Association of Fas/APO-1 gene polymorphism with systemic lupus erythematosus in Japanese

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Abstract

Objectives. This study was undertaken to investigate the possible association of Fas gene mutation(s) or polymorphism(s) with systemic lupus erythematosus (SLE) in Japanese.

Methods. Screening for structural defects of the Fas gene was performed by using reverse transcriptase-polymerase chain reaction (RT-PCR)/single-strand conformation polymorphism (SSCP) analysis in 57 patients with SLE, followed by direct sequencing for the aberrantly migrating bands. The frequency of Fas polymorphism was determined by sequence-specific oligonucleotide probe (SSOP) hybridization in 82 SLE patients and 132 ethnically matched healthy individuals.

Results. We found a novel polymorphism at nucleotide 297 (T297C), which was linked to Fas polymorphism at nucleotide 416 (A416G). The 297C/416G genotype was present in four of the 132 (3.0%) healthy controls, none of whom was homozygous for the genotype. The allele frequency for 297C/416G in the controls was 1.5%. In contrast, 10 of the 82 (12.2%) SLE patients carried the 297C/416G allele, including one patient homozygous for the genotype. The allele frequency in SLE patients was 6.7%. The 297C/416G allele was significantly frequent in SLE patients (P = 0.01, χ2) with a relative risk of 5.00.

Conclusion. As the polymorphism 297C/416G is silent at the amino acid level, it may affect the expression of Fas itself or be linked to a neighbouring genetic abnormality that is responsible for the pathogenesis of SLE.

Key words: Fas, Polymorphism, Systemic lupus erythematosus, Polymerase chain reaction, Single-strand conformation polymorphism, Sequence-specific oligonucleotide probe hybridization.

Systemic lupus erythematosus (SLE) is an inflammatory disorder affecting multiple organ systems; it is characterized by the presence of antibodies reactive against various self-antigens. The breakdown of self-tolerance in SLE is caused by a defect in the elimination of autoreactive T or B cells [1].

Apoptosis (programmed cell death) is a physiological process that regulates normal tissue homeostasis. Failure of apoptosis is likely to contribute to the development of autoimmune disorders by the impaired elimination of autoreactive T or B cells. Fas, also known as APO-1 or CD95, is a type I membrane protein and is expressed in various cell types. Fas is considered to play a critical role in the regulation of the immune system by deleting autoreactive peripheral lymphocytes [2]. Defects in Fas and its ligand (FasL) have been reported in mouse models of human SLE: MRL-lpr, MRL-lpr<sup>rg</sup> and C3H-gld mice [3–5]. In patients with SLE, increased levels of the soluble form of Fas have been reported which may interfere with the apoptosis of autoreactive lymphocytes [6–8]. However, a lack of correlation between soluble Fas levels and autoimmune diseases has also been reported [9, 10]. Structural defects in the genes for Fas and FasL have been identified in humans as well as mice. The defects in the Fas gene are identified in the disease known as autoimmune lymphoproliferative syndrome (ALPS) or Canale–Smith syndrome, which is characterized by massive lymphadenopathy, hepatosplenomegaly, and autoimmune manifestations such as haemolytic anaemia and thrombocytopenia, with increased peripheral double-negative T cells [11–14]. In one SLE patient, a defect in FasL was reported to be associated with defective activation-induced cell death (AICD) and FasL-mediated apoptosis [15]. These lines of evidence prompted us to investigate the structural defects in the Fas molecule in SLE patients. We did not...
identify any mutations in the Fas gene with reverse transcriptase-polymerase chain reaction (RT-PCR)/single-strand conformation polymorphism (SSCP) analysis; however, we identified a novel polymorphism, T297C, which corresponds to the first nucleotide of the codon for amino acid residue 19 (nucleotide and amino acid numbering based on that reported previously) [16], which was apparently linked to one of the two reported Fas gene polymorphisms, A416G [17]. Both of the two polymorphisms were located in the coding region, but were silent at the amino acid level. We found a strong association of Japanese SLE with the 297C/416G genotype, suggesting that this genotype may affect the expression of the Fas gene or may be in linkage disequilibrium with a disease-causing mutation within an adjacent gene.

