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Transforming Growth Factor Beta 1 Gene Polymorphism in Japanese Patients with Systemic Lupus Erythematosus

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To determine the role of transforming growth factor beta 1 (TGF β 1) gene polymorphisms in the pathogenesis of systemic lupus erythematosus (SLE), we investigated the polymorphisms of T869C of the TGF β 1 gene in 196 patients with SLE and 106 healthy controls by analyzing polymerase chain reaction-restriction fragment length polymorphism. The genotype and allele frequencies of T869C of the TGF β 1 gene did not differ between SLE and healthy controls. However, compared to the TC and CC genotypes, the TT genotype was associated with anti-SS-A/Ro antibody production ($p=0.029$) and a higher incidence of aseptic necrosis ($p=0.0097$); the CC genotype had a higher frequency of anti-RNP antibody production compared to the patients with the TC and TT genotypes ($p=0.023$). These results suggest that T869C polymorphism of the TGF β 1 gene is involved in the development of autoantibodies and the occurrence of aseptic necrosis in patients with SLE. Thus, TGF β 1 polymorphism might be one of the genetic factors that explain the heterogeneity seen with SLE.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies against a spectrum of nuclear antigens. The clinical consequences of SLE are extremely heterogeneous, and can potentially lead to inflammatory damage in a variety of different organ systems. Although the factors responsible for the initiation of SLE are poorly understood, genetic predisposition is firmly established as a key element in susceptibility, and several susceptibility loci have been reported (10, 21, 26). Certain major histocompatibility complex (MHC) and some polymorphic non-MHC genes, such as complement component, immunoglobulin receptors, cytokines, and the molecules involved in apoptosis, have also been reported to be associated with SLE or the disease phenotype (32). Such data highlights the complexity of the genetic interactions involved in SLE.

TGF β 1, which belongs to the TGF β family of proteins, is secreted by a variety of cell types with unique and potent immunomodulatory properties in the maintenance of normal immunological homeostasis (18). TGF β 1 displays multiple functions which, depending on the responding cell type and the state of differentiation, are sometimes opposing (33). TGF β 1 knockout mice developed an SLE-like disorder with various autoantibodies and Sjögren's syndrome-like lymphoproliferative disease, suggesting the possible involvement of TGF β 1 in the pathogenesis of SLE (8).

TGF β 1 gene has several polymorphisms, including C-988A, G-800A, and C-509T in the promoter region, position 72 (C insertion) in the non-translated region, and C263T (Thr/Ile), T869C (Leu/Pro), G915C (Arg/Pro) in the coding region (3). Among them, three polymorphisms, C-509T, T869C, G915C have been shown to be associated with the serum level of TGF β 1 (2,12). Among them, G915C polymorphism was not found in the Japanese population, and C-509T polymorphism has been shown to be in a linkage disequilibrium with T869C polymorphism (20,22,24). Thus, we studied the relationship of T869C polymorphisms of the TGF β 1 gene to disease susceptibility and the clinical features of SLE in Japanese patients.

PATIENTS AND METHODS

Patients

A total of 196 Japanese patients with SLE (179 female and 17 male; average age, 39.6 years) followed at Kobe University Hospital and Juntendo University Hospital were enrolled. All patients met the 1982 American College of Rheumatology (ACR) revised criteria for SLE (29). The patients who fulfilled the preliminary criteria for the classification of Sjögren's syndrome (SS) (31) were excluded from this study. 106 healthy Japanese volunteers (65 female and 41 male; average age 37.2 years) served as controls. Written informed consent was obtained from all subjects.

TGF β 1 genotyping

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll-Hypaque gradient centrifugation. Genomic DNA was extracted from the PBMC by a standard procedure. T869C polymorphism of the TGF β 1 gene was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as previously described (22). The PCR primers that were used were: forward primer: 5'-TTCCCTCGAGGCCCTCCTA and reverse primer: 5'-GCCGC-AGCTTGGACAGGATC. Sample DNA (100 ng) was amplified in 25 μ l of a reaction mixture containing 0.5 units of Taq DNA polymerase, dNTPs (2.5 μ M each) (Perkin-Elmer, Norwalk, CT, USA), using an automated PCR thermal cycler (GeneAmp PCR System 2700; Applied Biosystems; Foster City, CA USA). The samples were denatured at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min, and then incubated at 72°C for an additional 10 min to complete the final extension step after the last cycle. The PCR products were digested for 2 h at 37°C with MspAII (10,000 units/ml) (New England Biolabs, Hitchin, U.K) and 10 μ l amplicon, run on a 3% agarose gel, and then visualized with ethidium bromide. The T allele yields fragments of 161, 67, 40, and 26 bp, while the C allele yields fragments of 149, 67, 40, 26, and 12 bp, which allowed the genotypes to be determined; the PCR-RFLP results were confirmed in selected cases by direct genotyping (ABI PRISM 310 Genetic Analyzer; Applied Biosystems; Foster City, CA USA).

