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THE C20068T GENE VARIANT IN THE 3` END OF THE PROTHROMBIN GENE AND RECURRENT PREGNANCY LOSS: A PILOT STUDY

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Recurrent pregnancy loss (RPL) is a health problem affecting up to 5% of women of reproductive age. Several thrombophilic risk factors might contribute to RPL.To investigate relationship between a novel C20068T gene variant in the 3` end of prothrombin gene and RPL, we tested 153 women with RPL and 111 controls for the presence of this gene variant. In patients, we have detected four heterozygous (2.61%) and no homozygous carriers. In controls, no carriers were detected. Our results indicate higher prevalence of C20068T gene variant in women with RPL but this difference was not statistically significant. However, in patients who suffered 5 or more RPL, frequency of C20068T gene variant was significantly increased compared to controls (12.5% *vs.* 0%, P=0.02).

This is the first study which points out a possible role of C20068T gene variant in etiology of RPL, but larger studies should be carried out to confirm our findings.

Key words: recurrent pregnancy loss, 3` end of the prothrombin gene, synonymous gene variant,thrombophilia

INTRODUCTION

Fetal loss (FL) is a common health problem affecting up to 20% of women in reproductive age. Five percent of women experience two or more fetal losses, while 1-2% of women experience three or more losses (BRENNER, 2003).The cause of recurrent pregnancy loss

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(RPL) is very poorly understood. While several causes were implied in RPL, such as chromosomal abnormalities, anatomical alterations of the uterus, infectious, endocrinological and autoimmune disorders, and the majority of cases of RPL still remain unexplained (COOK &PRIDHAM, 1995; CLIFFORD *et al*., 1994).

Pregnancy itself is hypercoagulable state, with changes occurring in all aspects of haemostasis, including increase in concentrations of most clotting factors, and decrease in concentrations of natural anticoagulants and activity of fibrinolytic system (BRENNER, 2004). These changes might predispose to thrombosis and placental vascular disorders (ELDOR 2001; DI MICCO *et al.*, 2007). There is a growing pool of evidence implicating congenital and acquired thrombophilia in pathophysiological processes underlying the thrombotic damage in placental bed, which can lead to disorders such as RPL, intrauterine growth restriction or pre-eclampsia (PRANDONI, 2001). For several hereditary thrombophilic conditions, such as deficiencies of antithrombin, proteins C and S, factor V Leiden (FVL) and factor II G20210A (FII G20210A) mutations has been shown to be involved in etiology of RPL (CARP *et al*., 2002; KOVAC *et al.,* 2014; MITIC *et al.,* 2010).

In our previous study, we screened for sequence variants in the 3` end of the prothrombin gene in thrombophilic patients with elevated prothrombin level and FII 20210GG genotype and identified a novel C20068T gene variant. The consecutive study which included patients with thrombotic disorders and healthy blood donors showed increased prevalence of heterozygous C20068T carriers in the patient group compared to controls (3% and 1%, respectively) (DJORDJEVIC *et al*., 2011).

In order to elucidate the possible role of novel C20068T prothrombin gene variant in etiology of pregnancy loss, in this study we have determined the prevalence of C20068T in the group of women who suffered from RPL, as well as in control group of healthy women.

PATIENTS AND METHODS

Our study included 264 women divided in control and study groups. The control group consisted of 111 healthy women (39±12 years) with no history of miscarriages and at least one successful delivery. Women with history of arterial or venous thrombosis, or inherited thrombophilia (deficiencies of antithrombin, protein C and protein S and carriers of FV Leiden and FII G20210A mutations) were excluded from the control group.

The study group was formed by searching a database of over 4000 patients, referred to Institute of Molecular Genetics and Genetic Engineering for thrombophilia testing in period from 1998 to 2013. The following inclusion criteria were employed: history of RPL (two or more consecutive miscarriages) with no thrombotic manifestations (deep vein thrombosis, pulmonary thromboembolism, cerebral and myocardial infarction), absence of infective, immunological, endocrinological and gynaecological disorders and chromosomal abnormalities, as well as absence of recognized thrombophilic risk factors such as deficiencies of antithrombin, protein C and proteins S, FV Leiden and FII G20210A mutations. Overall, 153 women (33±5 years) were included in the study group.

This study was approved by the local hospital ethic committee.

