

A Functional Polymorphism in Fas (CD95/APO-1) Gene Promoter Associated with Systemic Lupus Erythematosus

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ABSTRACT. Objective. To investigate whether Fas promoter polymorphisms show a genetic contribution to the development of systemic lupus erythematosus (SLE) in a Japanese population, and to study the functional difference in promoter activity of the polymorphisms.

Methods. In 109 SLE patients and 140 controls, the frequencies of A/G polymorphisms at -670 nucleotide position and G/A at -1377 nucleotide position were determined by allele-specific polymerase chain reaction (PCR) or PCR-single strand conformation polymorphism analysis. The functional significance of the -670A/G polymorphism in the Fas gene was evaluated by a combination of Fas transcriptional activity in the reporter gene assay and binding activity of signal transducer and activator of transcription (STAT)1 protein in the electrophoretic mobility shift assay.

Results. SLE patients exhibited significantly higher frequency of A allele at nucleotide position -670 ($p = 0.004$). There was no significant difference in the nucleotide position -1377 in Fas promoter gene between SLE patients and controls. The electrophoretic mobility shift assay demonstrated that the oligonucleotide with -670A in the Fas promoter had a higher binding ability to a GAS binding protein, STAT1, than that with -670G, although there was no statistically significant difference in the reporter gene assay.

Conclusion. Fas promoter -670A/G polymorphism was significantly associated with SLE, suggesting a possibility that Fas promoter contributes, at least in part, to the pathogenesis of SLE. (J Rheumatol 2002;29:1183-8)

Key Indexing Terms:

FAS PROMOTER GENE POLYMORPHISM SYSTEMIC LUPUS ERYTHEMATOSUS

The etiopathogenesis of systemic lupus erythematosus (SLE) is multifactorial, including genetic, hormonal, environmental, and immunoregulatory factors. Genetic factors associated with SLE consist of major histocompatibility complex class II, complement component, Fc receptor II/III, T cell receptor, apoptosis, and cytokine genes¹⁻⁵. Apoptosis-related candidate genes include those encoding Fas^{6,7}, FasL⁸, bcl-2⁹ and DNase1¹⁰.

Recent reports have suggested that impaired clearance of apoptotic cells plays a major role in the etiopathogenesis of SLE^{11,12}. Defective clearance of apoptotic cells might reflect

dysfunction of phagocytes/complements and/or excessive production of apoptotic cells, either of which could result in the disease. With respect to clearance, opsonization with complement components participates in the *in vivo* clearance of apoptotic cells, and patients with complement deficiencies frequently develop SLE. In patients with SLE, the *in vivo* expression of Fas gene was elevated¹³⁻¹⁵, and the rate of apoptosis was increased in some reports^{12,15,16}, but normal in others^{14,17}. In addition, the high levels of circulating DNA, which possibly represented the uncleared cleavage products of apoptosis, were often detected^{18,19}. In murine studies, the intravenous administration of high numbers of apoptotic cells generated antinuclear antibody in normal mice²⁰. Thus, enhanced apoptosis might be also involved in SLE development through excessive production of apoptotic cells and DNA fragments in peripheral blood, resulting in the production of antinuclear/DNA autoantibodies.

On the other hand, only a few with autoimmune lymphoproliferative syndrome (ALPS) Ia (Fas deficiency) or Ib (FasL deficiency) out of many patients with ALPS showed a lupus-like syndrome^{6,8}. Thus, defects in Fas/FasL system appear to work as a potentiator in the development of at least a fraction of SLE patients, possibly through the failure of elimination of potentially autoreactive lymphocytes after *in vivo* activation.

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Although the association of Fas-mediated apoptosis with SLE has not been fully delineated, recent reports have shown that signaling through Fas can induce apoptosis in SLE cells^{21,22}. Recently, 2 polymorphisms in the promoter region of the Fas gene have been reported²³ and it was speculated that they modulate transcription of the Fas message. One is located at the nucleotide position of -670 from the transcriptional start site, and the other is at the nucleotide position of -1377²³.

To determine whether each polymorphism in the Fas promoter gene modulates the transcription of Fas gene expression and contributes to the pathogenesis of SLE, we performed an association study between these polymorphisms and SLE in a Japanese population, and then evaluated the biological significance of the -670A/G polymorphism by reporter gene and electrophoretic mobility shift assays.