Measurement of Serum TGF- β 1 Concentration

Sera were randomly obtained from 48 of the 196 SLE patients and stored at -20°C until the assays were done. The serum concentration of activation TGF- β 1 protein was determined with an enzyme-linked immunosorbent assay kit (Quantikine; R&D systems, Minneapolis, USA). The detection limit of this assay was 4.61 pg/ml.

Serological measurements

Sera were obtained from the 196 SLE patients and stored at -20°C until assays were done. Antibodies against dsDNA, Sm, nuclear RNP (nRNP), SS-A/Ro, and SS-B/La, were measured by enzyme-linked immunosorbent assays (ELISA; Mesacup-ANA; Medical and

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Biological Laboratories (MBL), Nagoya, Japan). A λ -phage-derived purified double-stranded DNA was used as an antigen for anti-dsDNA antibody ELISA. Antigens were purified from bovine spleen and thymus for anti-Sm antibody ELISA. A mixture of recombinant 70 kDa, A and C antigens was used for anti-RNP antibody. A mixture of recombinant 60 kDa antigen and purified antigens from bovine spleen and thymus was used for the anti-SS-A/Ro antibody. For the anti-SS-B/La antibody, recombinant SS-B/La antigen was used.

Statistical analysis

Chi-square analysis on 3x2 or 2x2 tables with Fisher's exact tests was used to study the differences in genotype and allele frequencies between SLE patients and controls. The 2x2 tables with Fisher's exact tests were used for the study of the association between the genotypes and the clinical features, as well as the antibodies of SLE patients. The Wolf procedure was used to estimate the odds ratio (OR) and the 95% confidence interval (CI). The Mann-Whitney *U* test was used to compare the serum concentrations of TGF β 1 among the T869C genotypes.

RESULTS

T869C polymorphisms of the TGF β 1 gene in patients and controls

We first compared the frequency of the genotype and allele frequencies of the T869C polymorphisms of the TGF β 1 gene in our patients and controls (Table 1). There were no significant differences in the genotype and allele frequencies between SLE patients and healthy controls; non-significant trends towards an increased frequency of the TT genotype were observed in SLE patients. We concluded that T869C polymorphism of the TGF β 1 gene is not associated with susceptibility to SLE.

TGF β 1 polymorphisms and the serum level of TGF β 1

The serum concentrations of TGF β 1 in 48 patients (n=11 for CC, 29 for CT, and 8 for TT) were measured and examined for their association with TGF β 1 polymorphisms. Though the serum TGF β 1 level was higher in patients with the CC genotype, and lower in patients with the TT genotype, the difference was not statistically significant (CC 38.26 $\pm$ 3.19 ng/ml, CT 37.21 $\pm$ 1.99 ng/ml, TT 30.13 $\pm$ 3.18 ng/ml; *P* = 0.089 between the CC and TT genotypes).

Table 1. Genotype and allele frequencies of the T869C TGF β 1 polymorphism in SLE patients and healthy volunteers

	SLE (n=196)	Controls (n=106)	P
Genotypes			
TT (%)	46(23.5)	18(17.0)	0.3
TC (%)	106(54.1)	58(54.7)	
CC (%)	44(22.4)	30(28.3)	
Alleles			
T (%)	198(50.5)	94(44.3)	0.1
C (%)	194(49.5)	118(55.7)	

P-values were determined by chi-square analysis of 3x2 or 2x2 tables with Fisher's exact test.

