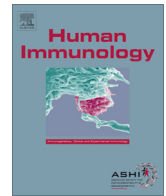




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Polymorphisms in tumor necrosis factor-alpha ($-863C>A$, $-857C>T$ and $+488G>A$) are associated with idiopathic recurrent pregnancy loss in Korean women ^{☆,☆☆}



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ABSTRACT

Polymorphisms in *TNF-α* have been reported as genetic risk factors for recurrent spontaneous abortion and *TNF-α* may be immunologically important. We therefore examined the contribution of several *TNF-α* mutations to this phenomenon. The study participants consisted of 388 patients with idiopathic recurrent pregnancy loss (RPL), which was diagnosed on the basis of at least two consecutive spontaneous abortions; control subjects were 224 healthy women with a history of successful pregnancies. Polymerase chain reaction-restriction fragment length polymorphism analysis was performed to determine the *TNF-α* $-863C>A$, $-857C>T$, and $+488G>A$ genotypes. The *TNF-α* $-863C>A$ variants correlated with increased risk of RPL (CA + AA; adjusted odds ratio [AOR], 2.142; 95% confidence interval [CI], 1.493–3.074). These data did not differ in a stratified analysis according to number of consecutive spontaneous abortions. In haplotype analysis, there were similar trends of data for combination analysis, but in patients with 3+ pregnancy losses, a stratified analysis revealed that this correlation did not increase directly with the number of pregnancy losses. The *TNF-α* $-863C>A$ variant is a possible genetic risk factor for idiopathic RPL in Korean women.

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1. Introduction

Recurrent pregnancy loss (RPL) is usually defined as three or more consecutive pregnancy losses before a gestational age of

Abbreviations: TNF, tumor necrosis factor; RPL, recurrent pregnancy loss; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

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20 weeks; however, the American Society for Reproductive Medicine (ASRM) has recently redefined this condition as two or more consecutive pregnancy losses [1,2]. Pregnancy loss occurs in one out of eight pregnant women, most often within 2–3 months of conception [3]. The likelihood of pregnancy loss is 5% higher for women who suffered a miscarriage during their first pregnancy than for women who did not [4]. Secretion of cytokines from helper T cells (Th) is essential in maintaining homeostatic balance, and it is associated with pregnancy, immune disorders, blood coagulation, angiogenesis, and cell death [5–7]. In the 1990s, maternal tolerance toward fetal antigens was explained by the predominance of Th2-type immunity during pregnancy, which protected the fetus from maternal Th1-cell attack [8]. Indeed, predominant Th1-type immunity has been observed in recurrent spontaneous abortion and in preeclampsia [9–11]. Other reports found that Th1 cytokines could exert a deleterious effect on pregnancy and inhibit the growth and development of the fetus [12]. On the other hand, Th2 cytokines are associated with successful pregnancy

[12,13]. Th1-type cytokines also seem to be related to the down-regulation of cell-mediated immunity, which appears as delayed-type hypersensitivity, weakened resistance to natural killer cell (NK cell) activity, and intracellular infection. It has been suggested that pregnancy is associated with the strengthening of Th1-type activity and down-regulation of Th2-type activity [14]. Therefore, the expression of Th2 cytokines may be required to induce pregnancy successfully, but the over-expression of Th1 cytokines may be associated with RPL [15,16].

Tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine produced by lymphocytes, antigen stimulated T cells, mononuclear phagocytes, and NK cells [17,5]. Certain polymorphisms in the TNF- α gene affect cytokine production levels. Thus, cytokine gene polymorphisms have been hypothesized to play a role in idiopathic RPL. These polymorphisms have been associated with altered TNF- α secretion and are linked with pregnancy complications, including idiopathic miscarriage [18]. The TNF- α -863C>A, -857C>T, +488G>A polymorphisms are located on the short arm of chromosome 6 (6p21) next to the major histocompatibility complex; TNF- α +488G>A is located in intron 1 and TNF- α -857C>T, TNF- α -863C>A are located in the proximal promoter region of the gene [19–21]. Gene expression of TNF- α is regulated at the transcriptional level [22] and polymorphisms in the promoter region can influence TNF- α gene expression. TNF- α -863C, -857C, and +488G alleles have been associated with significantly higher levels of circulating TNF- α [23–25]. TNF- α -863A, -857T, and +488A alleles are stronger transcriptional activators and these alleles are associated with increased TNF- α expression at the protein level [23–26]. In this study, we investigated the association between RPL and the TNF- α polymorphisms -863C>A, -857C>T, and +488G>A in a population of Korean women. TNF- α polymorphisms -857C>T, -863C>A, and +488G>A have been reported to be associated with a variety of diseases, but their association with RPL has yet to be studied. To our knowledge, this is the first study examining whether TNF- α polymorphisms -863C>A, -857C>T, +488G>A associate with recurrent pregnancy loss. We demonstrate that the TNF- α -863C>A polymorphism is associated with RPL in this patient cohort.

