

Interleukin-18 Promoter Gene Polymorphisms in Chinese Patients With Systemic Lupus Erythematosus: Association With CC Genotype at Position –607

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Abstract

Introduction: Interleukin-18 (IL-18) is a Th1 cytokine, which is postulated to play a role in systemic lupus erythematosus (SLE). Two single nucleotide polymorphisms (SNPs) in the IL-18 promoter gene region were found to influence the quantitative expression of the IL-18 protein. The aim of this study was to determine whether IL-18 promoter gene polymorphisms are associated with SLE. **Materials and Methods:** One hundred and thirteen Chinese SLE patients and 218 Chinese healthy individuals were recruited. Genomic DNA was extracted from peripheral venous blood. Sequence-specific primer PCR and restriction fragment length polymorphism (RFLP) analysis were used to genotype the DNA samples for SNP-137 and SNP-607. The following genotypes were obtained: SNP(-607) AA, AC, CC and SNP(-137) GG, GC, CC. Plasma IL-18 concentrations of patients and control subjects were measured by enzyme-linked immunosorbent assay. **Results:** the frequency of SNP-607/CC genotype was significantly higher in SLE patients ($P < 0.05$) while genotype SNP-607/AC was significantly decreased in SLE patients compared to the control group ($P < 0.05$). Plasma IL-18 concentrations were significantly higher in SLE patients than in control subjects ($P < 0.05$). Both patients and control subjects with CC and AC genotypes have significantly higher IL-18 concentrations than those with AA genotype. **Conclusion:** The IL-18 promoter gene polymorphism SNP-607 C allele is associated with SLE and may result in the enhanced production of IL-18 protein in SLE and normal individuals.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the production of high titres of autoantibodies and associated with a diverse array of clinical manifestations that include arthritis, vasculitis and nephritis. It is a complex disease, and genetic and environmental factors contribute to the disease pathogenesis. Abnormal Th1 and Th2 cytokines profiles might be involved in the pathogenesis of SLE. Peripheral blood mononuclear cells of SLE patients show decreased production of the Th1 cytokines IL-2, IFN- γ , TNF- α and IL-12 and upregulation of the Th2 cytokines IL-4 and IL-10.¹⁻³ Such cytokines profiles may account for the polyclonal B-cell activation observed in SLE. Other studies, on the contrary, have

demonstrated that the serum levels of Th1 cytokines IL-12, TNF- α and IFN- γ are significantly higher in SLE patients.^{4,6}

Interleukin-18 (IL-18) was characterised as a Th1 cytokine because of its property in inducing IFN- γ .⁷ IL-1 β -converting enzyme (caspase-1) cleave pro-IL18 to an active 18-kDa glycoprotein that recognises a heterodimeric receptor, consisting of unique α (IL-1Rrp) and non-binding β (AcPL) signalling chains.^{8,9} IL-18 is expressed by various cell types, including macrophages, dendritic cells, keratinocytes, osteoblasts, pituitary gland cells, adrenal cortical cells, intestinal epithelial cells, skin cells and brain cells.^{10,11}

The primary functions of IL-18 include the induction of IFN- γ and TNF- α in T cells and natural killer cells, up-regulation of Th1 cytokines including IL-2, granulocyte

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macrophage colony-stimulating factor (GM-CSF) and IFN- γ , stimulation of the proliferation of activated T cells, and enhancement of Fas ligand expression in NK and cytotoxic T lymphocytes.

IL-18 shares some biological activities with IL-12, e.g. promoting IFN- γ production in mice and in humans and serving as a co-stimulatory factor in the activation of Th1 cells.^{7,12} IL-18 affects Th1 cell development by inducing IL-12R expression and thus synergises with IL-12 in elevating IFN- γ production.^{13,14} In the absence of IL-12, IL-18-mediated effects on T cells may extend beyond Th1 differentiation to include Th2 cytokines production.¹⁵

Because of its multiple functions in cytokine networks, IL-18 is likely to participate in the development of diseases. Elevated IL-18 levels have been reported in the urine of nephrotic patients, the serum of patients with multiple sclerosis, adult-onset Still's disease, type I diabetes mellitus, viral infection, sepsis, allergic asthma and inflammatory rheumatic disease.