Peripheral blood was taken on 3.8% Na-citrate as anticoagulant. Genomic DNA was extracted from 200μL of whole blood using QIAamp DNA blood mini kit (QIAGEN, Germany) according to manufacturer's protocol. All DNA samples were stored at -20^{o}C until further

investigations. All participants were tested for the presence of C20068T gene variant by PCR-RFLP analysis.

PCR-RFLP analysis and DNA sequencing

For detection of the C20068T gene variant, we designed a new PCR-RFLP method. The following primers were used: FIIf (5`-GCTTCTACACACATGTGTTGC-3`) and FIIr (5`-TCAATGCTCCCAGTGCTATTC-3`). The PCR reaction was carried out in 25µL reaction volume containing 2.5µL 10X KapaTaq Buffer B, 2.5µL dNTPs (2mM), 0.5µL FIIf primer (10pmol/µL), 0.5µL FIIr primer (10pmol/µL), 1U of Kapa Taq DNA polymerase (Kapa Biosystems, Boston, USA) and 200ng of DNA template. PCR program was carried out as follows: 95°C 5 min; 94°C 1 min/61°C 0.5 min/72°C 1 min/x 30 cycles and 72°C for 10 minutes. PCR products (190bp) were digested using the *HhaI* (New England Biolabs, USA) restriction enzyme. Digestion reactions were incubated overnight at 37°C. Normal (170+20bp) and mutant allele (190bp) were analyzed on 10% acrylamide gel electrophoresis (Figure 1A).

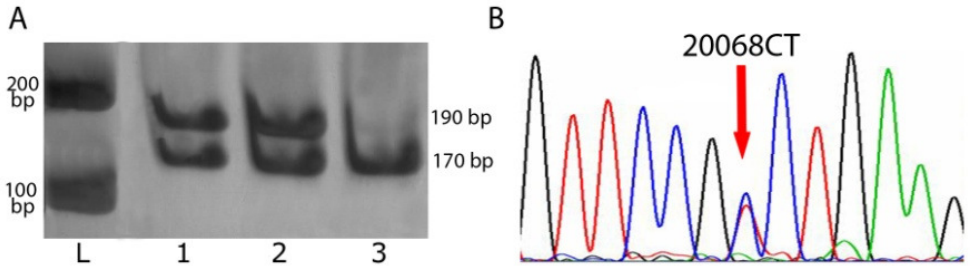

Fig 1. A. PCR-RFLP analysis: 1, 2-20068CT genotype; 2-20068CC genotype; L-Ladder size marker (100 bp)
B. Part of the prothrombin gene sequence with the C20068T gene variant in heterozygous form

The PCR-RFLP method was validated by DNA sequencing (Figure 1B). The fragment of 715bp, which includes the last intron and exon, 3' untranslated region and flanking region of the prothrombin gene (primers: 5'-GGAAACGAGGGGATGCCTGT-3' and 5'-GTGAGAG GAAAGATGGCAGG-3'), was amplified by PCR. PCR reactions were performed in 25µL reaction volume containing 2.5µL 10X KapaTaq Buffer B, 2.5µL dNTPs (2mM), 1 U of Kapa Taq polimerase (Kapa Biosystems,USA) and 1µL (10 pmol/µL) of forward and reverse primers and 200ng of DNA. The thermal cycle profile was: initial denaturation at 95 °C for 5 minutes and 37 cycles consisting of denaturation at 95 °C for 1 minute, annealing at 61 °C for 1 minute and polymerization at 72 °C for 1 minute were applied. Final extension of PCR products was at 72 °C for 10 minutes. The sequencing of the PCR products was performed according to manufacturer's protocol, using the BigDye™ Terminator Version 3.1 Ready Reaction Kit (Applied Biosystems, USA) on a 3130 Genetic Analyzer (Applied Biosystems, USA). Two sequencing reactions for the fragment of interest were performed for each sample (using forward primer: 5'-TCTAGAAACAGTTGCCTGGC-3' or reverse primer: 5'-TCAATGCTCCCAGTGCTATTC-3').

Statistical analysis

Statistical analysis was performed using MedCalc 12.2.1.0 statistical software (MedCalc Software bvba, Belgium). The prevalence of C20068T gene variant was compared between patients and controls with the use of Fisher's exact test. The odds ratio (OR) and 95%CI (Confidence Interval) were also estimated. Value p□0.05 was considered to be statistically significant. Deviations of genotypes distributions from Hardy-Weinberg equilibrium (HWE) were assessed by χ^2-test.