Patients and methods

SLE patients and control subjects

Eighty-two patients with SLE (77 females, five males) who were diagnosed according to the 1982 revised criteria of the American College of Rheumatology [18] were studied. The control samples were obtained from 132 ethnically matched healthy individuals.

DNA and RNA preparation

Peripheral blood mononuclear cells (PBMC) were prepared by Lymphocyte Separation Medium (LSM®) (Organon Teknika Corp., Durham, NC, USA) from the heparinized blood of subjects. Total RNA and DNA was prepared from the PBMC as described [19].

RT-PCR/SSCP analysis for the Fas gene

The primers used in the present study were synthesized on the basis of the nucleotide sequence of Fas cDNA [16] and are listed in Table 1. The schematic presentation of Fas cDNA, along with the position of the primers, is shown in Fig. 1. Fifty-seven out of the 82 SLE patients were studied. The control samples were obtained from 132 ethnically matched healthy individuals.

RT-PCR/SSCP analysis

The primers used in the present study were synthesized (New England Biolabs, Inc., Beverly, MA, USA) from the heparinized blood of subjects. Total RNA and DNA was prepared from the PBMC as described [19].

Sequencing analysis

Primers were labelled using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA, USA) and [α32P]ATP at 37°C for 20 min. For the analysis of the Fas gene, DNA fragments of interest were excised from the SSCP gels, purified on Suprec-01 (Takara Shuzo Co., Otsu, Japan) and reamplified for 20 cycles consisting of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. The PCR products were diluted with formamide dyes (95% formamide, 20 mm Na2EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and heat denatured at 80°C for 5 min. They were then subjected to electrophoresis on 5% polyacrylamide gel at 25°C with 5% glycerol, or at 4°C without glycerol at 13 V/cm, using 45 mm Tris–borate and 1 mm EDTA buffer (pH 8.3). DNA fragments were visualized by exposing the gels to Fuji RX film (Fuji Photo Film Co., Kanagawa, Japan).

Table 1. Oligonucleotides for amplification of human Fas cDNA

<table>
<thead>
<tr>
<th>Fragments</th>
<th>5’ Oligonucleotide (5’ to 3’)</th>
<th>3’ Oligonucleotide (5’ to 3’)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CACTTGGGAGGGATTGTCCCAACA</td>
<td>TGTTGACTCCTTCCTCCCTTTG</td>
<td>301</td>
</tr>
<tr>
<td>B</td>
<td>ATAGGGATGAACCCAGACTGC</td>
<td>CCCAACTAATTGTGGAATTG</td>
<td>343</td>
</tr>
<tr>
<td>C</td>
<td>GATCCGATCTAATCTTTGGGT</td>
<td>TCTTCCATGAAGTTCAGC</td>
<td>358</td>
</tr>
<tr>
<td>D</td>
<td>GCAGAAGCAAGAGTTCAACTGC</td>
<td>TATTTGCGGCTTTCAGCGCTA</td>
<td>331</td>
</tr>
</tbody>
</table>

*These two nucleotide sequences were previously designed by Cheng et al. [6].
Sequence-specific oligonucleotide probe hybridization

A sequence-specific oligonucleotide probe (SSOP) hybridization was performed to assess the frequency of Fas polymorphisms in SLE patients and healthy controls. As shown in Fig. 2, three pairs of SSOP were synthesized (Takara Shuzo Co.), and were used for this analysis as described [21]. Briefly, genomic DNA of exons 2, 3 and 7 of the Fas gene was amplified by PCR and used as a template. The primer pairs for amplifying these exons were designed based on the Fas genomic sequences reported previously [22]: 5'-GGTTTACA-CGGTGTCTACTTC-3', 5'-GACCTTCACTGTGAAATTCTG-3' for exon 2; 5'-CTGGTCAACACATT-GCTCCTT-3', 5'-CCAAGATTGGCCTTTCAAT-3' for exon 3; 5'-TCATGCTTTCTACAAGGCT-3', 5'-GCAAGACTCCATCTCAAAAC-3' for exon 7. The product sizes were 265, 213 and 269 bp, respectively.