MATERIALS AND METHODS

Study subjects. The study population consisted of 109 unrelated Japanese patients with SLE (100 female and 9 male) and 140 unrelated Japanese healthy volunteers (81 female, 59 male). This study population comprised only Japanese, excluding minor distinct ethnic groups (Ainu in Hokkaido and Ryukyu in Okinawa), and immigrants from Korea and China. All the healthy Japanese volunteers were recruited without selection. There was no difference in racial background/ancestry between patients and controls. All SLE patients fulfilled the American College of Rheumatology 1982 revised criteria for SLE²⁴. The mean \pm standard deviation (SD) age at onset of SLE was 32.0 ± 17.2 years. Informed consent was obtained from patients and/or their parents.

DNA extraction. Genomic DNA was extracted from peripheral blood lymphocytes using a QIAamp Blood Kit (Qiagen, Tokyo, Japan).

Analysis of -670A/G and -1377G/A genotypes of Fas gene. The genotyping of -670A/G and -1377G/A was performed by both allele-specific polymerase chain reaction (AS-PCR) and PCR-single strand conformation polymorphism (PCR-SSCP) analysis. AS-PCR was carried out on Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden) in a total volume of 25 μ l containing 20 ng of genomic DNA. The PCR profile to detect -670A/G polymorphism was as follows: initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and the final extension at 72°C for 7 min. The PCR to detect -1377 G/A polymorphism was carried out under the same conditions for -670A/G polymorphism except for the annealing temperature of 65°C. Combination of PCR primers were either A-specific primer (AS-P₁) or G-specific primer (AS-P₂) with common primer (AS-P₃) for -670A/G polymorphism and either G-specific primer (AS-P₄) or A-specific primer (AS-P₅) with common primer (AS-P₆) for -1377G/A, as shown in Table 1. Amplified products were separated on 2.5% agarose gels, stained by ethidium bromide, and visualized under ultraviolet light (Figures

1A, 2A). To determine an accuracy of the AS-PCR, we performed PCR-SSCP analysis for 50 samples randomly selected from the whole samples. PCR primers of SSCP-P₇ and SSCP-P₈ were used to amplify a 163-bp product including -670 nucleotide position of the Fas promoter region. PCR primers of AS-P₇ and SSCP-P₉ were employed to amplify a 461 bp product including -1377 nucleotide position. The PCR profile for the amplification of -670A/G region was as follows: initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 15 s, and the final extension at 72°C for 8 min. The PCR profile for -1377G/A region was the same for -670A/G, with the exception of the extension time of 30 s. Each sample was denatured for 5 min at 94°C, chilled quickly on ice, and loaded onto a GeneGel Excel 12.5/24 Kit (Amersham Pharmacia Biotech). The products were separated by electrophoresis with 15 mA at 20°C on GenePhor (Amersham Pharmacia Biotech), and gels were silver-stained with Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech) and photographed. A and G alleles were clearly separated and the results confirmed the data from ASPCR procedure (Figures 2A, 2B).

Linkage disequilibrium. We performed the haplotype analysis using the estimating haplotype frequencies software program. The linkage disequilibrium was calculated in the control and patient populations according to the method of Lewontin²⁵. Linkage disequilibrium coefficients $D' = D/D_{\max}$ and chi-square values were calculated for pairs of the most common alleles at each locus.

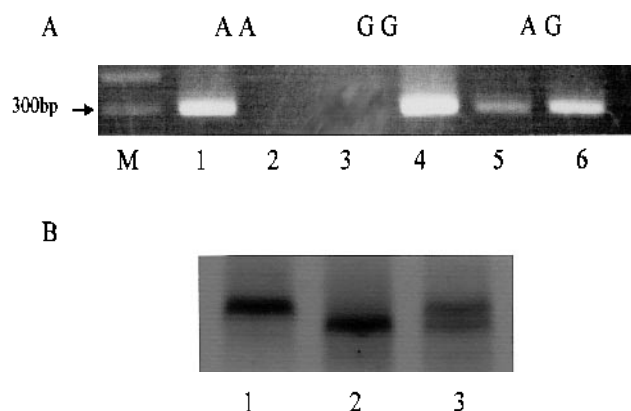


Figure 1. Fas promoter gene -670A/G genotype analysis. A. Allele-specific PCR analysis of -670A/G genotype. The 318 bp PCR products amplified with A allele-specific primer set (A: Lanes 1, 3, 5) or G allele-specific primers set (G: Lanes 2, 4, 6) are displayed. AA, GG, and AG indicate genotypes. B. SSCP analysis of -670A/G genotype. A and G polymorphisms at -670 nucleotide position show different mobility on gels. Lane 1: AA genotype, Lane 2: GG genotype, Lane 3: AG genotype.

