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The association of idiopathic recurrent pregnancy loss with polymorphisms in hemostasis-related genes ☆

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ABSTRACT

Recurrent pregnancy loss (RPL) is a complex, multifactorial condition. Inherited thrombophilia is the leading cause of thromboembolism and is associated with an increased risk of RPL. The aims of the current study were to investigate the effects of polymorphisms in hemostasis-related genes antithrombin (SERPINC1), thrombomodulin (THBD), tissue factor pathway inhibitor (TFPI), factor *V*, factor *II* and annexin A5 (ANXA5), involved in reproductive failure in 94 RPL cases with two or more consecutive pregnancy losses prior to 20 weeks of pregnancy and 169 healthy controls who had at least one term delivery and no history of pregnancy loss. The genotypes of SERPINC1 G786A, THBD C1418T, TFPI T-33C, factor *V* G1628A, factor *II* A19911G and ANXA5 G76A were assayed by the Sequenom MassARRAY system. Genotype and allele frequencies for SERPINC1 (rs2227589), TFPI (rs8176592), factor *V* (rs6020), factor *II* (rs3136516) and ANXA5 (rs113588187) in cases and controls were similar. The distribution of THBD C1418T allele showed significant differences between RPL cases and healthy controls (odds ratio (OR): 1.58, 95%, confidence interval (CI): 1.05–2.39, $P = 0.027$). In univariate logistic regression analyses, carriers of THBD 1418T allele (CT + TT) had an increased risk of RPL (OR: 1.83, 95%, CI: 1.10–3.06, $P = 0.020$). This indicated that THBD 1418T allele was associated with increasing the risk of RPL.

1. Introduction

Recurrent pregnancy loss (RPL) is a multifactorial disorder with a polygenic background. Recently, it has been redefined as two or more consecutive spontaneous loss before a gestational age of 20 weeks by the American Society for Reproductive Medicine (ASRM) (2013). RPL is estimated to occur in 2%–4% of reproductive-aged women, of which 40%–55% are caused by undetermined causes (Anon., 2013; Li et al., 2002). The known etiologies of RPL include parental chromosome abnormalities, uterine anomalies, endocrinology disorders, immune disorders, thrombophilia, maternal infections, nutritional, and environmental factors (Li et al., 2002; Okon et al., 1998; Stephenson and Kutteh, 2007). Thrombophilia was identified as a major cause of RPL, after parental chromosomal abnormalities with a rate of up to 40%, especially in the first half of pregnancy (Brenner et al., 1999). In the clinical context thrombophilia is distinguished into three types: inherited thrombophilia, acquired thrombophilia and combined thrombophilia (Franchini and Veneri, 2005; Martinelli, 2001). Mutations in hemostasis-related genes were considered as risk factors for hereditary thrombophilia and can act either in the first half of pregnancy or later in pregnancy, causing possible miscarriages (Rai and Regan, 2006).

The tissue factor pathway inhibitor (TFPI) gene encoding an endothelial-associated protein that down-regulates the initial phase of coagulation by inhibiting tissue factor–factor VIIa complex in a factor Xa-dependent manner (Crawley and Lane, 2008). Thrombomodulin (THBD) converts protein C (PC) zymogen into its active form; termed as activated protein C (APC), PC pathway can control the generation of thrombin, and thus affect the coagulation process (Dahlback, 2008). Antithrombin (SERPINC1) is a multifunctional serine protease inhibitor that inhibits almost all the active enzymes of the coagulation pathway (Dahlback, 2008). The relation between SNPs in TFPI, THBD and SERPINC1 and the risk of RPL had been reported by Guerra-Shinohara et al. for the first time (Guerra-Shinohara et al., 2012). Guerra-Shinohara et al. suggested that SERPINC1 G786A variant increases the risk of RPL, while TFPI T-287C variant is protective. Annexin A5 (ANXA5) is both a Ca^{2+} and phospholipid-binding protein that is localized at the surface of the placental syncytiontrophoblast layer and performs a vital anticoagulation function in the maternal blood at the intervillous space (Gerke and Moss, 2002). It has been shown that polymorphisms in the promoter region of the ANXA5 gene are significantly associated with RPL, that women with

Abbreviations: RPL, recurrent pregnancy loss; SERPINC1, antithrombin; THBD, thrombomodulin; TFPI, tissue factor pathway inhibitor; ANXA5, annexin A5; APC, activated protein C; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; BMI, body mass index.