A correlation between the expression of IL-18 mRNA and the active stage of the disease has been observed in the development of autoimmune Th1-dependent insulinitis in non-obese diabetic mice.¹⁶ The development of experimental autoimmune encephalomyelitis can be prevented by the administration of neutralising anti-IL-18 antibodies,¹⁷ whereas in IL-18-deficient mice, the administration of exogenous IL-18 restores autoreactivity by means of IFN- γ induction.¹⁸ IL-18 also promotes collagen-induced inflammatory arthritis,¹⁹ whereas the incidence and severity of the disease are strongly reduced in IL-18 knockout mice.²⁰ Elevated concentrations of IL-18 proteins have been found in the affected tissues of patients with Crohn's disease and rheumatoid arthritis (RA).^{21,22} Increased plasma IL-18 protein levels were reported in SLE patients.²³ High concentrations of circulating IL-18 also correlate with the disease activity.^{24,25} IL-18 accelerates spontaneous autoimmune lupus disease in MRL/lpr mice.²⁶ Daily injections of IL-18 or IL-18 plus IL-12 resulted in accelerated proteinuria, glomerulonephritis, vasculitis and raised levels of proinflammatory cytokines in MRL/lpr mice. IL-18 treated MRL/lpr mice developed a "butterfly" facial rash resembling clinical SLE.²⁷

Giedraitis et al²⁸ first reported that IL-18 promoter gene polymorphisms influenced the expression of IL-18 mRNA and potentially also IFN- γ expression after stimulation with PMA/ionomycin. Since then, many studies have been carried out to explore the association between IL-18 promoter gene polymorphisms and various diseases.²⁸⁻³³ Two single nucleotide polymorphisms (SNPs) at position -607 and -137 in the promoter gene region have been considered to be significant because the change from cytosine to adenine (C to A) at position -607 disrupts a

potential cAMP-responsive element binding (CREB) protein-binding site and a change at position -137 from guanine to cytosine (G to C) changes the H4TF-1 nuclear factor binding site to a binding site for an unknown factor found in the GM-CSF promoter.^{28,34,35}

In the present study, we hypothesise that specific genotypes of these 2 SNPs in the IL-18 promoter gene region are associated with SLE.

Materials and Methods

Patient Recruitment and Sample Preparation

One hundred and thirteen Chinese SLE patients were recruited from the Department of Rheumatology and Immunology, Singapore General Hospital, Singapore. They fulfilled the American Rheumatism Association criteria for the classification of SLE.³⁶ Two hundred and eighteen Chinese healthy individuals were recruited from among laboratory personnel, undergraduates, medical and nursing staff, and blood donors. Consent was obtained prior to venepuncture (IRB Approval Reference: #47/2003). About 5 mL of peripheral venous blood was collected under aseptic conditions into sodium-citrate tubes and processed within the same day.

Genomic DNA Extraction

The genomic DNA was extracted using a salting-out method.³⁷ The purity and concentration of DNA was determined using spectrophotometry. Following deproteinisation, the quality of DNA was reflected by a consistent ratio of 1.8 to 2.0. The coded genomic DNA solution was stored at 4°C.

Specific Sequence Primer Polymerase Chain Reaction (PCR)

The -137 SNPs were detected using sequence specific PCR, described by Giedraitis et al.²⁸ A common reverse primer 5'-AGGAGGGCAAATG CACTGG-3' and 2 sequence specific forward primers, 5'-CCCCAACTTTT ACGGA AGAAAAG-3' and 5'-CCCCAACTTTTACGG AAG AAAAC-3', were used. An amplification product of 261-bp was detected. A control forward primer 5'-CCAA TAGGACTGATTATTCGCA-3' was used to amplify a 446-bp fragment covering the polymorphic site to serve as an internal positive amplification control.

Restriction Fragment Length Polymorphism (RFLP) Analysis

For the -607 SNPs, RFLP analysis was used to genotype the polymorphism. Generally, PCR for genotyping was performed with mutated primers: the forward primer 5'-GTTGCAGAAAGTGTA AAAAAT TAGTA-3' introduced a restriction site for the *RsaI* enzyme. The reverse primer

Table 1. Allelic Frequencies of IL-18 Promoter Gene Polymorphisms at Position -607 and -137 in SLE Patients and Controls