Table 1. Primers to detect 2 sites of polymorphisms in the promoter region of the Fas gene.

Polymorphism	Method	Name	Primer Sequence	
-670A/G	AS-PCR	AS-P ₁	5'-GGTAACTGTCCATTCCA CA-3'	
		AS-P ₂	5'-GGTAACTGTCCATTCCA CG-3'	
		AS-P ₃	5'-TGCACAAATGGGCATTCTTG-3'	
	PCR-SSCP	SSCP-P ₇	5'-TCCCTTTTCAGAGCCCTATG-3'	
		SSCP-P ₈	5'-CTGGAGTCACTCAGAGAAAAG-3'	
-1377G/A	AS-PCR	AS-P ₄	5'-GTGTGCACAAGGCTG GCAAG-3'	
		AS-P ₅	5'-GTGTGCACAAGGCTGGCAAA-3'	
		AS-P ₆	5'-CAGTTCAGACTGGACACAC-3'	
	PCR-SSCP	SSCP-P ₉	5'-CAGAGATAATACAGAAATGCC-3'	

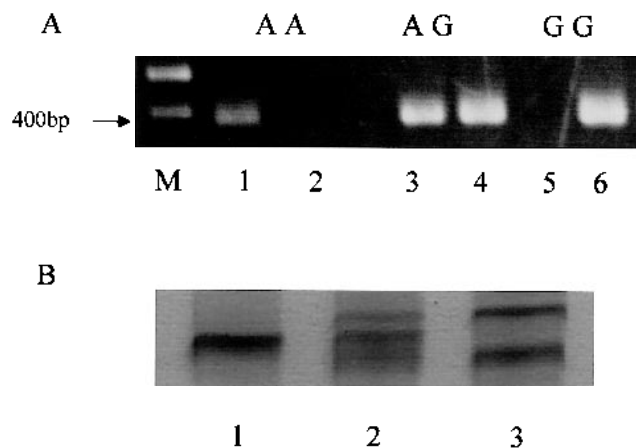


Figure 2. Fas promoter gene -1377G/A genotype analysis. A. Allele-specific PCR analysis of -1377G/A genotype. The 387 bp PCR products amplified with A allele-specific primer set (A: Lanes 1, 3, 5) or G allele-specific primers set (G: Lanes 2, 4, 6) are displayed. AA, AG, and GG indicate genotypes. B. SSCP analysis of -1377G/A genotype. G and A polymorphisms at -1377 nucleotide position show different mobility on gels. Lane 1: GG genotype, Lane 2: AG genotype, Lane 3: AA genotype.

Nuclear extract preparation. Human U937 cells (JCRB9021) were obtained from the Health Science Resources Bank (HSRRB; Osaka, Japan). After stimulation with 50 U/ml of IFN- γ and 100 ng/ml of lipopolysaccharide for 30 min, the cells were collected and resuspended in 200 μ l of ice cold hypotonic buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.2 mM ethyleneglycoltetraacetic acid (EGTA) (pH 8.0), 1 mM dithiothreitol and 0.5 mM phenylmethylsulphonyl fluoride, and were incubated on ice for 15 min. The cells were then lysed using a homogenizer, and the lysate was centrifuged for 40 s at 4°C. The resulting nuclear pellet was resuspended in 30 μ l of ice cold hypertonic buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA (pH 8.0), 0.2 mM EGTA (pH 8.0), 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, and 25% (v/v) glycerol, incubated for 30 min on ice with frequent gentle mixing, and then centrifuged for 20 min at 4°C to remove insoluble material. The nuclear extract was stored at -80°C, and its protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, California, USA).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts (5 μ g of protein) were incubated for 30 min at 4°C in a final volume of 10 μ l reaction mixture containing 20 mM Hepes (pH 7.9), 10 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 2 μ g of salmon sperm DNA, and 1 \times 10⁴ cpm of a ³²P-labeled oligonucleotide probe in the absence or presence of competitors. The sequence of Fas/GAS oligonucleotide was 5'-TGTCATTCAGN^AACGTCTG-3' (nucleotide -662 to -682 of the Fas gene, N:G/A). The consensus GAS oligonucleotide, 5'-AAGTACTTTCAGTTTCATATTACTCTA-3', was purchased from Santa Cruz Biotechnology, Inc. For competitive assay with an anti-signal transducer and activator of transcription (STAT)1 α ⁹¹ antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA), 2 μ g of antibody was incubated with nuclear extract for 30 min at 4°C before adding the probe. To ensure the specificity of interactions, a 50 or 200-fold excess of unlabeled oligonucleotide was used in cold competition reactions. Then, electrophoresis was performed on 6% polyacrylamide gels (polyacrylamide/bis-acrylamide ratio, 29:1) in a Tris-borate buffer (pH 8.0). The gel was directly analyzed using a Fujix BAS 2000 bioimage analyzer (Fuji Photo Film Co., Tokyo, Japan).