Association of clinical features with TGFβ1 genotypes in SLE patients

SLE is a heterogeneous disease with various clinical features, which in part may be ascribed to genetic factors. Therefore, we examined the genotype distribution of the TGFβ1 gene in SLE patients with different clinical features of SLE (Table 2). When the TT genotype was compared to the combined TC and CC genotypes, patients with the TT genotype had a higher incidence of aseptic necrosis (20.0% vs. 6.5%; $p=0.0097$, OR 3.75, 95%CI: 1.306 to 10.767), which suggests an association of the TT genotype with aseptic necrosis in SLE patients.

Table 2. The association of TGFβ1 T869C genotypes with the clinical features of SLE patients

	TT	TC	CC
	%positive	%positive	%positive
Clinical features	(positive/total)	(positive/total)	(positive/total)
Discoid rash	21.4(9/42)	21.6(21/97)	17.0(7/41)
Malar rash	69.0(29/42)	70.2(66/94)	61.5(24/39)
Oral ulcer	41.5(17/41)	31.3(30/96)	27.0(10/37)
Raynaud's	26.8(11/41)	40.6(39/96)	35.1(13/37)
Arthritis	61.9(26/42)	72.5(74/102)	67.5(27/40)
Photosensitivity	39.5(17/43)	41.7(40/96)	41.7(15/36)
Pleuritis	7.1(3/42)	11.1(11/99)	7.9(3/38)
Pericarditis	11.6(5/43)	9.5(9/95)	10.5(4/38)
Interstitial pneumonia	0(0/43)	7.0(7/100)	4.9(2/41)
Persistent proteinuria	45.5(20/44)	35.4(35/99)	33.3(13/39)
Nephrotic syndrome	20.9(9/33)	16.5(14/89)	15.4(6/39)
Renal failure	2.3(1/43)	4.0(4/103)	0(0/40)
CNS disease	20.5(9/44)	9.9(10/101)	12.5(5/40)
Aseptic necrosis	20.0(8/40)*	4.4(4/91)	10.8(4/37)
Hemolytic anemia	9.3(4/43)	10.(10/100)	12.2(5/41)
Leukopenia	46.7(21/45)	47.9(46/96)	46.2(18/39)
Lymphocytopenia	62.2(28/45)	67.3(66/98)	59.0(23/39)
Thrombocytopenia	20.5(9/44)	24.7(24/97)	22.5(9/40)

* $P=0.0097$ (TT vs. TC+CC), OR= 3.75, 95%CI (1.306- 10.767). P value was determined by chi-square analysis of 2x2 tables with Fisher's exact test. CNS: central nervous system

Association of autoantibody profile with TGFβ1 genotypes in SLE patients

Next, we examined the association of autoantibody production with TGFβ1 gene polymorphism in SLE patients (Table3). The TT genotype was associated with a higher incidence of the anti-SS-A/Ro antibody than the combined TC and CC genotypes ($p=0.029$, OR 2.34, 95%CI: 1.077 to 5.068). Unexpectedly, we also found that the percentage of

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anti-RNP antibody-positive patients was significantly higher in patients with the CC genotype than in patients with the TC and CC genotypes ($p=0.023$, OR 2.51, 95%CI: 1.120 to 5.619). This suggests that TGF β 1 polymorphism may play a role in autoantibody production in these SLE patients.

Table 3. The association of TGF β 1 T869C genotypes with autoantibody production in SLE patients

	TT	TC	CC
	%positive	%positive	%positive
Antibodies	(positive/total)	(positive/total)	(positive/total)
Anti-dsDNA	52.6(20/38)	48.8(42/86)	37.5(12/32)
Anti-Sm	25.0(10/40)	12.4(12/97)	16.2(6/37)
Anti-RNP	29.7(11/37)	21.3(20/94)	43.8(14/32)**
Anti-SS-A/Ro	52.9(18/34)*	32.6(29/89)	32.3(10/31)
Anti-SS-B/La	15.2(5/33)	10.3(9/87)	6.5(2/31)
aCL	40.9(9/22)	25.8(16/62)	25.9(7/27)
RF	26.9(7/26)	23.7(18/76)	37.0(10/27)

* $p=0.029$ (TT vs. TC+CC), OR=2.34, 95%CI (1.077-5.068); ** $p=0.023$ (CC vs. TC+TT), OR=2.51, 95%CI (1.120-5.619). P values were determined by chi-square analysis from 2x2 tables with Fisher's exact test. RF: rheumatoid factor; aCL: anticardiolipin antibodies

DISCUSSION

In this study we examined T869C polymorphisms of the TGF β 1 gene in SLE patients and healthy volunteers. The T869C polymorphism was not associated with susceptibility to SLE. However, the T869C polymorphism in SLE patients was associated with the production of autoantibodies, such as the anti-SSA/Ro antibody and the anti-RNP antibody, and the development of aseptic necrosis. Thus, this is the first report to show that T869C polymorphisms of the TGF β 1 gene may be involved in the heterogeneity of clinical features seen in SLE patients.

