

Fas/FasL promoter gene polymorphism in patients with rheumatoid arthritis

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SUMMARY

Objectives. Fas/FasL is significantly involved in the pathogenesis of rheumatoid arthritis (RA). Fas/FasL gene polymorphism may be associated with susceptibility to rheumatoid arthritis and disease severity.

Aim. To investigate the Fas 670 G/A and FasL 843 C/T genotype and allele frequency in patients with RA, and determine its potential association with susceptibility to the disease and the clinical parameters.

Methods. One hundred and one patients with RA and 105 healthy control subjects were enrolled in the study. Fas 670 A/G and FasL 843C/T genotype polymorphism was investigated by PCR-RFLP. Chi-square test was used for determining the genotype distribution and the allele incidence.

Results. There was no statistically significant difference between the patients with RA and the healthy subjects with respect to Fas-670 A/G and FasL-843C/T genotype distribution and allele frequency ($P>0.05$). While there was no statistically significant difference in disease severity and various clinical parameters, a correlation was detected between Fas-670 polymorphism and anti-CCP antibody and anemia ($P<0.01$ and $P<0.03$, respectively).

Conclusions. Fas-670A/G and FasL-843C/T promoter gene polymorphisms are not considered to represent a major genetic risk factor for RA susceptibility and disease severity. However, based on these results, Fas-670 promoter polymorphism may modulate anti-CCP antibody synthesis and response in patients with rheumatoid arthritis.

Key words: Rheumatoid arthritis, Fas/FasL, gene polymorphism, association, manifestations.

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■ INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, multisystemic, autoimmune disease with no well-established etiology, which manifests itself with synovial hyperplasia, articular cartilage and bone destruction. In RA synovium, pannus formation occurs as a result of permanent and abnormal synoviocytes and various inflammatory cell proliferations (1, 2). The cause of this chronic inflammation is not yet clear; however, one of the basic, major hypothesis is inadequate apoptosis (3, 4). Many various molecules and receptors have been defined in the regulation of cell apoptosis; however, Fas-Fas ligand death receptor pathway is significantly involved in the pathogenesis of RA (5). Fas(CD95) is a molecule that is expressed on the cell surface and initiates the signaling of death after binding its ligand (FasL). While Fas is expressed

in many cells, FasL is expressed in exclusive regions such as the active T cells, the natural killer cells, the tumor cells and the eye. The Fas-FasL interaction accounts for the T-lymphocyte activation-mediated cell death, the natural killer cell and the T-lymphocyte cytotoxicity. In addition, they play an important role in lymphocyte homeostasis (6, 7); *in vitro* studies demonstrated a high level of Fas/FasL expression and secondary Fas-mediated susceptibility to apoptosis in synoviocytes, synovial T cells and macrophages (8). Increased intra-synovial and/or serum sFas prevents the apoptosis of synoviocytes. Cytokines of stromal cell origin (*e.g.*, TGF- β , TNF- α , IL-1, FGF) protect the RA synoviocytes against Fas-mediated apoptosis (9, 10). RA T-lymphocytes are protected against apoptosis as a result of close connection with the fibroblast-like synoviocytes. In addition, RA synovial macrophages and fibroblasts pro-

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vide the up-regulation of the FLIP and the sentrin (11-13). At the same time, RA inhibits the nitric oxide (NO), caspase-3 and stromelysin-1 (14, 15). Different intra- and extracellular pathways lead to impairment of Fas-mediated apoptosis in the RA joint. Fas and FasL play a significant role in the pathogenesis of various autoimmune diseases such as RA and SLE (16). The impaired mechanisms of apoptosis and the increased Fas gene expression may result in a number of genetic disorders. An association was demonstrated between the Fas gene promoter polymorphism and the pathogenesis of diseases such as SLE and multiple sclerosis (17). In the Fas gene promoter region, 2 single nucleotide polymorphisms were commonly demonstrated, namely -1377 G-A and -670 A-G. The biological impact of these polymorphisms is not well-established; while Fas-1377 polymorphism probably binds the transcription factor SP-1 sequences, Fas-670 is associated with the nuclear transcription factor GAS. Based on our hypothesis, the genetic variations occurring in the Fas/FasL promoter region may be significantly involved in the pathogenesis of RA by affecting apoptosis. The current study was designed to investigate the Fas/FasL promoter gene polymorphism, and demonstrate the association with disease activity, clinical and laboratory findings.