2. Subjects and methods

2.1. Subjects

Blood samples were collected from 388 patients with idiopathic RPL (age range, 22–45 years; mean \pm SD age, 33.21 \pm 4.55 years; mean body mass index [BMI], 24.15 \pm 7.07) and 224 control participants (age range, 23–43 years; mean \pm SD age, 33.37 \pm 5.81 years; BMI, 21.69 \pm 3.37). The RPL patients were enrolled between March 1999 and February 2012 in the Department of Obstetrics and Gynecology or the Fertility Center, both located at the CHA Bundang Medical Center in Seongnam, South Korea. The women in the control group were also recruited from the CHA Bundang Medical Center and met the following criteria: pregnant, regular menstrual cycles, a history of at least one naturally conceived pregnancy, no history of pregnancy loss, and karyotype 46, XX. The Institutional Review Board of CHA Bundang Medical Center approved the study, and all patients gave written informed consent. All patients were diagnosed with RPL if they had a history of at least two consecutive spontaneous abortions. Pregnancy loss was diagnosed by analysis of human chorionic gonadotropin levels, ultrasonography, and physical examination before 20 weeks of gestational age. The average gestational age was 8.26 \pm 7.64 weeks and number of miscarriages per patient was 3.17 \pm 1.68. No participant had a history of smoking or alcohol use. Patients with miscarriage due to anatomic, hormonal, chromosomal, infectious, autoimmune, or thrombotic causes were excluded from the study

group. Anatomical causes of miscarriage were determined using hysterosalpingogram, hysteroscopy, computerized tomographic scanning, and magnetic resonance imaging to define intrauterine adhesions, septate uterus, or uterine fibroids. Hormonal causes of miscarriage included hyperprolactinemia, luteal insufficiency, and thyroid disease, and were evaluated by determining blood levels of the appropriate hormones. To determine a chromosomal cause for miscarriage, chromosome analysis was performed using standard protocols [27], and metaphase chromosomes were stained by the GTG banding method. Miscarriages caused by infection with *Ureaplasma urealyticum* or *Mycoplasma hominis* infections were evaluated by bacterial culture. Autoimmune causes of miscarriage, defined as lupus and antiphospholipid syndrome, were evaluated using lupus anticoagulant and anticardiolipin antibodies. Thrombotic cause was defined as thrombophilia and was evaluated by testing for protein C and protein S deficiency, and by presence of anti-beta-2 glycoprotein.

2.2. Genotyping

DNA was extracted using the G-DEX blood extraction kit (iNtRON Biotechnology Inc., Seongnam, Korea). Three TNF- α polymorphisms were selected using the human genome SNP database (dbSNP, <http://www.ncbi.nlm.nih.gov/snp>): -857C>T (rs1799724), -863C>A (rs1800630) and +488G>A (rs1800610). Samples were genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay with the following primers and PCR conditions: for TNF- α -863C>A: forward 5'-AGG AAT GGG TTA CAG GAG ACC ACT-3', reverse 5'-TCT ACA TGG CCC TGT CTT CGT TGA G-3'; Initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s – annealing at 61 °C for 30 s – extension at 72 °C for 30 s; and final extension at 72 °C for 5 min; for TNF- α -857C>T: forward 5'-TGG AAG TCG AGT ATG GGG ACC CCC CAT TAA-3', reverse 5'-CAG TGT GTG GCC TAT CTT C-3'; Initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s – annealing at 57 °C for 30 s – extension at 72 °C for 30 s; and final extension at 72 °C for 5 min; for TNF- α +488G>A: forward 5'-GCC AGA CAT CCT GTC TCT CC-3', reverse 5'-CAG AGG GAA GAG GTG AGT GC-3'; Initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s – annealing at 56 °C for 30 s – extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. The PCR products were digested with the restriction enzymes *MnII*, *AseI*, and *NlaIII* (New England Biolabs, Beverly, MA, USA) at 37 °C for 16 h.