Plasmid constructs. Genomic DNA was obtained from 2 healthy individuals, who have a homozygous genotype of A or G alleles at the -670 nucleotide

position. Their sequences were confirmed by direct sequencing. The Fas promoter fragment was initially isolated by PCR using following primers; Fas-5: 5'-GCGGCTAGCAGTAATGATGTCATTATCC-3', Fas-3: 5'-CTGAAGCTTGGTTGTTGAGCAATCCTC-3'. The primers were originally described by Rudert, *et al*²⁶, and we modified their original sequences to contain a *Nhe* I site at the 5'-end of Fas-5 and a *Hind* III site at that of Fas-3 primers, respectively. Amplified fragments were then digested with *Nhe* I and *Hind* III. The resulting 1008 bp fragments were introduced in front of a luciferase gene in the pGL3-Basic Vector (Promega, Madison, Wisconsin, USA) digested with *Nhe* I and *Hind* III. These reporter constructs were designated pGL3-FAS(A) and pGL3-FAS(G), according to the -670A and G genotypes, respectively.

Luciferase-reporter assay. For transfection, human cervix carcinoma cell line, HeLa cells (JCRB9004) were obtained from the HSRRB. The transfection was performed with LipofectAMINE2000 (LF2000; Gibco BRL Life Technologies, Rockville, Maryland, USA) according to the manufacturer's instruction. Briefly, the day before transfection, 5 \times 10⁵ cells were plated in each well of 6 well plates and incubated with serum-free MEM for 24 h. The cells were co-transfected with 5 μ g of pGL3-FAS (A) or pGL3-FAS (G) and 100 ng of pRL-CMV vector (Promega) by using LF2000. After transfection, the cells were cultured with serum-free MEM at 37°C for 6 h, and sequentially incubated in the medium with 10% fetal bovine serum for 24 h. Then the transfectants were harvested and assayed for luciferase activity. The measurement of luciferase activity of transient transfectants was performed using a Dual-Luciferase assay protocol (Promega). The data obtained from the luciferase assay were quantified and expressed relative to the basal expression levels produced by the promoterless pGL3-Basic vector.

Statistical analysis. Allele and genotype frequencies were analyzed with chi-square test at a significance level $p < 0.05$. Transcriptional activity was analyzed with unpaired t test at a significance level of $p = 0.05$.

RESULTS

Allele and genotype frequencies of -670A/G and -1377G/A polymorphisms in Fas promoter gene. The frequencies of -670A/G and -1377G/A polymorphisms in patients and controls are shown in Table 2. There were no significant differences in the allele and genotype frequencies of -1377G/A polymorphism between SLE and controls. In contrast, the frequency of -670A allele was significantly

Table 2. Genotype frequencies in Fas promoter gene.

	SLE, n = 109 (%)	Controls, n = 140 (%)	P
-670A/G			
Genotype frequencies			
AA	35 (32)	26 (18)	
AG	49 (45)	94 (46)	0.03*
GG	25 (23)	50 (36)	
Allele frequencies			
A	119 (55)	116 (41)	0.004
G	99 (45)	164 (59)	
-1377G/A			
Genotype frequencies			
GG	42 (39)	45 (32)	
AG	42 (39)	62 (44)	0.29**
GG	25 (22)	33 (24)	
Allele frequencies			
G	126 (58)	152 (54)	0.43
A	92 (42)	128 (46)	

* AA and AG genotypes vs GG genotype.