RESULTS

The baseline characteristics of patients and controls are given in Table 1. No statistical difference regarding the age of patients and controls was observed. There were 159 successful pregnancies in controls, while there were none in patients. In the study group, more than 459 miscarriages were detected, with average of 3 fetal losses per patient. One hundred and forty seven patients had 2-4 fetal losses, while 16 patients had 5 or more consecutive miscarriages. The family history of thrombotic disorders was markedly noticeable in the group of patients (27.4%, P<0.001). The study group was in HWE (χ^2=0.03). HWE was not calculated for control group, as no carriers of C20068T variant were detected.

Table 1. Demographic data of recurrent pregnancy loss (RPL) patients and controls

	Study group (n=153)	Control group (n=111)	P
Age (mean±SD)	33±5	39±12	1
Number of successful pregnancies	/	159	
Number of miscarriages	>449	/	
Family history of thrombotic disorders	42 (27.4%)	2 (1.8%)	P<0.001

SD: standard deviation

P: probability

The results of C20068T genotyping are given in Table 2. In the group of women who suffered RPL we have detected four heterozygous carriers of C20068T gene variant (2.61%), while no homozygous carriers were detected. In controls, no carriers of C20068T gene variant, whether heterozygous or homozygous, were detected. The prevalence of C20068T gene variant was higher in group of women with RPL, but this frequency increase was not statistically significant (P=0.30). Women who were heterozygous carriers of C20068T gene variant had 6.71-fold increased risk of RPL occurrence compared to controls (95% CI 0.36-125.97).

Additionally, based on the number of RPL they suffered, we stratified the study group into four subgroups: women with two (78 patients), three (41 patients), four (18 patients) and 5 or more consecutive miscarriages (16 patients). The prevalence of C20068T gene variant in group of women who suffered 2-4 RPL was not significantly increased. However, in the group of 16 women who suffered 5 or more consecutive miscarriages, the frequency of C20068T gene variant was significantly increased (P=0.02) compared to controls.

Table 2. Genotype frequencies of C20068T gene variant in patients with recurrent pregnancy loss (RPL) and controls

	C20068T		P	OR
	C/C	C/T		95% CI
Study group n=153	149 (97.39%)	4 (2.61%)	0.20	6.71 0.36-125.97
Subgroup 1 (2 PL) n=78	76 (97.44%)	2 (2.56%)	0.20	7.29 0.34-153.93
Subgroup 2 (3 PL) n=41	41 (100%)	0 (0%)	-	-
Subgroup 3 (4 PL) n=18	18 (100%)	0 (0%)	-	-
Subgroup 2 (≥5 PL) n=16	14 (87.5%)	2 (12.5%)	0.02	38.44 1.75-841.04
Control group n=111	111 (100%)	0 (0%)	-	-

PL: pregnancy loss,P: probability,OR: Odds ratio ,CI: confidence interval

DISCUSSION

The present study was designed to explore the implications of C20068T gene variant, located in the 3`end of prothrombin gene, in etiology of RPL. The 3' end region of the prothrombin gene is considered as very dynamic because of its non-canonical architecture. Therefore, it could be a potential region for finding new variants (DANCKWARDT *et al*., 2004; DANCKWARDT *et al*., 2006, DANCKWARDT *et al*., 2008; GVOZDENOV *et al.,* 2015).

Our previous study, in which we screened the 3`end of the prothrombin gene, indicated the possible role of novel FII C20068T gene variant in pathogenesis of thrombophilia. In the NCBI/SNP database, this gene variant is registered under the number rs3136532 (http://www.ncbi.nlm.nih.gov/projects/SNP). In the PGA-European-Panel group, which included 22 Caucasians, one heterozygous carrier was detected. In the HapMap project, which included 60 European subjects, one heterozygous carrier was also detected (http://hapmap.ncbi.nlm.nih.gov). In our study, we did not detect carriers of C20068T among 111 controls. On the other hand, among 153 women with RPL, we detected four heterozygous carriers of C20068T gene variant. The higher C20068T frequency in the patients was not statistically significant compared to controls, likely due to the restricted size of study groups. Carriers of 20068CT genotype have ~6-fold increased risk for RPL occurrence compared to controls, indicating that C20068T gene variant in heterozygous form could represent a risk factor for RPL. Additionally, the presence of 20068CT genotype was significantly increased (P=0.02) in women who suffered five or more pregnancy losses. To the best of our knowledge, no data regarding association of C20068T gene variant with other disorders is available.