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an M2 haplotype have more than a 2-fold higher risk of pregnancy loss than non-carriers (Bogdanova et al., 2007). For both factor *V* Leiden and factor *II* G20210A polymorphisms, an association with the risk of RPL has been proven by statistical meta-analysis (Rey et al., 2003). Factor *V* Leiden, a mutation that leads to an R to Q substitution at position 506 of the factor *V* amino acid sequence, is responsible for more than 75% of inherited activated protein C resistance, hence, an increased level of thrombin generation and a pro-coagulation state (Kalafatis et al., 1994). The G to A transition at position 20210 in the 3′-untranslated region of the factor *II* gene eventually results in elevated mRNA production and an increased synthesis of prothrombin (Gehring et al., 2001). Considering the significant effect of factor *V* Leiden and factor *II* G20210A polymorphisms on RPL, we speculate other polymorphisms near the above two loci in factor *V* and factor *II* gene may also have an effect on RPL.

Given the key role of SERPINC1, THBD, TFPI, factor *V* and factor *II* in regulating the generation of thrombin and the vital anticoagulation function of ANXN5, we investigated the effects of SERPINC1 G786A, THBD C1418T, TFPI T-33C, factor V G1628A, factor II A19911G and ANXA5 G76A on the risk of RPL in a Chinese population for the first time.

2. Materials and methods

2.1. Subjects

This study included 94 RPL women (mean ± SD age, 28.370 ± 3.742 years; body mass index (BMI), 20.943 ± 2.241 kg/m^2) with at least two consecutive pregnancy losses before 20 week's gestational age as case and 169 ethnically-matched healthy controls (mean ± SD age, 28.07 ± 3.611 years; BMI, 20.079 ± 2.281 kg/m^2) with regular menstrual cycles, at least one naturally conceived pregnancy and no history of pregnancy loss or other pregnancy complication. The cases were enrolled at the Maternal and Child Health Center in Kunshan City, the First People's Hospital and the Second People's Hospital affiliated with Soochow University. Patients were identified and recruited when they visited the above-mentioned hospitals for repeated (two or more), consecutive unexplained terminations of pregnancy before 20 weeks of gestation or expulsions of a fetus weighing <500 g. Other inclusion criteria included an age between 20–40 years and a recruitment date less than two weeks after the most recent miscarriage. Subjects with the following characteristics were excluded: anatomical disorders, endocrine disorders or autoimmune disorders. One hundred sixty-nine pregnant women aged 20–39 years with no history of miscarriage were recruited as control subjects (2 for each case patient) with the following matching criteria: age (±2 year), gestational age (±1 week), and residence (the same district). None of the controls had a history of pregnancy complications, miscarriage, still birth or pre-eclampsia, and none had given birth to a child who was small for gestational age. Written informed consent was obtained from all the participants before their enrollment in the study. The study was approved by the ethics committee of Soochow University.

Interviews to RPL patients and controls were all carried out through a structured questionnaire. The informations on demographic characteristics, lifestyle, obstetric history, and other RPL risk factors were obtained. In the following two weeks after the day of recruitment, anticoagulated peripheral blood samples were collected from the 94 RPL cases and 169 health controls by vein puncture. All the blood samples were stored at −80° C until genomic DNA isolation was undertaken.