■ MATERIALS AND METHODS

One hundred and one patients with RA under follow-up at a single center and 105 age and sex matched healthy control individuals were enrolled in the study. After obtaining a consent from all RA patients and the healthy subjects, peripheral blood samples were drawn into Na-EDTA containing tubes and stored at -80°C. The study was approved by the Regional Committee of Ethics.

Patients

One hundred and one (86 females) patients diagnosed with RA according to ACR classification criteria were enrolled in the study.

Table I - Demographic and clinical features of RA patients of the current study.

Age (median)	55.8 (19-72)
Disease duration (years)	8.3
Sex (f/m)	86/15
Tenosynovitis	60 (59.4%)
Lung involvement	7 (6.9%)
Sicca (s/m)	41 (40.6%)
Rheumatoid nodule	5 (5.0%)
Hand deformity	24 (23.8%)
Anemia	51 (50.5%)
DAS28	
>5.1	50 (49.5%)
3.2-5.1	15 (14.9%)
<3.2	36 (35.6%)
Amiloidosis	1 (1%)
Felty (s/m)	1 (1%)
RF positivity	90 (89.1%)
Anti-CCP positivity	73 (72.3%)

RA = rheumatoid arthritis.

The mean patient age was 55.8 years, the mean disease duration was 8.3 years. The demographic and clinical features of RA patients are presented in Table I.

Genomic DNA preparation

Genomic DNA was extracted from EDTA-anticoagulated whole blood samples using a commercially available genomic DNA purification kit (Nucleospin Blood L, Macherey-Nagel, Germany) following manufacturer's instructions. DNA concentration used by the PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR, USA) using manufacturer's instructions which were diluted as 100 ng/μL.

PCR-restriction fragment length polymorphism (PCR-RFLP) genotyping for the Fas gene 670A/G polymorphism

The Fas-670A/G polymorphism was described previously with some minor modifications. Briefly, amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA) in a 25 μL reaction mixture in 0.2 mL thin-wall PCR strip tubes (Axygen Scientific, Inc., Union City, CA, USA) containing 1 μL genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris-HCl, pH 8.0, 50 mmol/l KCl; PE Applied

Biosystems), 1.5 mmol MgCl₂, 50 µmol/l each of the deoxyGTP (dGTP), deoxy-ATP (dATP), deoxythymidine triphosphate (dTTP), and deoxyCTP (dCTP) (Promega, Madison, WI), 25 pmol each forward and reverse primers and 1.0U AmpliTaq Gold polymerase (PE Applied Biosystems). The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 45 s, 62°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 7 min.

Primers used for PCR-RFLP

For Fas -670A/G polymorphism forward PCR-RFLP primer was 5'-CTA CCT AAG AGC TAT CTA CCG TTC-3' and reverse PCR-RFLP primer was 5'-GGC TGT CCA TGT TGT GGC TGC-3', respectively.

Digest conditions

Amplified 331 bp PCR product (3 µL) was digested in a 10-µL final reaction volume using 1 µL of Reaction Buffer 2 and 5 units of MvaI restriction enzyme (New England Biolabs, Beverly, MA, USA), at 37°C overnight. Controls of known genotype were included for every set of digestions carried out. After digestion allele G yielded three fragments of 99, 188, and 44 bp, whereas allele A yielded two fragments of 99 and 232 bp. Digested fragments were separated on 3% agarose gels and visualized after ethidium bromide staining in the BioDoc-It System (UVP, Upland, CA, USA) Biolmaging systems.