	Patients (n = 226)		Controls (n = 436)		P
	n	Frequency	n	Frequency	
Position -607					
A	46	0.20	149	0.34	<0.01
C	180	0.80	287	0.66	
Position -137					
G	187	0.83	380	0.87	ns
C	39	0.17	56	0.13	

n: number of alleles; frequency: allelic frequency; ns: not significant

was: 5'-TAACCTCATTTCAGGACT TCC-3'. Biometra T3 thermocycler was used in PCR with the following conditions: 1 cycle of 95°C for 5 minutes followed by 35 cycles denaturing at 94°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The final extension step was done at 72°C for 10 minutes. 30 uL of PCR reaction contained 50 ng genomic DNA, 0.2 uM each of forward and reverse primers; 0.1 mM of dNTPs, 1x reaction buffer and 0.2 units of DyNAzyme™ II DNA polymerase (FinnZymes). After confirmation of a successful PCR process, 6 uL of PCR product was incubated with *RsaI* enzyme and the buffer recommended by the supplier for 60 minutes at 37°C. Visualisation of RFLP was done in ethidium bromide stained 3% agarose gel in 0.5x TBE buffer.

ELISA

The plasma IL-18 concentrations of 76 SLE patients and 113 control subjects who were randomly selected were measured by enzyme-linked immunosorbent assay (ELISA) using the human bioactive IL-18 ELISA kit (Medical and Biological laboratories, Nagoya, Japan).

Statistics

Data were analysed by Statistical Package for Social Science (SPSS) software (version 11.0). The allelic, genotypic frequencies of the IL-18 promoter gene polymorphisms in SLE patients and controls were compared using Chi-square (χ^2) test and a corrected P (*Pc*) value of <0.05 was considered statistically significant. The Fisher's exact test was used if any of the cells contained less than 5% of the cohort analysed. The Bonferroni method³⁸ was used to correct for multiple testing. Expected population genetic frequencies were calculated using the Hardy-Weinberg equilibrium. A P value of <0.05 was considered as indicating a significant difference.

Results

Subjects

One hundred and thirteen SLE patients (age range, 15 to 74 years) and 218 healthy unrelated controls (age range, 20

Table 2. Genotypic Frequencies of IL-18 Promoter Gene Polymorphisms at Position -607 and -137 in SLE Patients and Controls

	Patients (n = 113)		Controls (n = 218)		Pc
	n	Frequency	n	Frequency	
Position -607					
AA	8	0.07	32	0.15	ns
AC	30	0.27	85	0.39	0.048
CC	75	0.66	101	0.46	<0.01
Position -137					
CC	10	0.09	2	0.01	0.003
GC	19	0.17	52	0.24	ns
GG	84	0.74	164	0.75	ns

n: number of subjects; frequency: genotypic frequency; ns: not significant, Pc: corrected P value

Table 3. Correlation Between SNP -607 Genotypes and Plasma IL-18 Protein Concentrations

	n	Mean ± SD (pg/mL)	SE	P	
Patients	AA	6	75.3 ± 66.7	27.2	0.02
	AC	22	214.2 ± 139.3	29.7	
	CC	48	217.3 ± 165.3	23.9	
Controls	AA	21	65.9 ± 45.6	9.96	0.002
	AC	45	143.7 ± 107.7	16.0	
	CC	47	136.7 ± 141.1	20.6	

n: number of subjects; SD: standard deviation; SE: standard error

to 45 years) were studied. Both groups comprised individuals of Chinese descent. The female-to-male ratio in SLE patients and controls were 11.3:1 and 3.4:1, respectively.

Allelic Frequencies of IL-18 Promoter Gene Polymorphisms at Position -607 and -137 in SLE Patients and Controls

The allelic frequencies of the A and C at position -607 as well as G and C alleles at position -137 are shown in Table 1. For SNP-607, there is a significant difference between the distribution of the allelic frequency in SLE patients and in normal controls. But no significant differences at position-137 were found between the 2 groups.

Genotypic Frequencies of IL-18 Promoter Gene Polymorphisms at Position -607 and -137 in SLE Patients and Controls

The genotypic frequencies of the 2 SNPs are shown in Table 2. The differences reflect an increase of genotype -607/CC and a decrease of genotype -607/AC in SLE patients. The frequency of genotype -607/AA was also lower in SLE patients, but was found not to be statistically significant after correction for multiple testing. For position -137, the frequency of genotype CC showed a significant increase in SLE patients (n = 10, frequency was 0.09) when compared to control individuals (n = 2, frequency was 0.01) (*Pc* < 0.003). The other 2 genotypes were only slightly different without any statistical significance. The calculated genotypic frequencies (based on Hardy-Weinberg

equilibrium) for position -607 are AA:0.04, AC:0.32, CC:0.64 for SLE patients and AA:0.12, AC:0.45, CC:0.43 for normal controls. For position -137, the frequencies are CC: 0.03, GC: 0.28, GG: 0.69 for SLE patients and CC: 0.01, GC: 0.23, GG: 0.76 for normal controls.