PCR was performed for the Fas gene for 30 cycles of 1 min at 95°C and 2 min at 60°C. Fifty nanograms of amplified DNA in 2 μl were dot-blotted onto nylon filter membranes, alkaline denatured with 0.4 N NaOH, and incubated at 54°C for 1 h with hybridization buffer (50 mM Tris–HCl, pH 8.0, 3 mM tetramethylammonium chloride, 2 mM EDTA, 5 × Denhardt’s solution, 0.1% SDS, 100 μg/ml heat-denatured salmon sperm DNA) containing 5–10 pmol of SSOP that was end-labelled by [γ-32P]ATP using T4 polynucleotide kinase. After rinsing with 2 × SSPE (0.3 M NaCl, 0.02 M sodium phosphate, 2 mM EDTA, pH 7.4) containing 0.1% SDS at room temperature, the filters were washed in TMAC solution (50 mM Tris–HCl, pH 8.0, 3 mM tetramethylammonium chloride, 2 mM EDTA, 0.1% SDS) at room temperature for 10 min, followed by washing in TMAC solution at 58°C for 10 min twice. The filters were then subjected to autoradiography.

Analysis of Fas expression on freshly isolated PBMC

The experimental procedure has been described previously [23]. The expression of Fas was studied by FACScan. The immunofluorescence analysis was performed using mouse monoclonal IgM antibody against Fas (MBL, Nagoya, Japan).

Statistical analysis

Statistical analysis was performed using the χ² test.

Results

Detection of Fas polymorphism by RT-PCR/SSCP analysis

To investigate the presence of Fas gene mutations in SLE patients, we first analysed mRNA transcripts of the Fas gene by RT-PCR/SSCP. As shown in Fig. 1, four pairs of primers were used to amplify four overlapping regions of Fas cDNA (fragments A, B, C and D), which covered the entire coding region of Fas along with the part of the 5' and 3' untranslated region of Fas cDNA. The product size of PCR was between 301 and 358 bp. As the 3' primer for fragment B and the 5' primer for fragment C were designed to locate in the transmembrane domain of the Fas gene, fragments B and C detected only the transmembrane form. Among 57 SLE patients, six (patients numbers 1, 4, 27, 38, 39 and 43) displayed aberrant bands in fragment A, and five (patients numbers 3, 14, 16, 36 and 42) in fragment C, while none of the patients showed aberrant bands in fragments B and D. As both of the bands detected in fragment A of patient no. 4 migrated differently from those of healthy controls, this patient appeared to be homozygous for the variation. The other patients carried two aberrantly migrated bands in addition to those found in healthy controls, suggesting the heterozygosity for the variation. The aberrant bands were excised from the SSCP gel and directly sequenced in their entirety. The nucleotide sequence of the aberrant bands in fragment A was identical with that reported previously [16], except that nucleotide 297 was a C instead of a T (T297C), and nucleotide 416 was a G instead of an A (A416G). In fragment C, the aberrantly migrated band encoded the nucleotide sequence identical to that previously described except that nucleotide 836 was a T instead of a C (C836T). These polymorphisms at nucleotides 297, 416 and 836 did not lead to amino acid substitution.

Association of 297C/416G Fas polymorphism with SLE

We next analysed the frequencies of the three polymorphisms in SLE patients and healthy controls. PCR amplification was performed with intron-based primers for exons 2, 3 and 7 for 82 SLE patients, followed by genotyping by SSOP analysis. As the polymorphisms 297C and 416G always existed simultaneously in the samples examined, both were considered to be located on the same haplotype. As shown in Table 2, the 297C/416G allele was present in 24 of the 132 (18.0%) healthy controls, none of whom was homozygous for the genotype. The allele frequency for 297C/416G in the controls was 1.5%. In contrast, 10 of the 82 (12.2%) SLE patients carried the 297C/416G allele, including one patient homozygous for the genotype. The allele
frequency in SLE patients was 6.7%. The 297C/416G allele was significantly frequent in SLE patients \((P = 0.01, \chi^2)\), with a relative risk of 5.00. The expression of Fas was not altered in the PBMC from the SLE patient homozygous for the 297C/416G genotype (data not shown). No significant differences in clinical features were observed between the patients with or without the 297C/416G allele. On the other hand, there was no significant difference in the frequency of the polymorphisms at nucleotide 836 (Table 2).