PCR-RFLP genotyping for the -843C/T polymorphism of FasL gene

Genomic DNA was amplified using PCR carried out on a Gene-Amp PCR System 9700 (PE Applied Biosystems) in a 25 µL reaction mixture in 0.2 mL thin-wall PCR strip tubes (Axygen Scientific, Inc.) containing 2 µL genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris-HCl, pH 8.0, 50 mmol/l KCl; PE Applied Biosystems), 1.5 mmol MgCl₂, 50 µmol/l each of the dGTP, dATP, dTTP, and dCTP (Promega, Madison, WI, USA), 5 pmol each forward and reverse primers and 1.5 U AmpliTaq Gold polymerase (PE Ap-

plied Biosystems). The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. Forward (5'-CAA TGA AAA TGA ACA CAT TG-3') and reverse (5'-CCC ACT TTA GAA ATT AGA TC-3') primers were used according to the published sequence (Genbank accession number AF027385).

Digest conditions

Amplified 114 bp PCR product (7 µL) was digested in a 20-µL final reaction volume using 2 µL of Reaction Buffer 2 and 5 units of DraIII restriction enzyme (New England Biolabs, Beverly, MA, USA), at 37°C 3h. After digestion allele T yielded two fragments of 98 and 16 bp, whereas allele C wasn't digested and yielded 114 bp. Digested electrophoresis on gels containing a mixture of 1.5% Agarose (Sigma, St. Louis, MO, USA) and 1.5% NuSieve GTG (BMA, Rockland, ME, USA) was run for 1.5 h at 70 V and visualized after ethidium bromide staining in the BioDoc-It System Biolmaging systems (UVP).

Statistical analysis

The Hardy-Wainberg principle was used to test whether the patient and the control groups were balanced. Then, using the χ^2 -test, the patients and the subjects genotype distribution and allele frequency were evaluated. The association of the genotype and allele frequency with the disease parameters was assessed by χ^2 -test. The statistical analysis was performed using SPSS and P<0.05 was considered statistically significant.

■ RESULTS

Fas-670 G/A polymorphism

Fas/670 genotype distribution and the allele frequency were detected to be similar between the RA patient group and the healthy subject group (Tab. II). GA genotype and A allele were more frequent in both groups. Whilst no association among

Fas gene polymorphism, the disease severity and certain clinical findings was determined, there was a statistically significant difference with anemia and anti-CCP antibodies ($P < 0.01$, $P < 0.03$, respectively) (Tab. III).

FasL-843 C/T polymorphism

FasL/844 genotype distribution and the

allele frequency did not show a statistically significant difference between the RA patients and the healthy subject group (Tab. IV). CT genotype was more frequent in both groups (39.6% and 41.6%). There was no statistically significant difference in FasL gene polymorphism and the disease severity and the clinical parameters (Tab. V).

Table II - Fas/670 genotype distribution and allele frequencies of RA patients and healthy controls.

Groups	Genotypes			Alleles	
	GG	GA	AA	G	A
RA	24 (24.7%)	50 (48.5%)	27 (26.7%)	99 (98%)	103 (12%)
Control	14 (13.3%)	52 (49.5%)	39 (37.2%)	80 (76.1%)	130 (123%)

RA = rheumatoid arthritis.

Table III - Clinical and laboratory analysis of the RA patients according to the Fas 670 genotypes.

	Fas-670 Genotypes			P
	AA(N=27)	GA(N=49)	GG(N=25)	
Tenosynovitis	16	26	18	0.169
Lung involvement	4	1	2	0.102
Sicca (s/m)	13	19	9	0.646
Rheumatoid nodule	4	1	0	0.021
Hand deformity	5	12	7	0.671
Anemia	16	18	17	0.010
DAS28>5.1	9	28	13	0.219
RF positivity	24	43	23	0.445
Anti-CCP positivity	19	33	21	0.030

RA = rheumatoid arthritis.

Table IV - FasL/843 genotype distribution and allele frequencies of RA patients and healthy controls.

Groups	Genotypes			Alleles	
	CC	CT	TT	C	T
RA (n=105)	30 (29.7%)	40 (39.6%)	31 (30.7%)	100	102
Controls (n=96)	33 (34.5%)	40 (41.6%)	23 (23.9%)	106	86

RA = rheumatoid arthritis.