2.3. Flow cytometry analysis of CD3–CD16+CD56+ peripheral NK cells

Flow cytometry was performed using a BD FACSCalibur device (BD Biosciences, Inc., Seoul, Korea) that was compensated by a single fluorochrome. Data were analyzed using CellQuest software (BD Biosciences). The fluorescent labels specific for CD56, CD3, and CD16, were monoclonal antibodies (fluorescein isothiocyanate/PE/PerCP antibody/APC) obtained from BD Biosciences, Inc. AntiNKG2A-PE was obtained from Immunotech (Beckman Coulter, Fullerton, CA). PBMCs (2.5×10^5) were stained for cell surface antigen expression at 4 °C in the dark for 30 min, then washed twice in 2 mL phosphate buffered saline containing 1% bovine serum albumin and 0.01% sodium azide (FACS wash buffer) and subsequently fixed in 200 μ L of 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) prior to sorting [28,29].

2.4. Measurement of plasma homocysteine, folate, total cholesterol, and urate

Uric acid, plasma homocysteine, folic acid, and cholesterol were measured in blood samples collected from RPL cases after 12 h of

Table 1
Clinical characteristics of RPL patients and control subjects.

Characteristic	Control (n = 224)	RPL (n = 388)
Age (year)	33.37 ± 5.81	33.21 ± 4.55
BMI (kg/m ²)	21.69 ± 3.37	24.15 ± 7.07
Previous pregnancy losses (N)	None	3.02 ± 1.51
RPL < 14 weeks (%)	None	98.9%
Live births (N)	1.72 ± 0.72	None
Mean gestational age (weeks)	39.28 ± 1.67	7.53 ± 1.93
Homocysteine (μmol/L)	NA	6.98 ± 2.10
Folate (mg/mL)	NA	14.21 ± 11.94
Total cholesterol (mg/dL)	NA	187.73 ± 49.42
Urate (mg/dL)	NA	3.80 ± 0.84
PAI-1 (ng/mL)	NA	10.53 ± 5.72
PT (sec)	NA	11.58 ± 0.86
aPTT (sec)	NA	32.24 ± 4.33

Abbreviations: RPL, recurrent pregnancy loss; BMI, body mass index; NA, not applicable; PAI-1, plasminogen activator inhibitor-1; PT, prothrombin time; aPTT, activated partial thromboplastin time.

fasting. Homocysteine was measured using a fluorescence polarization immunoassay using the Abbott IMx analyzer (Abbott Laboratories, Abbott Park, IL, USA). Folic acid was measured using a competitive immunoassay using the ACS 180 Plus Automated Chemiluminescence System (Bayer Diagnostics, Tarrytown, NY). Uric acid and total cholesterol were measured using commercially available enzymatic colorimetric tests (Roche Diagnostics, GmbH, Mannheim, Germany).

2.5. Statistical analysis

Differences in *TNF-α* gene frequencies between cases and controls were assessed using logistic regression models and Fisher's exact test. Odds ratio (OR), adjusted odds ratio (AOR), and 95% confidence intervals (CIs) were used to examine the association between *TNF-α* polymorphisms and RPL risk. The data are presented as mean ± SD (continuous variables) or percentages (categorical variables). Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA) or MedCalc version 12.1.4 (MedCalc Software bvba,

Mariakerke, Belgium). The HAPSTAT program (v.3.0, www.bios.unc.edu/~lin/hapstat/) was used to estimate haplotype frequencies for the polymorphisms determined to have strong synergistic effects. *P*-values less than or equal to 0.05 were considered statistically significant. False discovery rate (FDR) was also used to adjust for multiple comparison; associations with an FDR-corrected *P*-value < 0.05 were considered statistically significant. The statistical power of the study was calculated using G*POWER 3.1.9 (Franz Faul, Universitat Kiel, Germany).