The changes in haemostatic status which lead to thrombophilia are frequently associated with RPL (ROBERTSON *et al*., 2006; KOVAC *et al.,* 2010). Pregnancy *per se* represents hypercoagulable state and thrombophilic risk factors could alternate the sensitive haemostatic balance towards increased hypercoagulation and miscarriages (RAI & REGAN, 2006, R2). A meta-analysis (KOVALEVSKY *et al*., 2004) pointed out FV Leiden and FII G20210A mutations as risk factors for RPL, with odds ratios being 2 for both FV Leiden (95% CI 1.5-2.7; P=0.001) and FII G20210A (95% confidence interval, 1.0-4.0; P=0.03). Our results show that, compared to FV Leiden and FII G20210A mutations, C20068T gene variant is less frequent, but associated with higher odds ratio (Table 2), suggesting 20068CT genotype should be considered as risk factor in patients with RPL, especially where no other risk factors are established.

The C20068T gene variant is located in the last exon of the prothrombin gene and leads to the replacement of CGC codon for arginine to CGT codon which also codes for arginine on the position 608 in the protein. Therefore, it is a synonymous gene variant and does not lead to amino acid replacement in the protein. Silent (synonymous) single nucleotide polymorphisms (SNPs) do not alter coding sequences and are not expected to influence the function of the protein in which they occur (HURST, 2011; SHABALINA *et al*., 2013). However, there is a growing pool of evidence that synonymous SNPs indeed do change protein activity through changes in mRNA level, mRNA folding that could influence splicing, processing or translational control and regulation (SHABALINA *et al*., 2013; NACKLEY *et al*., 2006). The mechanism by which C20068T gene variant alters the expression of prothrombin gene remains to be elucidated.

The major limitation of our study is a relatively small group size. In order to obtain well defined group, including only women with idiopathic RPL, we implemented very strict selection criteria. We have defined RPL as one or more consecutive miscarriages and included women who will potentially have more miscarriages in the future, considering the fact that the risk of an abortion progressively grows after each successive pregnancy loss (RAI & REGAN, 2006). We also excluded patients with well-defined causes of RPL such as chromosomal abnormalities, anatomical alterations of the uterus, endocrinologic and autoimmune disorders, infections, as well as patients with thrombophilic conditions (antithrombin, protein C and protein S deficiencies, homozygosity/heterozygosity for FV Leiden and FII G20210A mutations).

In conclusion, our pilot study has shown a trend of higher prevalence of 20068CT genotype in women with RPL, while the frequency was significantly increased in women with five or more RPL. Even though further prospective studies are needed to confirm our findings, this is the first study which points out C20068T gene variant as potential thrombophilic marker for RPL.

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ZNAČAJ GENSKE VARIJANTE C20068T U 3' KRAJU GENA ZA PROTROMBIN U ETIOLOGIJI PONOVLJENIH SPONTANIH POBAČAJA: PILOT STUDIJA

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Izvod

Ponovljeni spontani pobačaji predstavljaju zdravstveni problem koji pogađa oko 5% žena u reproduktivnoj dobi. U etiologiju ovog oboljenja uključen je i veći broj trombofilnih faktora rizika. Cilj ove studije bio je da se utvrdi značaj nove genske varijante C20068T, locirane u 3' kraju protrombinskog gena, za nastanak spontanih pobačaja. U studiju je uključeno ukupno 264 ispitanice, od čega 153 pacijentkinje sa ponovljenim spontanim pobačajima i 111 ispitanica kontrolne grupe, koje su testirane na prisustvo genske varijante C20068T.

U grupi pacijentkinja detektovana su četiri heterozigotna nosioca (2,61%) ove genske varijante, dok homozigotni nosioci nisu detektovani u ovoj studiji. U kontrolnoj grupi nije detektovan nijedan nosilac genske varijante C20068T. Naši rezultati pokazuju da postoji povećana učestalost genske varijante C20068T kod pacijentkinja sa ponovljenim spontanim pobačajima, ali razlika u učestalosti u odnosu na kontrolu grupu nije statistički značajna. Sa druge strane, učestalost genske varijante C20068T kod pacijentkinja koje su imale 5 i više spontanih pobačaja bila je statistički značajno povećana u odnosu na kontrolnu grupu (12,5% i 0%,respektivno, P=0,02).

Ovo je prva studija koja ističe značaj i moguću ulogu genske varijante C20068T u etiologiji spontanih pobačaja. Da bi se potvrdili rezultati ove pilot studije, potrebno je sprovesti dalja istraživanja koja obuhvataju veći broj ispitanica.

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