoter region, and  $330 T > G$  and  $334 A > T$  in the exon2 region), including 3 novel SNP ( $-278 A > C$ ,  $330 T > G$ , and  $334 A > T$ ) in the *IL-6* gene. Further, we found that the rare allele of the  $-278 A > C$  and the  $334 A > T$  SNP, and the  $-373 A_{10}T_{11}$  allele of the  $-373 A_nT_n$  tract polymorphism were associated with a significantly higher disease susceptibility. Our results suggest that

the human *IL-6* gene plays an important role in the development of SLE.

Two promoter polymorphisms in the *IL-6* gene were not in linkage disequilibrium. Among the polymorphisms,  $-278 A > C$  promoter polymorphism was associated with genotype and phenotype (thrombocytopenia). Therefore, we focused on the functional effects of the  $-278 A > C$  polymorphism. To investigate the effects of the  $-278 A > C$  polymorphism on *IL-6* expression, we used a functional assay of promoter activity in reporter structures that contained mutant type or polymorphic promoters in the Hep3B cell line and HeLa cell line. Because the Hep3B cell line originates from human hepatocellular carcinoma and *IL-6* is primarily synthesized in the liver, it is an appropriate cell line for this purpose. To replicate the Hep3B cell line results, we also tested the promoter activity in the HeLa cell line from human cervical carcinoma<sup>21,23</sup>. The promoter reporter structure carrying the  $-278 C$  allele displayed higher promoter activity than the structure carrying the  $-278 A$  allele in Hep3B cells and HeLa cells. Characteristically, patients with SLE have elevated autoantibody production and overexpression of *IL-6*. These autoantibodies attack platelets and cause thrombocytopenia. Our results showed that the  $-278 C$  allele, which was associated with thrombocytopenia, displayed significantly higher promoter activity.

To determine if the genetic variants created a transcription factor binding site, sequences were submitted to the TFSEARCH online program, which revealed that the  $-278 A > C$  polymorphism might be a potential AP-1 binding motif. Moreover, several reports have suggested that the human *IL-6* promoter contains multiple regulatory elements such as those binding transcription factors belonging to the NF- $\kappa$ B, C/EBP, and AP-1 families<sup>24,25</sup>. The AP-1 transcription factors are homodimers and heterodimers composed of basic region-leucine zipper proteins that belong to the Jun and Fos subfamilies. Several lines of evidence suggest that members of the AP-1 transcription factor work in concert to regulate the *IL-6* promoter in a cell-type or inducer-specific fashion<sup>25,26,27,28,29</sup>. Our results indicate that the binding affinity of AP-1 may be increased in patients with SLE who carry the  $-278 C$  allele.

In the 334  $A > T$  polymorphism, the genotype frequency of the minor allele was significantly higher in the patients with SLE compared to the controls. Although this mutation did not lead to an amino acid change, we think that there might be an indirect association between this mutation and SLE.

The genotype frequency of  $-373 A_{10}T_{11}$  was significantly higher in patients with SLE than in the controls. However, patients with SLE had significantly lower  $-373 A_{10}T_{10}$  genotype frequencies than did the controls. These results demonstrated that  $-373 A_nT_n$  tract polymorphism was associated with the disease susceptibility of SLE. Specifically, the  $-373 A_{10}T_{11}$  genotype might play an important role in

*IL-6* expression. These findings are consistent with the results of previous studies that have shown that different  $A_nT_n$  patterns influence this differential expression<sup>16,23</sup>. It is possible that individual  $A_nT_n$  tract genotypes make differing contributions to *IL-6* expression, acting to either enhance or repress transcription<sup>30</sup>.

Our results demonstrated that the  $-572 C > G$  polymorphism was associated with anti-dsDNA positivity and that the  $-278 A > C$  polymorphism was associated with thrombocytopenia. These findings suggest that the disease phenotype was more common in patients with SLE who had minor allele  $-572 C > G$  and  $-278 A > C$  polymorphisms than in those who had the major homozygous genotype.

In the haplotype analysis, there was significant difference between SLE and controls in the observed haplotype HT3 ( $CA_{10}T_{11}ATA$ ) that was associated with decreased arthritis, and increased leukopenia, anti-dsDNA, and complementemia. Our results suggest that patients with SLE have elevated autoantibodies that acted against leukocytes, DNA, and complements. Particularly,  $-373 A_{10}T_{11}$  may have a powerful influence between HT3 and lupus phenotypes.

The most frequently investigated polymorphism is the  $-174 G > C$  polymorphism in the promoter region of the *IL-6* gene. In a German SLE study,  $-174 G > C$  polymorphism did not contribute significantly to disease susceptibility, but did predispose to distinct clinical and immunological features<sup>20</sup>. In a study of Whites and African Americans, the  $-174 G > C$  polymorphism was not associated with SLE<sup>31</sup>. Interestingly, the present study revealed that there was no  $C$  allele in the  $-174 G > C$  polymorphism, which is a common polymorphism in Whites<sup>19</sup>. Our results support those of studies that have found decreased frequencies of the  $C$  allele in Asians such as southern Chinese coal workers with pneumoconiosis ( $C$  allele 0.20%)<sup>32</sup>, Japanese women with hypertension ( $C$  allele 0.0%)<sup>33</sup>, and Korean IgA nephropathy patients ( $C$  allele 0.48%)<sup>34</sup>. The reason for the decreased occurrence of the  $C$  allele is unclear; however, considering the very low frequencies of the  $-174 C$  allele in the Korean, Japanese, and Chinese, it likely reflects the genetic characteristic of Far East Asian populations. In addition, the major alleles of  $-373 A_nT_n$  and  $-572 C > G$  were found to be  $-373 A_{10}T_{10}$  and  $-572 C$  in Koreans, but were  $-373 A_8T_{12}$  and  $-572 G$  in other ethnic groups, which further demonstrates that genetic variations in SLE are associated with ethnic backgrounds.

It is important to note that our study has the following limitations. First, our study was performed in a single population of patients without replication. In addition, the studied population was relatively small, which likely prevented identification of small differences in the genetic susceptibility of SLE. Therefore, further studies with larger populations are needed. Second, we did not evaluate the functional effects of the  $-572 C > G$  polymorphism. Although there was no significant difference between SLE and controls in

genotype analysis in the case of -572, it was associated with anti-dsDNA antibody. Further studies will address the functional effects of the -572 C > G polymorphism and the double functional effects of the -278 A > C and -572 C > G polymorphism. Although the -572 and -278 were not in linkage disequilibrium, both polymorphisms were located in the promoter site, which is important for gene expression.

These data suggest that *IL-6* genetic polymorphisms are associated with disease susceptibility and clinical manifestations of SLE in Koreans. Specifically, promoter polymorphisms may be involved in regulation of *IL-6* expression.

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