** GG and AG genotypes vs AA genotype.

higher in SLE patients than that in controls (55% vs 41%, $p = 0.004$), as shown in Table 2. There were no associations between Fas promoter -1377G/A or -670A/G polymorphism, and clinical characteristics of SLE patients such as gender (100 female vs 9 male), age at onset (> 15 yrs, $n = 95$ vs ≤ 15 years, $n = 14$), and the presence or absence of renal disorder (presence, $n = 30$), photosensitivity ($n = 15$), and oral ulcer ($n = 5$) (data not shown).

Linkage disequilibrium between -670A/G and -1377G/A polymorphisms. Because these polymorphisms are located about 700 bp nucleotides apart, it was speculated that these polymorphisms may be under linkage disequilibrium. As shown in Table 3, there was a significant linkage disequilibrium between the A allele of -670A/G polymorphism and G allele of -1377G/A polymorphism in both groups. Haplotype analysis demonstrated that there were mainly 3 haplotypes in

these 2 polymorphic sites, and that a significant difference was observed in haplotype distribution between patients with SLE and controls (Table 3, $p = 0.0099$).

Nuclear factor STAT1 binding activity to Fas/GAS region. The -670A/G polymorphism is located in the consensus sequence of nuclear transcription element GAS binding site, so we performed EMSA to investigate whether this polymorphism affects the STAT1 binding activity to the GAS region in the Fas promoter. The intensity of the STAT1 binding complex was 3.5-fold higher in A sequence than in G sequence (Figure 3, lanes 2 and 9). Further, a 50-fold excess of unlabeled consensus GAS oligonucleotide almost completely inhibited the binding of STAT1 to the GAS sequence with -670G, but not to that with -670A (Figure 3, lanes 3 and 10), and a 200-fold excess of consensus GAS or Fas/GAS oligonucleotide with -670A or -670G inhibited both probes' binding (lanes 4-

Table 3. Estimated haplotype frequencies and linkage disequilibrium coefficients.

Population	Haplotype (-670A/G)-(-1377G/A)				D'	Disequilibrium*		
	A-G	A-A	G-A	G-G		χ^2	p	
Controls	0.374	0.039	0.417	0.170	**	0.792	67.0	< 0.00001
SLE	0.453	0.093	0.329	0.125		0.597	45.0	< 0.0001

Data given for pairs of Fas loci in control and SLE populations.

* $D' = D/D_{max}$ for association of most common alleles at each locus.

** Whole distributions of alleles between patients with SLE and controls were evaluated by chi-square test for a 2×4 table ($p = 0.0099$)

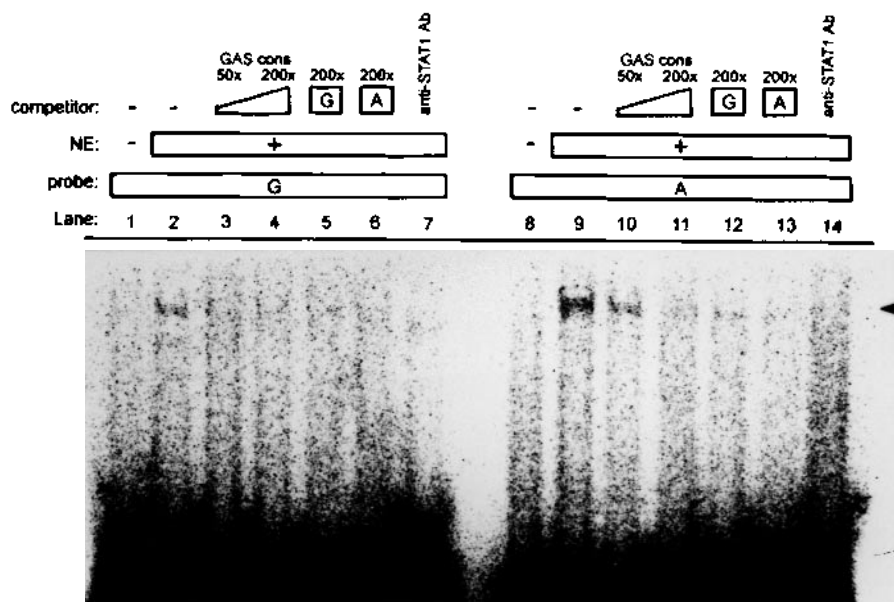


Figure 3. STAT1 binding to the GAS-like element on the Fas gene promoter. Nuclear extracts from cytokine-stimulated U937 cells were incubated with 2 kinds of double-stranded oligonucleotide probes, and electrophoretic mobility shift assays were performed. Each probe has the GAS element on the Fas promoter containing single nucleotide polymorphism, G or A. Arrowhead indicates the probe-STAT1 complexes. The anti-STAT1 α^{p91} antibodies specifically inhibit the complex forming (lanes 7 and 14). The radiointensity of the probe (A)-STAT1-formed complex is higher than that of the probe (G)-STAT1 complex (lanes 2 and 9). The competitive assay using radioisotope-free GAS consensus or Fas/GAS oligonucleotides also shows that the probe (A) binds with STAT1 more strongly than probe (G) (lanes 3-6 and 10-13).