Table V - Clinical and laboratory analysis of the RA patients according to the FasL-843 genotypes.

	FasL-843 Genotypes			P
	CC(N=30)	CT(N=40)	TT(N=31)	
Tenosynovitis	20	25	15	0.305
Lung involvement	0	3	4	0.137
Sicca (s/m)	14	13	14	0.404
Rheumatoid nodule	2	0	3	0.154
Hand deformity	8	8	8	0.770
Anemia	14	21	16	0.880
DAS28>5.1	12	24	14	0.199
RF positivity	29	36	25	0.130
Anti-CCP positivity	25	28	20	0.239

RA = rheumatoid arthritis.

■ DISCUSSION

Rheumatoid arthritis is a systemic, autoimmune disease mediated by various inflammatory cells that leads to synovial tissue hyperplasia, articular cartilage and bone destruction. Although its pathogenesis is not well-established, the potential role of certain genetic factors is discussed. Certain non-HLA genes as well as the known conventional HLA-gene association are considered to be potentially involved in the pathogenesis (18).

Apoptosis is an important process that provides cell homeostasis under physiological conditions. Impairment in the elimination of autoreactive T and B cells may lead to various autoimmune diseases (19). The Fas molecule accounts for the deletion of autoreactive peripheral lymphocytes and is thus significantly involved in the regulation of the immune system. Therefore, Fas, an apoptosis gene may play an important role in the pathogenesis of RA.

Studies demonstrated that synoviocytes and the lymphocytes present in the rheumatoid synovium exhibited Fas expression (20). Based on these findings, Fas-mediated apoptosis may be significantly involved in the regression of synovial hyperplasia. Huang *et al.* investigated the Fas promoter-670 polymorphism in patients with SLE and RA. Although an increased Mval genotype incidence was detected in 103 Australian patients with RA, SLE patients had the same Mval genotype distribution with the control group.

In addition, AA genotype was more frequent in SLE patients with photosensitivity and oral ulcer (17). In another study, Fas-670 promoter gene polymorphism was associated with development of anti-RNP antibodies in patients with SLE (21). Coakley *et al.* investigated the correlation between RA and Felty syndrome or large granular lymphocyte leukemia and the Fas promoter gene polymorphism (22). There was no significant difference between the groups in genotype and allele incidence. No evidence of Fas gene polymorphism that would cause susceptibility to these diseases could be detected. Mullighan *et al.*

investigated the Fas gene promoter polymorphism in patients with primary Sjögren's syndrome (SS) (23). Although there was no difference between the SS and the control group with respect to genotype and allele distribution, 670A allele was more frequent in Ro/La negative patients. These results suggest that the Fas gene expression modulates the Ro/La antibody response in SS patients. Donn *et al.* investigated whether a polymorphism in the Fas-670 promoter region confers susceptibility to juvenile idiopathic arthritis (JIA). No statistically significant differences were found between the JIA cases and the controls. Similarly no differences were seen between the JIA subgroups, or when the patients were divided on the basis of rheumatoid factor or antinuclear antibody positivity (24).

The present study detected no association between the RA patients and the healthy control subjects with respect to Fas-670 and FasL-843 genotype and allele distribution.

A potential polymorphism in these genes was shown not to cause a susceptibility to the disease. In addition, a potential correlation between the Fas-670 and FasL-843 gene polymorphisms and the clinical parameters of the disease was detected. Whilst no correlation was detected for many parameters investigated, there was a statistically significant correlation between the Fas promoter gene -670 gene and anemia and anti-CCP antibody positivity.

These results suggest that the Fas promoter -670 gene modulated anti-CCP antibody synthesis and antibody response in patients with RA. In conclusion, while the present study detected no association between the Fas/FasL promoter gene polymorphism and RA, the Fas promoter -670 polymorphism was detected to be associated with anti-CCP antibody synthesis. The exact role of the Fas promoter -670 polymorphism is not yet well-established, and either the geographical and ethnical distribution of this polymorphism is not known. Multi-center, controlled trials are required to elucidate the significance and the role of the Fas gene polymorphism in different ethnical groups.

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