3. Results

Demographic and clinical characteristics of RPL cases and controls are presented in Table 1. Various clinical variables stratified by *TNF-α* genotype in RPL cases can be found in Supplementary Table 1. Previous studies suggest that *TNF-α* contributes to RPL, and some results have shown that uric acid levels are higher in patients with RPL than normal subjects [30–32]. In our study, patients with the *TNF-α* –857C>T variant showed an increase in uric acid levels, but the difference between the two groups was not statistically significant. The distribution of *TNF-α* polymorphisms in RPL cases and controls is shown in Table 2. We found a significant association between the *TNF-α* –863C>A polymorphism and RPL. However, the –857C>T and +488G>A polymorphisms were not significantly associated with RPL. We investigated this association more closely using various other statistical methods. First, we performed a *TNF-α* haplotype analysis using the three polymorphisms to see whether specific haplotypes were associated with RPL in Table 3. When analyzing all three polymorphisms (–863/–857/+488), we identified significantly increased odds of RPL in association with three haplotypes: A–C–G (OR 1.738; 95% CI 1.206–2.506; *P* = 0.003), A–C–A (OR 2.410; 95% CI 1.037–5.602; *P* = 0.046), and A–T–A (OR, 4.998; 95% CI 1.141–21.890; *P* = 0.024). When analyzing haplotypes comprised of just two polymorphisms, we identified three associated with RPL: A–C for –863/–857 (OR 1.911; 95% CI 1.355–2.697; *P* = 0.0002) and A–G (OR 1.791; 95% CI 1.275–2.518; *P* = 0.001) and A–A (OR 3.669; 95% CI 1.272–5.603; *P* = 0.008) for –863/+488. In contrast, no 857/+488 haplotypes were associated with

Table 2
Genotype analysis for the *TNF-α* polymorphisms –863C>A, –857C>T, and +488G>A in RPL patients and controls.

Genotype	Controls (n = 224)	RPL patients (n = 388)	AOR (95% CI) ^a	<i>P</i>	FDR- <i>P</i>
<i>TNF-α</i> –863C>A					
CC	166 (74.1)	221 (57.0)	1.000 (reference)		
CA	53 (23.7)	148 (38.1)	2.076 (1.429–3.017)	0.0001	0.0003
AA	5 (2.2)	19 (4.9)	2.846 (1.041–7.780)	0.042	0.125
Dominant (CC vs. CA + AA)			2.142 (1.493–3.074)	<0.0001	0.0003
Recessive (CC + CA vs. AA)			2.259 (0.832–6.137)	0.110	0.110
HWE- <i>P</i>	0.753	0.359			
<i>TNF-α</i> –857C>T					
CC	154 (68.8)	277 (71.4)	1.000 (reference)		
CT	60 (26.8)	98 (25.4)	0.906 (0.619–1.326)	0.612	0.878
TT	10 (4.5)	13 (3.4)	0.723 (0.310–1.688)	0.453	0.453
Dominant (CC vs. CT + TT)			0.883 (0.615–1.268)	0.501	0.729
Recessive (CC + CT vs. TT)			0.740 (0.319–1.718)	0.484	0.419
HWE- <i>P</i>	0.193	0.242			
<i>TNF-α</i> +488G>A					
GG	168 (75.0)	286 (73.7)	1.000 (reference)		
GA	53 (23.7)	93 (24.0)	1.031 (0.698–1.523)	0.878	0.878
AA	3 (1.3)	9 (2.3)	1.802 (0.480–6.766)	0.383	0.453
Dominant (GG vs. GA + AA)			1.069 (0.732–1.563)	0.729	0.729
Recessive (GG + GA vs. AA)			1.724 (0.461–6.445)	0.419	0.484
HWE- <i>P</i>	0.605	0.660			

Abbreviations: RPL, recurrent pregnancy loss; AOR, adjusted odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; FDR-*P*, false-positive discovery rate-corrected.

