Tumor Necrosis Factor- α Gene Polymorphisms in Korean Patients With Recurrent Spontaneous Abortion

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Abstract

The objective of this study was to investigate the contribution of the tumor necrosis factor- α (*TNF- α*) gene polymorphisms to recurrent spontaneous abortion (RSA). The study participants consisted of 357 Korean women with RSA and 236 fertile women controls. Four *TNF- α* gene variants of all participants were analyzed by polymerase chain reaction–restriction fragment length polymorphism assay. The *TNF- α* -1031T>C and *TNF- α* -238G>A variants increased the risk of RSA *TNF- α* -1031TC+CC; adjusted odds ratio [AOR], 2.292; 95% confidence interval [CI], 1.547-3.395; $P < .001$; *TNF- α* -238GA+AA; AOR, 2.327; 95% CI, 1.038-5.217; $P = .040$), and these data were not different in a stratified analysis according to the number of consecutive spontaneous abortions. Also, the mutant genotypes of *TNF- α* -1031 and *TNF- α* -238 showed synergistic effects on increased RSA risk (-1031TC+CC/-238GA+AA; AOR, 4.054; 95% CI, 1.520-10.812; $P = .005$). In haplotype analysis, there were similar trends of data for combination analysis. In conclusion, the *TNF- α* -1031T>C and *TNF- α* -238G>A variants are possible genetic risk factors for RSA.

Keywords

tumor necrosis factor- α , recurrent spontaneous abortion, polymorphism, risk factor

Introduction

Recurrent spontaneous abortion (RSA) is a primary reproductive problem affecting 1% to 3% of otherwise healthy women¹ and occurs in 10% to 20% of pregnant woman.² The clinical diagnosis of RSA is based on several tests that detect parental chromosomal anomalies and maternal thrombophilic, endocrine, and immunological disorders.³ The homeostatic balance between helper T 1 (TH1) and helper T 2 (TH2) cytokines is essential to maintain pregnancy⁴⁻⁶ as well as in autoimmune abnormalities, coagulation, angiogenesis, vascular tone, and apoptosis.³ Overexpression of TH1 cytokines induces RSA, while elevated TH2 cytokine expression induces successful pregnancy.^{7,8}

Tumor necrosis factor (TNF) is a proinflammatory cytokine produced by mononuclear phagocytes, natural killer (NK) cells, and antigen-stimulated T cells and lymphocytes.⁹ Tumor necrosis factor was shown to mediate a number of pregnancy complications including RSA.^{10,11} Mechanistically, increased *TNF- α* secretion led to RSA through inducing proapoptotic gene expression in human fetal membrane and apoptosis of trophoblast cells. These phenomena also resulted in accelerated membrane degradation and increased infertile susceptibility.¹²⁻¹⁴ Although some data suggest that *TNF- α* affects human

pregnancy through the activation of NK cells and macrophages,¹⁵ it has also been argued that NK cells facilitate placental formation, rather than promotion of trophoblast apoptosis.¹⁶

The *TNF- α* is located within the human leukocyte antigen class III region in chromosome 6p21.3.^{17,18} Variants in the *TNF- α* promoter region result in lipopolysaccharide

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stimulation and were previously implicated in the pathogenesis of RSA.^{7,19,20} The purpose of this study is to evaluate the contribution of the single nucleotide polymorphisms (SNPs) of *TNF- α* (-1031T>C, -376G>A, -308G>A, and -238G>A) to RSA susceptibility in the Korean population.

Materials and Methods

Patients

Blood samples were collected from 357 patients with idiopathic RSA (mean age $\pm$ standard deviation [SD], 32.8 $\pm$ 4.29 years; age range: 22-45, body mass index [BMI]: 21.5 $\pm$ 3.88) and 236 control participants (mean age $\pm$ SD, 33.4 $\pm$ 5.79 years; age range: 23-43, BMI: 21.7 $\pm$ 3.41). All patients with RSA were diagnosed on the basis of at least 2 consecutive spontaneous abortions, and their average gestational age and number of miscarriages were 8.26 $\pm$ 7.64 weeks and 3.17 $\pm$ 1.68, respectively. The patients with RSA were enrolled between March 1999 and February 2010 in the Department of Obstetrics and Gynecology, and Fertility Center of CHA Bundang Medical Center in Seongnam, South Korea. Patients with a pregnancy loss (<20 weeks of gestational age) were identified by the analysis of human chorionic gonadotropin levels, ultrasound, and/or physical examination. None of the patients had a history of smoking or alcohol use. Patients with recurrent pregnancy losses due to anatomic, hormonal, chromosomal, infectious, autoimmune, or thrombotic causes were excluded from the study group. Gravidity was experienced in 2.8% of the patients, and 7.8% had a parity history. The women in the control group were recruited from CHA Bundang Medical Center and met the following enrollment criteria: regular menstrual cycles, a history of at least 1 naturally conceived pregnancy, no history of pregnancy loss, and karyotype 46, XX. The institutional review board (IRB) of CHA Bundang Medical Center approved the study in 2009, and all patients gave written informed consent.

