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Short communication

Interleukin-18 promoter polymorphisms and risk of idiopathic recurrent pregnancy loss in a Tunisian population

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ABSTRACT

IL-18 is a pro-inflammatory cytokine that regulates the differentiation and effector functions of CD4+ (Th1) and CD8+ (CTL) T cells, which are implicated in the pathogenesis of recurrent pregnancy loss (RPL). We investigated the association of the *IL-18* gene promoter single nucleotide polymorphisms (SNPs) −656C/A (rs1946519), −137G/C (rs187238), −119A/C (rs360718), and −105G/A (rs360717), by TaqMan assays in analysis in 470 Tunisian women comprising 235 RPL cases and 235 multi-parous controls. The association of *IL-18* alleles, genotypes, and haplotypes with RPL was evaluated by Fisher's exact test and regression analysis. The frequency of minor alleles −105G/A ($P < 0.001$) and −656C/A ($P < 0.001$), but not −119A/C ($P = 0.93$) or −137G/C ($P = 0.32$), were higher in RPL cases. Significant differences were also noted in the genotype distribution of −105G/A ($P < 0.001$) and −656C/A ($P < 0.001$) between cases and controls. Four-locus (−656C/A, −137G/C, −119A/C, −105G/A) *IL-18* haplotype analysis identified AGAA (corrected $P < 0.001$), and CGAA (corrected $P < 0.001$) haplotypes to be associated with increased RPL risk, after adjusting for age and BMI. These results demonstrate that −105G/A and −656C/A *IL-18* variants are significantly associated with RPL.

1. Introduction

Interleukin 18 (IL-18) is an 18-kDa pro-inflammatory cytokine produced by macrophages and monocytes (Boraschi and Dinarello, 2006; Srivastava et al., 2010), and mediates a myriad of inflammatory responses, including induction of interferon-γ expression, and maturation of T cells and NK cells (Srivastava et al., 2010), and also indirectly by stimulating IL-1β expression (Srivastava et al., 2010; Lebel-Binay et al., 2000). Alterations of IL-18 expression and secretion were implicated in increased susceptibility to autoimmune and inflammatory disorders (Aizawa et al., 2005; Roland et al., 2010), and were linked with pregnancy complications, including preeclampsia (Roland et al., 2010; Szarka et al., 2010), preterm delivery (Daskalakis et al., 2009), in vitro fertilization embryo transfer failure (Lédée-Bataille et al., 2004), implantation failure, and recurrent miscarriage (Laird et al., 2006; Wilson et al., 2004a,b).

Recurrent pregnancy loss (RPL) is a major reproductive problem with poorly understood etiology, reportedly affecting 1.5–2.0% of total pregnancies (Chaouat et al., 2004). Several hypotheses were proposed to explain its etiology (Chaouat et al., 2004; Pandey et al., 2005), which included acquired/life style factors (smoking, diet, obesity) (Pandey et al., 2005; Metwally et al., 2010), inherited predisposition (Pandey et al., 2005; Pfeiffer et al., 2001), coagulation abnormalities (Paidas et al., 2005), and dysregulated immunity highlighted by altered

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cytokine production and the increased frequency of anti-phospholipid antibody positivity among RPL cases (Sater et al., 2011a,b; Zammiti et al., 2009). Whereas the immunological basis underlying idiopathic RPL was independently confirmed in several populations (Laird et al., 2006), the exact molecular mechanisms underlying RPL remain to be seen, thus prompting identification of additional risk factors.

Successful pregnancy depends on adequate fetal–maternal interaction, and embryonic implantation, which is controlled by a fine balance between maternally and fetally derived cytokines and growth factors (Huang, 2006). Insofar as RPL is linked to altered IL-18 levels (Ostojić et al., 2007; Wilson et al., 2004b; Huang, 2006), and given that the secretion of IL-18 is genetically determined (Barbaux et al., 2007), it is proposed that specific *IL-18* gene variants, acting by altering IL-18 levels, are likely candidates for increased RPL susceptibility. In this study, we evaluated −656C/A (rs1946519), −137G/C (rs187238), −119A/C (rs360718), and −105G/A (rs360717) *IL-18* promoter single nucleotide polymorphisms (SNPs) by allelic discrimination (real-time PCR) in RPL and control women, and identified specific four-locus haplotypes associated with increased risk of RPL.