^a Adjusted for age.

Table 3Haplotype analysis for the *TNF- α* polymorphisms –863C>A, –857C>T, and +488G>A in RPL patients and controls.

Haplotype	Controls (2n = 448)	RPL patients (2n = 776)	OR (95% CI)	P ^a	FDR-P
<i>TNF-α</i> –863/–857/+488					
C-C-G	308 (68.7)	493 (63.6)	1.000 (reference)		
C-C-A	7 (1.7)	4 (0.5)	0.357 (0.104–1.230)	0.119	0.208
C-T-G	27 (6.1)	29 (3.7)	0.671 (0.390–1.155)	0.158	0.221
C-T-A	42 (9.4)	64 (8.3)	0.952 (0.629–1.441)	0.832	0.832
A-C-G	46 (10.2)	128 (16.5)	1.738 (1.206–2.506)	0.003	0.021
A-C-A	7 (1.5)	27 (3.5)	2.410 (1.037–5.602)	0.046	0.106
A-T-G	8 (1.8)	15 (2.0)	1.171 (0.491–2.796)	0.830	0.832
A-T-A	2 (0.5)	16 (2.0)	4.998 (1.141–21.89)	0.024	0.084
<i>TNF-α</i> –863/–857					
C-C	316 (70.5)	496 (63.9)	1.000 (reference)		
C-T	69 (15.4)	94 (12.1)	0.868 (0.617–1.221)	0.430	0.430
A-C	52 (11.6)	156 (20.1)	1.911 (1.355–2.697)	0.0002	0.001
A-T	11 (2.5)	30 (3.9)	1.738 (0.858–3.517)	0.140	0.209
<i>TNF-α</i> –863/+488					
C-G	335 (74.8)	516 (66.5)	1.000 (reference)		
C-A	50 (11.2)	74 (9.5)	0.961 (0.654–1.411)	0.845	0.845
A-G	54 (12.1)	149 (19.2)	1.791 (1.275–2.518)	0.001	0.002
A-A	9 (2.0)	37 (4.8)	2.669 (1.272–5.603)	0.008	0.011
<i>TNF-α</i> –857/+488					
C-G	354 (79.0)	621 (80.0)	1.000 (reference)		
C-A	14 (3.1)	31 (4.0)	1.262 (0.663–2.405)	0.529	0.793
T-G	35 (7.8)	44 (5.7)	0.717 (0.451–1.138)	0.182	0.546
T-A	45 (10.0)	80 (10.3)	1.013 (0.688–1.494)	1.000	1.000

Abbreviations: RPL, recurrent pregnancy loss; OR, odds ratio; CI, confidence interval; FDR-P, false-positive discovery rate-corrected.

^a Fisher's exact test.**Table 4**Combined genotype analysis for the *TNF- α* polymorphisms –863C>A, –857C>T, and +488G>A in RPL patients and controls.

Combined genotype	Controls (n = 224)	RPL patients (n = 388)	AOR (95% CI) ^a	P	FDR-P
<i>TNF-α</i> –863/–857					
CC/CC	114 (50.9)	150 (38.7)	1.000 (reference)		
CC/CT + TT	52 (23.2)	71 (18.3)	1.047 (0.677–1.618)	0.835	0.950
CA + AA/CC	40 (17.9)	127 (32.7)	2.447 (1.588–3.772)	0.0001	0.0003
CA + AA/CT + TT	18 (8.0)	40 (10.3)	1.679 (0.910–3.095)	0.097	0.146
<i>TNF-α</i> –863/+488					
CC/GG	125 (55.8)	166 (42.8)	1.000 (reference)		
CC/GA + AA	41 (18.3)	55 (14.2)	1.015 (0.636–1.619)	0.950	0.950
CA + AA/GG	43 (19.2)	120 (30.9)	2.143 (1.406–3.265)	0.0004	0.0006
CA + AA/GA + AA	15 (6.7)	47 (12.1)	2.323 (1.240–4.355)	0.009	0.026
<i>TNF-α</i> –857/+488					
CC/GG	142 (63.4)	251 (64.7)	1.000 (reference)		
CC/GA + AA	12 (5.4)	26 (6.7)	1.209 (0.590–2.477)	0.603	0.950
CT + TT/GG	26 (11.6)	35 (9.0)	0.735 (0.421–1.281)	0.277	0.277
CT + TT/GA + AA	44 (19.6)	76 (19.6)	0.975 (0.636–1.493)	0.906	0.906

Abbreviations: RPL, recurrent pregnancy loss; AOR, adjusted odds ratio; CI, confidence interval; FDR-P, false-positive discovery rate-corrected.