Genetic Analysis

Four *TNF- α* SNPs were selected using the human genome SNP database (dbSNP, <http://www.ncbi.nlm.nih.gov/snp>): -1031T>C (rs1799964), -376G>A (rs1800750), -308G>A (rs1800629), and -238G>A (rs361525). DNA for genotyping was extracted from blood samples using the G-DEX blood extraction kit (Intron, Korea). The genotypes of all participants were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism assay.

The *TNF- α* -1031T>C Polymorphism

The PCR primers were forward 5'-AGC AAG AGC TGT GGG GAG AA-3' and reverse 5'-CCT GTA ACC CAT TCC TCA GAG CC-3'. The initial 5-minute denaturation at 95°C was followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR

products were cut using restriction enzyme *Bbs* I (New England Biolabs, Beverly, Massachusetts) at 37°C for 16 hours.

The *TNF- α* -376G>A Polymorphism

The PCR primers were forward 5'-CAC AGC TTT TCC CTC CAA CC-3' and reverse 5'-CCC CAT GCC CCT CAA AAC-3'. The initial 5-minute denaturation at 95°C was followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR products were cut using restriction enzyme *Tsp*509 I (New England Biolabs) at 65°C for 16 hours.

The *TNF- α* -308G>A Polymorphism

The PCR primers were forward 5'-AGG CAA TAG GTT TTG AGG GCC AT-3' and reverse 5'-TCC TCC CTG CTC CGA TTC CG-3'. The 5-minute initial denaturation at 95°C was followed by 38 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR products were cut using restriction enzyme *Nco* I (New England Biolabs) at 37°C for 16 hours.

The *TNF- α* -238G>A Polymorphism

The PCR primers were forward 5'-AGA AGA CCC CCC TCG GAA CC-3' (the underlined base is mismatch sequence) and reverse 5'-ATC TGG AGG AAG CGG TAG TG-3'. The initial 5-minute denaturation at 95°C was followed by 38 cycles of denaturing at 95°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR products were cut using restriction enzyme *Msp* I (New England Biolabs) at 37°C for 16 hours.

Estimation of Homocysteine and Folate Concentration

Blood was collected after 12 hours of fasting. The collection tube contained anticoagulant and was centrifuged for 15 minutes at 1000g to separate the plasma from the whole blood. The concentrations of homocysteine and folate in the plasma were measured by IMx fluorescent polarizing immunoassay (Abbott Laboratories, Abbott Park, Illinois) and radioassay kit (ACS:180; Bayer, Tarrytown, New York), respectively.

Statistical Analysis

Odds ratio (OR), adjusted OR (AOR), and 95% confidence intervals (CIs) were used to examine the association between *TNF- α* polymorphisms and RSA risk. The data are presented as mean $\pm$ SD (continuous variables) or percentages (categorical variables). Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc, San Diego, California) or MedCalc version 12.1.4 (MedCalc Software bvba, Mariakerke, Belgium). The HAPSTAT program (v.3.0,

Table 1. Genotype Frequencies of *TNF- α* -1031T>C, -376G>A, -308G>A, and -238G>A Polymorphisms in Korean Patients With Recurrent Spontaneous Abortion and in Controls.