The TGF β 1 gene has a number of polymorphisms, such as C-988A, G-800A, C-509T, 72 (C insertion), C263T, T869C, and G915C (3). Schotte et al. investigated the G915C polymorphism at codon 25 and did not find any association between the polymorphism and SLE in the German population (25); this polymorphism is not observed in the Japanese population. Lu et al. did not find an association between the TGF β 1 polymorphisms, including C-988A, G-800A, C-509T, T869C, and G915C, and susceptibility to SLE or lupus nephritis in the Taiwanese population (19). In agreement with this report, we also did not find an association between T869C polymorphisms and disease susceptibility. However, we did find an association between the T869C TGF β 1 polymorphism and several clinical features of SLE. Thus, our study is the first to show an association between TGF β 1 gene polymorphism and the clinical features of SLE.

Consistent with our results, the children with the TT T869C genotype of the TGF β 1 gene have been reported to be more susceptible to anti-SSA/Ro antibody-associated congenital heart block (7). Furthermore, T869C polymorphisms have been reported to be associated

with various diseases. In the Japanese population, rheumatoid arthritis, osteoporosis, and myocardial infarction have been associated with the TT genotype or the T allele (27, 36, 37), while systemic sclerosis, hypertension, and a reduced risk of breast cancer have been associated with the CC genotype (14, 22, 35). These findings suggest that both the TT and CC genotypes influence the susceptibility to various diseases. Therefore, the association between T869C polymorphisms and the production of different autoantibodies could be ascribed to different systemic or local TGF β 1 protein levels, as well as biological functions. It has been shown that the TT genotype is associated with a lower and the CC genotype with a higher serum level of TGF β 1 in healthy individuals in Japanese (35,37). However, in this study, we did not find an association between serum TGF β 1 levels and the T869C genotypes. The discrepancy could probably be due to either the small sample numbers in this study, or due to possible effects of disease activity and SLE therapies on the serum TGF β 1 levels of SLE patients.

A growing body of evidence has implicated TGF β 1 as a crucial factor during the clearance process of apoptotic cells by macrophages and has suggested that TGF β 1 may be involved in the production of various autoantibodies (4,9,15). Deficiency in the activation of TGF β 1 contributes to a reduced rate of phagocytosis of macrophages, as well as the development of autoantibodies in TGase^{-/-} mice (28). U1RNP 70-kD autoantigen has been shown to be produced from apoptotic cells (30). TGF β 1 can directly induce cell apoptosis *in vivo* and *in vitro* (5, 23); this leads to the modification of the 70-kD protein of the U1RNP antigen (6), which can be a relevant factor in the pathogenesis of SLE and other systemic rheumatic diseases (13).

In our study, the TT genotype of the T869C polymorphism of the TGF β 1 gene was also found to be significantly associated with aseptic necrosis, which is a well recognized complication of SLE. Steroid treatment and the presence of anti-phospholipid antibody are both considered to be risk factors for aseptic necrosis (1). Altered lipid metabolism may play a critical role in aseptic necrosis (17, 34), and TGF β 1 may be a potent regulator of lipoprotein lipase gene expression (16). Grainger *et al.* suggested an inverse correlation between serum LDL-cholesterol levels and the proportion of the active form of TGF β 1 (11). Thus, the TT genotype, which is the low TGF β 1 protein genotype, may be one of the risk factors for aseptic necrosis in SLE, through its effect on lipid metabolism.

In summary, T869C polymorphism of the TGF β 1 gene is likely one of the genetic factors involved in the production of anti-RNP and anti-SSA/Ro antibodies, and is associated with aseptic necrosis in SLE patients. Further studies are needed to help elucidate the mechanism by which the TGF β 1 polymorphism contributes to the pathogenesis of SLE.

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