2.2. Genotyping

Genomic DNA was extracted from leukocytes of the peripheral blood with anticoagulant using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality evaluation of DNA was performed by measuring the absorbance at 260 nm on the NanoDrop spectrophotometer (ThermoScientific). Each sample, with a DNA concentration of 30 ng/ul, was used for genotyping D260/D280 ranging from 1.8 to 2.0 and D260/D230 no less than 1.5.

SERPINC1 G786A, THBD C1418T, TFPI T-33C, factor V G1628A, factor II A19911G and ANXA5 G76A polymorphisms were assayed by utilizing the Sequenom MassARRAY iPLEX Platform. The assay consists of an initial locus-specific PCR reaction, followed by a single base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Using MALDI-TOF mass spectrometry, the distinct mass of the extended primer identifies the SNP allele (Gabriel et al., 2009).

2.3. Statistical analysis

Statistics were performed with SPSS (version 13.0). The case and control women have a similar age and BMI range (case: mean ± SD age, 28.370 ± 3.742 years; BMI, 20.943 ± 2.241 kg/m^2; control: mean ± SD age, 28.07 ± 3.611 years; BMI, 20.079 ± 2.281 kg/m^2), hence, the univariate logistic regression was used to investigate the risk of RPL in the SERPINC1 G786A, THBD C1418T, TFPI T-33C, factor V G1628A, factor II A19911G and ANXA5 G76A polymorphisms between case and control subjects (having RPL according to genotype distributions of investigated SNPs as independent variables). Hardy-Weinberg equilibrium was tested by the use of the goodness-of-fit chi-square test. Odds ratio (OR) and their 95% confidence intervals (CI) were calculated as a measure of the strength of the association between genotype and allele frequencies and RPL. The comparisons of genotype and allele frequencies were performed on the online software SHEsis (http://202.120.7.14/analysis/myAnalysis.php) (Shi and He, 2005). The power of analysis was calculated using the G*Power 3.1 program (Faul et al., 2009). A *P* value less than 0.05 was considered of statistical significance.

2.4. Prediction of SNP effects

The possible SNP effects on mRNA splicing were predicted using the Web-based prediction software ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) (Smith et al., 2006).

3. Results

To investigate the association between RPL and the polymorphisms in the hemostasis-related genes, genotype and allele frequencies of six SNPs were detected in 94 RPL women and 169 healthy controls. The power analysis showed that the statistical power of our sample to detect a significant association ($P < 0.05$) was 98.4% in genotypic comparison for RPL, when an effect size ($w = 0.5$) was presumed. This indicated that the sample size in our study was sufficient to achieve a relatively low risk of type II error. Women of RPL and controls presented similar age and BMI. The distribution of the six polymorphisms was all in Hardy–Weinberg equilibrium (data not shown).

The data of genotype and allele frequencies were shown in Table 1. As shown in Table 1, the genotype and allele frequencies for SERPINC1 G786A, TFPI T-33C, factor V G1628A, factor II A19911G and ANXA5 G76A were similar between RPL case and healthy control groups. The distribution of THBD C1418T allele showed significant differences between RPL cases and healthy controls (OR: 1.58, 95%, CI: 1.05–2.39, $P = 0.027$). In univariate logistic regression analyses, women with THBD 1418 T allele (CT + TT genotypes) had an increased risk of RPL (OR: 1.83, 95%, CI: 1.10–3.06, $P = 0.020$) when compared with CC genotype carriers (Table 2). These suggested that the THBD 1418 T allele was positively associated with the increased risk of RPL.

The mutant "T" allele of THBD C1418T was analyzed by ESEfinder; the data indicate that T allele can affect splicing regulation by altering ESE motifs. The ESEfinder analysis result showed that the mutant "T" results in the gain of SF2/ASF motif (3.32) (Fig. 1). ESEs are thought to serve as binding sites for specific serine/arginine-rich (SR) proteins. the members of the family of serine/arginine (SR)-rich proteins include