Characteristics	Controls, n = 236	Patients With RSA, n = 357	COR (95% CI)	AOR (95% CI)	P ^a
<i>TNF-α</i> -1031T>C					
TT	191 (80.9)	230 (64.4)	1.000		
TC	45 (19.1)	115 (32.2)	2.122 (1.430-3.149)	2.071 (1.392-3.081)	<.001
CC	—	12 (3.4)	20.770 (1.221-353.330)	NA	NA
TC+CC			2.344 (1.586-3.463)	2.292 (1.547-3.395)	<.001
HWE P	0.105	0.605			
<i>TNF-α</i> -376G>A					
GG	236 (100.0)	357 (100.0)			
GA	—	—	NA	NA	NA
AA	—	—	NA	NA	NA
<i>TNF-α</i> -308G>A					
GG	213 (90.3)	319 (89.4)	1.000		
GA	21 (8.9)	36 (10.1)	1.145 (0.650-2.015)	1.149 (0.652-2.024)	.631
AA	2 (0.8)	2 (0.5)	0.668 (0.093-4.779)	0.713 (0.099-5.119)	.737
GA+AA			1.103 (0.639-1.905)	1.111 (0.643-1.920)	.705
HWE P	0.083	0.379			
<i>TNF-α</i> -238G>A					
GG	228 (96.6)	330 (92.4)	1.000		
GA	8 (3.4)	26 (7.3)	2.245 (0.999-5.050)	2.237 (0.994-5.033)	.052
AA	—	1 (0.3)	2.074 (0.084-51.182)	NA	NA
GA+AA			2.332 (1.040-5.226)	2.327 (1.038-5.217)	.040
HWE P	0.791	0.526			

Abbreviations: HWE, Hardy-Weinberg equilibrium; COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval; NA, not applicable; RSA, recurrent spontaneous abortion; *TNF- α* , tumor necrosis factor α .

^a Adjusted by age of all participants.

Table 2. Combination Analysis of *TNF- α* -1031T>C, -308G>A, and -238G>A Polymorphisms in Korean Patients With Recurrent Spontaneous Abortion and in Controls.

Characteristics	Controls, n = 236	Patients With RSA, n = 357	AOR (95% CI)	P ^a
<i>TNF-α</i> -1031T>C/-308G>A				
TT/GG	171 (72.5)	197 (55.2)	1.000	
TT/GA+AA	20 (8.4)	33 (9.2)	1.425 (0.786-2.583)	.243
TC+CC/GG	42 (17.8)	122 (34.2)	2.467 (1.639-3.714)	<.001
TC+CC/GA+AA	3 (1.3)	5 (1.4)	1.420 (0.334-6.044)	.635
<i>TNF-α</i> -1031T>C/-238G>A				
TT/GG	188 (79.7)	228 (63.9)	1.000	
TT/GA+AA	3 (1.3)	2 (0.5)	0.526 (0.087-3.187)	.485
TC+CC/GG	40 (16.9)	102 (28.6)	2.037 (1.343-3.090)	.001
TC+CC/GA+AA	5 (2.1)	25 (7.0)	4.054 (1.520-10.812)	.005
<i>TNF-α</i> -308G>A/-238G>A				
GG/GG	205 (86.9)	292 (81.8)	1.000	
GG/GA+AA	8 (3.4)	27 (7.6)	2.366 (1.053-5.317)	.037
GA+AA/GG	23 (9.7)	38 (10.6)	1.169 (0.675-2.023)	.577

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; RSA, recurrent spontaneous abortion; *TNF- α* , tumor necrosis factor- α .

^a Adjusted by age of all participants.

www.bios.unc.edu/~lin/hapstat/) was used to estimate haplotype frequencies for the polymorphisms determined to have strong synergistic effects.

Results

The *TNF- α* SNPs were in complete Hardy-Weinberg equilibrium in the patient with RSA and control populations. The

genotype frequencies of *TNF- α* -1031T>C, -376G>A, -308G>A, and -238G>A between controls and patients with RSA are presented in Table 1. The frequencies of *TNF- α* -1031TC (AOR, 2.071; 95% CI, 1.392-3.081; $P < .001$), -1031TC+CC (AOR, 2.292; 95% CI, 1.547-3.395; $P < .001$), and -238GA+AA (AOR, 2.327; 95% CI, 1.038-5.217; $P = .040$) were significantly higher in patients with RSA. Although the *TNF- α* -376G>A polymorphism was first described in a

Table 3. Haplotype Frequencies of *TNF- α* -1031T>C, -308G>A, and -238G>A Polymorphisms in Korean Patients With Recurrent Spontaneous Abortion and in Controls.