2. Subjects and methods

2.1. Study subjects

This was a retrospective case–control study, performed at the Maternity Center of the Hospital Farhat Hached of Sousse, Tunisia. Data collection procedures were the same for patients and control subjects, all of whom were Tunisians, and were required to give informed consent prior to inclusion in the study. Cases comprised 262 fertile women with three or more unexplained pregnancy losses occurring in the first trimester with the same partner. After excluding non-Arab participants (17 Berber and 10 European women), the final number of eligible participants was 235 women. The gestational age was calculated on a clinical basis, and defined as the time between the date of the first day of the last normal menstrual period and the date of the first clinical symptom of pregnancy loss (evidence of bleeding, including assessment of vaginal discharge through inspection of sanitary pads, and presentation with pain and cramping). Most miscarriages (48.4%) occur before 12 weeks, with one-third of the cases (35.5%) occurring between 12 and 20 weeks of gestation; the remainder (16.1%) had both early and later miscarriages.

Exclusion criteria included non-Arab origin, parental karyotype aberrations, Rh incompatibility, >40 years of age at first miscarriage, preclinical miscarriages and/or biochemical pregnancy, and preeclampsia, given the strong link between RPL and preeclampsia (Trogstad et al., 2009). Patients were also excluded if they had systemic autoimmune disease, arterial hypertension, endocrine diseases (diabetes mellitus, thyroid dysfunction), anatomical disorders, infections (toxoplasmosis, HCMV, rubella, HIV, Group B streptococci, *Chlamydia trachomatis*, hepatitis B and C and bacterial vaginosis), liver function abnormalities, and hyper-prolactinemia prior to luteal phase defects. Transvaginal ultrasound was performed to confirm spontaneous miscarriage (no heartbeat detection).

The control group consisted of 235 age-matched ($P = 0.87$) multi-parous control women, who had had at least two children, and no known personal or immediate family history of pregnancy losses. Controls were matched with patients according to a number of risk factors (smoking, alcohol consumption, oral contraceptive use). The University of Monastir Research & Ethics Committee (Monastir, Tunisia), and the Hospital Farhat Hached (Sousse, Tunisia) approved the study protocol. Blood samples were taken in EDTA-containing tubes from study subjects by venipunture genomic DNA extraction.

2.2. IL-18 genotyping

We screened the 5′-UTR region of the *IL-18* gene for polymorphisms with a minor allele frequency of >10% in Caucasians, using SNPbrowser software (version 4.0, Applied Biosystems, Foster City, CA, USA). *IL-18* genotyping was performed using the allelic discrimination method using VIC- and FAM-labeled primers. TaqMan assays, as assay-on-demand, were obtained from Applied Biosystems: C_2898459_20 (rs1946519/−656CA), C_2408543_10 (rs187238/−137GC), C_2898461_10 (rs360718/−119AC), and C_2898462_10 (rs360717/−105GA). The reaction was performed in 6-μl volume on a StepOne real-time PCR system, according to the manufacturer's instructions (Applied Biosystems). Replicate-blinded quality control samples were included to assess the reproducibility of the genotyping procedure; concordance was >99%.

2.3. Statistical analysis

Statistical analysis was performed on SPSS v. 17.0 (SPSS, Chicago, IL, USA). After the power was computed for each SNP (Genetic Power Calculator; SGDP Statistical Genetics Group), the overall power (82.4%) was calculated as the average power over the SNPs genotyped. Data were expressed as percentages of the total (categorical variables) or as mean ± SD (continuous variables). Student's *t*-test was used to determine differences in means, and χ^2 test were used to assess inter-group significance. Allele frequencies were calculated by the gene-counting method, and each polymorphism was tested for Hardy–Weinberg equilibrium using HPlus 2.5 software (http://qge.fhcrc.org/hplus).

All analyses were conducted under additive genetic effect, using SNPStats software (bioinfo.iconcologia.net/snpstats/). Linkage disequilibrium analysis was performed using Haploview 4.1 (http://www.broad.mit.edu/mpg/haploview), and haplotype reconstruction was performed by the expectation maximization method using H-Plus 2.5. Bonferroni multiple-comparison correction method was employed in calculating the corrected *P* value, as per: $Pc = 1 - (1 - P)^n$, where n is the number of comparisons. Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95% confidence