Correlation Between Plasma IL-18 Protein Levels and Genotypes at SNP -607

The levels of plasma bioactive IL-18 in SLE patients and normal controls are shown in Table 3. In the patients group, the concentrations of IL-18 bioactive protein for 6 patients who had AA genotype at position -607 was 75.33 ± 66.72 (mean \pm SD) pg/mL, while the concentration level for the 22 patients who had AC genotype was 214.2 ± 139.3 pg/mL. The 48 patients with CC genotype had a concentration level of 217.3 ± 165.3 pg/mL. Significant difference was observed in the 3 subgroups ($P < 0.02$). In the control group, 21 subjects with AA genotype at SNP -607 had significantly lower IL-18 protein concentrations when compared to the 45 subjects with AC genotype and the 47 subjects with CC genotype ($P < 0.002$). The means for the 3 subgroups were 65.9 pg/mL, 143.7 pg/mL and 136.7 pg/mL respectively.

Discussion

In this article, we describe the IL-18 promoter gene polymorphisms in a cohort of Chinese SLE patients. Two SNPs at positions -607 and -137 of the IL-18 promoter gene were studied. We found the genotype SNP-607/CC (i.e. homozygous for C allele) at position -607 to be significantly higher in Chinese SLE patients when compared to control individuals. A significant decrease of genotype AC at position -607 was also observed in SLE patients compared to controls. For position -137, the genotype CC was significantly higher in SLE patients than in controls (Table 2). The increase in the SNP-137/CC genotype's frequency may be statistically significant but probably not clinically important, as the number of persons analysed is small, i.e. 10 patients and 2 controls.

The differences in the genotypic frequencies at position -607 may be more important. This homozygous state is associated with higher transcription activity by the CREB protein, thus resulting in potentially higher levels of IL-18 protein expression. This is consistent with the findings of several studies that demonstrate elevated IL-18 protein levels in SLE patients.^{24,25}

Takada et al²⁹ have reported that in patients with sarcoidosis, the C allele at position -607, which is associated with higher IL-18 promoter activity, was a risk factor for sarcoidosis in the Japanese population. In RA patients, Sivalingam et al³² did not find the C allele at position -607 to be associated with RA but found that the A allele at position -607, in the homozygous state, has a protective

effect against the development of RA. Our data indicate that higher promoter activity of IL-18 gene is associated with SLE. The possible mechanism underlying this is that genetic polymorphisms affect the transcriptional activity, hence changing the protein expression of IL-18. Generally, cAMP induces enhanced transcription of a gene through a specific transcription factor known as cAMP response element binding protein (CREB). CREB binds to a specific recognition site, an 8-base pair CRE consensus sequence,³⁴ in the enhancer/promoter region of a gene to activate transcription. In the promoter region of IL-18 gene, this CRE sequence is present around position -607. A change from nucleotide C to nucleotide A disrupts the CRE sequence. Therefore, the CC genotype results in higher transcription activity than the other genotypes, resulting in the expression of higher levels of the IL-18 protein.

For position -137, a change from G to C changes the H4TF-1 nuclear factor-binding site to a binding site for an unknown factor found in the GM-CSF promoter. Giedraitis et al²⁸ postulated that because H4TF-1 nuclear factor would not bind to C allele of position -137, the SNP-137/C allele would have lower IL-18 mRNA expression. But they also admit that the expression patterns observed were in actual fact not fully consistent. In the same study, the allelic frequency of C nucleotide at position -137 was found to be higher in Swedish multiple sclerosis patients than in controls.²⁸ In addition, in Type 1 diabetes, an increase in the GC genotypes and a decrease in GG genotypes at position -137 in the promoter region of IL-18 gene have been reported by Kretowski et al.³⁰ Hence, our hypothesis was that the change at position -137 from G to C, which changes the H4TF-1 nuclear factor binding site to a binding site for an unknown factor found in the GM-CSF promoter, may confer a higher IL-18 protein expression. This unknown factor in the GM-CSF promoter may play an important role.

In conclusion, we have provided evidence that SNP-607/C allele of the IL-18 gene is significantly associated with SLE. How this allele interacts with other possible genetic factors in influencing the pathogenesis of SLE will require further study.

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