Characteristics	Controls, 2n = 472	Patients With RSA, 2n = 714	OR (95% CI)	P ^a
<i>TNF-α</i> -1031T>C/-308G>A/-238G>A				
T-G-G	400 (84.7)	532 (74.5)	1.000	
T-G-A	4 (0.8)	2 (0.3)	0.376 (0.068-2.064)	.411 ^b
T-A-G	24 (5.2)	40 (5.6)	1.253 (0.743-2.113)	.396
C-G-G	40 (8.5)	114 (16.0)	2.143 (1.461-3.143)	<.001
C-G-A	4 (0.8)	26 (3.6)	4.887 (1.692-14.119)	.001
<i>TNF-α</i> -1031T>C/-308G>A				
T-G	402 (85.2)	536 (75.1)	1.000	
T-A	24 (5.2)	40 (5.6)	1.250 (0.741-2.108)	.402
C-G	46 (9.6)	138 (19.3)	2.250 (1.573-3.218)	<.001
<i>TNF-α</i> -1031T>C/-238G>A				
T-G	424 (89.9)	572 (80.1)	1.000	
T-A	4 (0.8)	2 (0.3)	0.371 (0.068-2.034)	.411 ^b
C-G	40 (8.5)	114 (16.0)	2.113 (1.442-3.094)	<.001
C-A	4 (0.8)	26 (3.6)	4.818 (1.669-13.913)	.001
<i>TNF-α</i> -308G>A/-238G>A				
G-G	440 (93.2)	646 (90.5)	1.000	
G-A	8 (1.6)	28 (3.9)	2.384 (1.076-5.280)	.028
A-G	24 (5.2)	40 (5.6)	1.135 (0.675-1.910)	.633

Abbreviations: OR, odds ratio; CI, confidence interval; RSA, recurrent spontaneous abortion; *TNF- α* , tumor necrosis factor- α .

^a Chi-square test.

^b Fisher exact test.

Korean RSA population, that SNP was not detected in this study, and it was excluded from additional statistical analyses.

Analysis of the combined genotype frequencies of *TNF- α* -1031T>C, -308G>A, and -238G>A polymorphisms also revealed significant differences between patients with RSA and controls (Table 2). The combined genotypes of *TNF- α* -1031TC+CC/-308GG (AOR, 2.467; 95% CI, 1.639-3.714; $P < .001$), *TNF- α* -1031TC+CC/-238GG (AOR, 2.037; 95% CI, 1.343-3.090; $P = .001$), *TNF- α* -1031TC+CC/-238GA+AA (AOR, 4.054; 95% CI, 1.520-10.812; $P = .005$), and *TNF- α* -308GG/-238GA+AA (AOR, 2.366; 95% CI, 1.053-5.317; $P = .037$) each increased the risk of RSA. The variants of *TNF- α* -1031 and -238 showed strong synergistic effects.

We constructed haplotypes with 2 or 3 *TNF- α* polymorphisms to determine whether any specific haplotypes were associated with RSA risk (Table 3). The C-G-G (OR, 2.143; 95% CI, 1.461-3.143; $P < .001$) and C-G-A (OR, 4.887; 95% CI, 1.692-14.119; $P = .001$) haplotype frequencies of *TNF- α* -1031T>C, -308G>A, and -238G>A were significantly different between patients with RSA and controls. Of the 2 SNP haplotypes, the *TNF- α* -1031C/-308G (OR, 2.250; 95% CI, 1.573-3.218; $P < .001$), -1031C/-238G (OR, 2.113; 95% CI, 1.442-3.094; $P < .001$), and -1031C/-238A (OR, 4.818; 95% CI, 1.669-13.913; $P = .001$) haplotypes increased RSA risk.

Stratified analysis of genotype frequencies according to the number of consecutive spontaneous abortions is presented in Table 4 and Supplementary Table 1. There were no significant differences between the stratified groups. In Supplementary Table 2, plasma homocysteine and folate levels according to

the *TNF- α* variants are presented. There were no significant associations. The *TNF- α* -1031T>C, -308G>A, and -238G>A genotype frequencies in previously published normal populations are presented in Supplementary Tables 3, 4, and 5. Racial differences are evident.

Discussion

We performed this case-control study to investigate the association of *TNF- α* polymorphisms with RSA risk. Etiologies of RSA are heterogeneous and genetic, physiological, and environmental risk factors have been postulated. The genetic etiologies of RSA are largely unknown. In the present study, the *TNF- α* -1031C allele was the strongest genetic risk factor and the *TNF- α* -238A allele was an additional risk factor for RSA.