6 and 11-13). The antibody against STAT1 αp^{91} blocked the DNA binding activity of the STAT complex. A supershifted complex, however, was not observed when this antibody was used.

The transcriptional activity of Fas promoter -670A/G polymorphism determined by luciferase assay. To examine whether this polymorphism affects the transcriptional activity of Fas promoter, we made 2 genotype constructs of Fas promoter and evaluated their transcriptional activities by the luciferase assay. As shown in Table 4, Fas promoter with -670A showed a slightly higher transcriptional activity than that with -670G allele, although there was no statistically significant difference.

DISCUSSION

Precise regulation of Fas gene expression appears to play a role in the avoidance of SLE development, since either enhanced or defective Fas-mediated apoptosis might result in an impaired clearance of apoptotic cells or failure in the elimination of autoreactive cells, and contribute as one of the susceptibility factors to SLE development. A recent report has shown that silent Fas gene polymorphism at the nucleotide position of 297 of C allele or 416 of G allele was associated with the development of SLE in a Japanese⁷, but not in an Italian population²⁷.

Our study showed that -670A polymorphism of Fas promoter gene was associated with the development of SLE. Recently, Huang, *et al* found that homozygous A allele at -670 was associated with clinical features such as photosensitivity and oral ulcers but not with SLE development in Caucasian SLE patients²⁸. Possible reasons for the inconsistency might include the differences in the genetic and environmental backgrounds and in the frequencies of photosensitivity and oral ulcer in each patient group. In contrast, the -1377G/A polymorphism was not associated with SLE susceptibility in our and their studies²⁹. Among these -670A/G and -1377G/A polymorphisms, there were 3 major haplotypes -670A/-1377G, -670G/-1377A, and -670G/-1377G. -670A was mainly associated with -1377G. Comparison of estimated haplotype frequencies demonstrated that there was a significant difference between SLE patients and controls. Taken together, genotype analysis of each polymorphism and the haplotype study of adjacent ones suggested that the promoter region, which includes these polymorphisms, was of functional

Table 4. Determination of the transcriptional activity of Fas promoter -670A/G genotype by luciferase assay.

Promoter construct	Relative fold	P
-670 A	4.05 ± 0.70	0.25
-670 G	3.52 ± 1.23	

Reporter gene activity of -670A or G genotype (mean ± SE) was expressed relative to that of promoterless pGL3-Basic vector (activity = 1).

importance for the transcription and that -670A/G polymorphism influenced the transcriptional activity of the Fas gene.

The transcriptional mechanism controlling Fas expression is largely unknown, but its gene expression can be regulated by a number of genetic elements located in the 5' upstream region of the gene. Recent studies have shown the transcription factors that bind to these elements and regulate transcription of Fas promoter³⁰⁻³². The promoter region of the Fas gene encompasses a 2000 bp sequence that consists of basal promoter, enhancer and silencer regions³³ and contains 2 polymorphisms, -670A/G polymorphism in the enhancer region and -1377G/A polymorphism in the silencer region²³. The -670A/G polymorphism results in a nucleotide substitution within the GAS sequence for the transcription factor termed STAT1. With EMSA and luciferase assays, we demonstrated that STAT1 binding activity was higher in -670A allele of Fas promoter than in -670G allele and that the transcriptional activity of A allele tended to be higher than that of G allele although there was no statistically significant difference. Therefore, in SLE, it was speculated that the -670A allele with higher STAT1 binding activity might result in the alteration of Fas gene expression, followed by the change in the sensitivity to Fas-mediated apoptosis in several decades after birth.

In conclusion, our findings that -670A allele with slightly higher STAT1 binding activity was significantly associated with SLE suggest that Fas gene might serve as a candidate gene for development of SLE in Japanese population.

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