^a Adjusted for age.

RPL. **Table 4** shows a combined genotype analysis for *TNF- α* polymorphisms. Several associations with RPL were observed, including –863CA + AA/–857CC (AOR 2.447; 95% CI 1.588–3.772; $P = 0.001$), –863CA + AA/+488GG (AOR 2.143; 95% CI 1.406–3.265; $P = 0.0004$), and –863CA + AA/+488GA + AA (AOR 2.323; 95% CI 1.240–4.355; $P = 0.009$). No significant associations with RPL were observed for other genotype combinations. We investigated whether the *TNF- α* –863C>A polymorphism occurred more frequently in women with 3 + pregnancy losses. **Supplementary Tables 2–4** show that the *TNF- α* –863C>A polymorphism was present at similar frequencies in women with RPL, regardless of the number of pregnancy losses they suffered. In other words, the *TNF- α* –863C>A polymorphism did not correlate more strongly as the number of pregnancies increased. **Supplementary Table 5** presents the statistical power of the study to detect positive genetic associations for each comparison.

4. Discussion

Causes of RPL include environmental risk factors, heterologous genes, and physiology, and although chromosomal abnormalities are responsible for very early pregnancy losses just before placentalation, the precise mechanism underlying the loss is not known [33]. *TNF- α* has anti-tumor activity in various tumor cell lines. *TNF- α* arrests cell cycle transition from G1 to S phase in carcinoma cells and induces apoptosis in tumor cells [34–36], and *TNF- α* is the primary inflammatory cytokine. Cytokines play an important role in the control of ovulation, atresia, luteolysis, and steroidogenesis. *TNF- α* is now known to play essential roles in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis, and morphogenesis [37].

Of the variants examined in this study, we found that the *TNF- α* –863A allele was the strongest genetic risk factor for RPL and that

the *TNF- α* –857C allele was a secondary risk factor for RPL. The *TNF- α* –863C>A and –857C>T polymorphisms, in combination, increase TNF promoter activity and lipopolysaccharide-induced TNF- α production [38,39]. In contrast, *TNF- α* –488G>A did not show any association with RPL. Increased TNF- α levels can cause high inflammatory cytokine activity prior to conception and in early pregnancy that may damage the placenta [40]. It is possible that TNF- α levels are influenced by particular genetic variants, thereby disrupting placentation. Several studies have shown that RPL can be induced by the TH1/TH2 imbalance or by dysregulated immunity. This mechanism was suggested as the underlying cause of a large proportion of idiopathic pregnancy loss cases [41,42]. Thus, TH1-mediated immunity may facilitate pregnancy failure, and TH2 immunity may promote successful pregnancy. The data from Supplementary Tables 2–4 show that –863A was observed in patients with increased RPL. From our findings, we hypothesize that *TNF- α* –863A and –857C alleles may lead to increased circulating TNF- α levels and that elevated TNF- α could elevate the RPL risk in Korean women. We also analyzed whether BMI, homocysteine levels, or folic acid levels were associated with pregnancy loss, given these may be risk factors associated with metabolic syndrome. However, we found no association among these variables. Therefore, the *TNF- α* –863C>A polymorphism was found to be associated with the RPL risk independent of these other variables.

Limitations of this study include lack of a survey for vascular risk factors, insufficient clinical data on controls, and a relatively small number of controls. Additionally, in previous studies, *TNF- α* –863C>A and –857C>T combination genotype frequencies were not significantly increased in women with RPL. These discrepancies may be attributed to differences in ethnic background, in the selection of RPL cases, or in sample size. In conclusion, this study showed significant associations between *TNF- α* –863C>A gene polymorphisms and RPL in Korean women.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.humimm.2016.04.012>.

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