The *TNF- α* has antitumor activity in various tumor cell lines, including breast cancer cell lines.²¹ The *TNF- α* arrests cell cycle transition from G1 to S phase in mammary carcinoma cells and induces apoptosis in tumor cells.^{22,23} The *TNF- α* -308G>A and -238G>A polymorphisms are associated with altered TNF expression in vivo and in vitro.²⁴⁻²⁶ The *TNF- α* -1031C SNP increases TNF promoter activity and lipopolysaccharide-induced *TNF- α* production.^{27,28}

Previous studies of association of *TNF- α* polymorphisms with RSA risk revealed that *TNF- α* -308G>A was associated with RSA in caucasian and Iranian women.²⁹⁻³¹ In our findings, *TNF- α* -308G>A did not show association with RSA. This discrepancy may be due to racial differences in genotype frequency, as summarized in Supplementary Tables 3, 4, and 5. Several studies support the concept that RSA can be induced

Table 4. Genotype Frequencies of *TNF-α* -1031T>C, -308G>A, and -238G>A According to the Number of Consecutive Spontaneous Abortions.

Genotypes	Controls, n = 236	SA = 2, n = 187	AOR (95% CI)	P ^a	SA ≥ 3, n = 170	AOR (95% CI)	P ^a
<i>TNF-α</i> -1031T>C							
TT	191 (80.9)	115 (61.5)	1.000		115 (67.6)	1.000	
TC	45 (19.1)	66 (35.3)	2.320 (1.479-3.638)	<.001	49 (28.8)	1.815 (1.133-2.907)	.013
CC	–	6 (3.2)	NA		6 (3.6)	NA	
TC+CC			2.534 (1.625-3.951)	<.001		2.043 (1.288-3.241)	.002
HWE P	0.105	0.345			0.783		
<i>TNF-α</i> -308G>A							
GG	213 (90.3)	165 (88.2)	1.000		154 (90.6)	1.000	
GA	21 (8.9)	21 (11.2)	1.292 (0.681-2.451)	.433	15 (8.8)	0.988 (0.493-1.978)	.973
AA	2 (0.8)	1 (0.6)	0.764 (0.068-8.563)	.827	1 (0.6)	0.705 (0.063-7.859)	.776
GA+AA			1.249 (0.671-2.325)	.482		0.963 (0.492-1.884)	.912
HWE P	0.083	0.711			0.353		
<i>TNF-α</i> -238G>A							
GG	228 (96.6)	172 (92.0)	1.000		158 (92.9)	1.000	
GA	8 (3.4)	14 (7.4)	2.338 (0.957-5.711)	.062	12 (7.1)	2.157 (0.861-5.406)	.101
AA	–	1 (0.6)	NA		–	NA	
GA+AA			2.515 (1.041-6.080)	.041		2.157 (0.861-5.406)	.101
HWE P	0.791	0.240			0.633		

Abbreviations: HWE, Hardy-Weinberg equilibrium; AOR, adjusted odds ratio; CI, confidence interval; SA; spontaneous abortion; *TNF-α*, tumor necrosis factor-α.

^a Adjusted by age of all participants.

by an altered TH1/TH2 balance.^{3,32} Increased *TNF-α* levels are associated with the activation of NK cells and subsequent placental damage,³³ and some studies have demonstrated high NK cell activity prior to conception and in early gestation.^{34,35} Besides, normal pregnancy does not result from altered TH1/TH2 cell ratio but rather from decreased NK1/NK2 and NKT1/NKT2 cell ratios.³⁶ We hypothesized that *TNF-α* -1031C and -238A alleles may lead to increased circulating *TNF-α* levels and that elevated *TNF-α* could elevate the RSA risk in Korean women. A limitation of this study was that we were unable to measure serum *TNF-α* levels in patients with RSA because we did not have IRB approval for that analysis.

In conclusion, this study showed significant associations between *TNF-α* gene polymorphisms and RSA in Korean patients. In particular, *TNF-α* -1031T>C and -238G>A are associated with increased RSA incidence. Because *TNF-α* gene frequencies differ among ethnic groups, there is a need for large and heterogeneous population-based genetic studies to confirm our findings and hypotheses.

Authors' Note

Supplementary material is available at <http://rsx.sagepub.com/supplemental>.

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

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