**Discussion**

SLE is a polygeneic autoimmune disorder affecting multiple organ systems. Genetic predisposition to SLE has been linked to genes located within major histocompatibility complex (MHC) class II (DR, DQ) and class III (C2, C4, TNF) on chromosome 6 [24]. Of these factors, the C4 null allele, especially C4A (C4AQ0), has been shown to be the strongest correlate among different ethnic groups [25]. Individuals with a deficiency of the other class III complement gene, C2, are also at increased risk for SLE. The precise mechanism by which these complement deficiencies contribute to the pathogenesis of SLE is unclear; however, there are explanations, such as a disturbance of immune complex clearance, the increased risk of pathogens causing an autoimmune response, or the linkage disequilibrium with the other MHC genes. Associations between DR2 or DR3 and susceptibility to SLE have also been noted in various ethnic groups [26]. It is suggested that the antigen for autoimmune response is presented by the critical amino acids in the DR region. Recent studies in both animal models and other human autoimmune diseases also suggest that non-MHC genes contribute to the susceptibility to autoimmune diseases. In SLE, an association has been shown with the deficiencies of the complement components C1q, C1r and C1s, and certain alleles of immunoglobulin heavy-chain (Gm) gene and Fcγ receptor genes [24–26].

In order to study the possibility that the Fas gene can be an additional gene candidate, we searched for mutations in SLE patients using RT-PCR/SSCP analysis. Although we were unable to identify any mutations in our 57 SLE patients, we found a novel polymorphism at nucleotide 297 in addition to the previously identified polymorphisms at nucleotides 416 and 836 [17]. The novel polymorphism 297C was apparently linked to the polymorphism 416G and, more importantly, the allele 297C/416G was significantly associated with SLE when studied in 82 SLE patients and 132 healthy controls (Table 2). This silent polymorphism does not seem to affect the steady-state expression of Fas by changing the secondary structure of the Fas message or by linkage to the structural defects in the Fas gene, because FACSscan analysis showed that the expression of Fas was not modulated on the PBMC from the patient homozygous for the 297C/416G allele (data not shown). This is compatible with the finding that no significant defects could be detected in Fas expression or function in SLE patients [27]. Alternatively, it could be speculated that the mutation or polymorphism, linked to the 297C/416G genotype, may reside in the promoter or in the intron region of the Fas gene, and that it modulates transcription or alternative splicing of the Fas message in a particular condition or cell population. In fact, such polymorphisms have been reported in the promoter region of the human Fas gene (−1377 nucleotide position in the silencer region and at −670 nucleotide position in the enhancer region) [28]. The former polymorphism resides in the binding site sequence of transcription factor SP-1, and the latter occurs in the binding site of the nuclear transcription element GAS. It is also probable that polymorphism(s) in the intron may affect the alternative splicing of the Fas gene, considering that at least five forms of soluble Fas molecules have been reported to be generated by alternative splicing and to prevent Fas-mediated apoptosis [29]. The association of silent polymorphism in the coding region with a human disease is not unprecedented. For example, 5-HT2A receptor polymorphism was implicated in the pathogenesis and pharmacotherapy of schizophrenia by a large-scale multicentre study in Europe (European Multicentre Association Study of Schizophrenia Group) [30]. The T to C transition at nucleotide 102 (T102C) in the coding region of the 5-HT2A receptor did not alter amino acid sequence or the steady-state expression of the 5-HT2A receptor [31].

Although a large-scale study in SLE patients, as well as in patients with autoimmune diseases other than SLE, is still needed, the 297C/416G polymorphism in the Fas gene or a neighbouring genetic abnormality may serve as a candidate for the pathogenesis of SLE.

**Acknowledgements**

This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan and the Foundation of the Advancement of Clinical Medicine.

**References**


**Table 2. Association of Fas polymorphisms and SLE**

<table>
<thead>
<tr>
<th>Fas polymorphism</th>
<th>SLEa</th>
<th>Controlb</th>
<th>RR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>297C/416G</td>
<td>12.2%</td>
<td>3.0%</td>
<td>5.00</td>
<td>0.01</td>
</tr>
<tr>
<td>836T</td>
<td>10.9%</td>
<td>8.3%</td>
<td>1.34</td>
<td>NS</td>
</tr>
</tbody>
</table>

RR, relative risk; NS, not significant.

aThe numbers of patients studied were 82 for 297C/416G and 46 for 836T.
bThe numbers of controls studied were 132 for 297C/416G and 36 for 836T.


