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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-a* 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-a* 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.

1. Introduction

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

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

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[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-alpha (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-a* + 488G>A is located in intron 1 and *TNF-a* −857C>T, *TNF-α* −863C>A are located in the proximal promoter region of the gene [19–21]. Gene expression of *TNF-a* is regulated at the transcriptional level [22] and polymorphisms in the promoter region can influence *TNF-a* gene expression. *TNF-a* −863C, −857C, and +488G alleles have been associated with significantly higher levels of circulating TNF-a [23–25]. *TNF-a* −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-a* 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 ± SD age, 33.21 ± 4.55 years; mean body mass index [BMI], 24.15 ± 7.07) and 224 control participants (age range, 23–43 years; mean ± SD age, 33.37 ± 5.81 years; BMI, 21.69 ± 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 ± 7.64 weeks and number of miscarriages per patient was 3.17 ± 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 *Mnl*I, *Ase*I, and *Nla*III (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 μL 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