GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 16, Number 4, 2012

Pp. 259–264
DOI: 10.1089/gtmb.2011.0173

Detection of Thrombophilic Mutations Related to Spontaneous Abortions by a Multiplex SNaPshot Method

Svetlana Madjunkova,[1] Marija Volk,[2] Borut Peterlin,[2] and Dijana Plaseska-Karanfilska[1]

Spontaneous abortion is a significant clinical problem of different etiologies. Certain thrombophilia gene mutations have been associated with an increased risk of spontaneous abortion. Also, mutations in folate-related genes can lead to abnormal chromosomal segregation during meiosis which is the most common cause of spontaneous abortion. We have developed a multiplex single-base extension reaction assay that allows simultaneous analysis of 10 different mutations in thrombophilia- and folate-related genes (*Factor V Leiden G1691A, Factor V H1299R, Factor II G20210A, Factor XIII V34L, PAI-I -675 4G/5G, FGB -455G/A, MTHFR C677T, MTHFR A1298C, MTR A2756G,* and *MTRR A66G*). Using this method we have studied 232 women who had a spontaneous abortion and 209 of their male partners. Prevalence of *Factor II G20210A* and *Factor V H1299R* mutations was significantly higher in the women than in their male partners (2.4% and 0.7%, respectively [$p = 0.0499$] for the Factor II mutation and 9.3% and 5.7%, respectively [$p = 0.0485$] for the *Factor V* mutation). The prevalence of *MTHFR C677T, MTHFR A1298C, MTR A2756G,* and *MTRR A66G* mutations did not differ between the studied groups. In conclusion, we have developed a rapid, simple, reliable, and inexpensive multiplex SNaPshot method for determination of 10 thrombophilic mutations that may result in spontaneous abortions.

Introduction

Spontaneous abortion is the most common complication of pregnancy, affecting approximately 10–15% of clinically recognized pregnancies. Recurrent spontaneous abortions, defined as the occurrence of three or more consecutive, clinically detectable pregnancy failures, are estimated to occur in 0.5–3% of all couples trying to conceive. After excluding anatomic, metabolic, endocrine, infectious, genetic, and environmental factors, the cause remains unknown in approximately 50% of women who experience spontaneous abortions (Frosst *et al.*, 1995).

There is growing evidence of involvement of inherited thrombophilia in the etiology of spontaneous abortion and placenta-related complications in pregnancy, including intrauterine growth restriction, intrauterine fetal death, placental abruption, and preeclampsia (Ford and Schust, 2009; Rodger *et al.*, 2010). Such thrombophilias augment the prothrombotic state of pregnancy and lead to inadequate fetomaternal circulation and affect the process of placentation in the developing embryo. The most commonly related to spontaneous abortions are the *Factor V Leiden* mutation *G1691A*, mutation in the promoter region of the *prothrombin gene G20210A*, and homozygosity of *C677T* mutation in the *methylene tetrahydrofolate reductase (MTHFR) gene* with general prevalence of 2–15%, 2–3%, and 11%, respectively. These mutations are associated with mild thrombotic risks, and their association with spontaneous abortion remains controversial (Brenner *et al.*, 1999; Pickering *et al.*, 2001; Rai *et al.*, 2001; Hefler *et al.*, 2004; Tranquilli *et al.*, 2004; Coulam *et al.*, 2006; Yenicesu *et al.*, 2010). Mutations that affect genes for factors involved in the common pathway of the coagulation cascade and the fibrinolytic system, such as in *Factor XIII*, *β Fibrinogen*, and *plasminogen activator inhibitor-1 (PAI-1)*, may also contribute to the thrombotic tendency (Coulam *et al.*, 2006; Yenicesu *et al.*, 2010).

Hyperhomocysteinemia leads to endothelial injury and vascular inflammation and is an independent risk factor for thrombosis. However, reduced regeneration of methionine caused by mutations in folate-related enzymes of one-carbon metabolism can lead to impaired segregation (nondisjunction) of chromatids during meiosis I or II. This nondisjunction produces aneuploid gametes that give rise of aneuploid embryos. Chromosomal aneuploidies are a major and well-defined cause of first trimester spontaneous abortion (Stephenson *et al.*, 2002). Cytogenetic evaluation of spontaneous abortions has revealed chromosomal abnormalities in 50–70% (Hogge *et al.*, 2003). The most common are *de novo* numerical abnormalities (94%), in particular autosomal trisomies for

[1]Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov," Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia.
[2]Department of Obstetrics and Gynecology, Institute of Medical Genetics, University Medical Center Ljubljana, Ljubljana, Slovenia.

chromosomes 13, 14, 15, 16, 21, and 22, followed by monosomy X and polyploidy (Hassold *et al.*, 1980; Strom *et al.*, 1992; Stephenson *et al.*, 2002).

Available genotyping methods for the thrombophilic mutations include polymerase chain reaction (PCR) with fragment length polymorphism (PCR-RFLP) analysis, real-time PCR with allele-specific Taqman probes (Ulvik and Ueland, 2001), allele-specific reverse hybridization (Yilmaz *et al.*, 2006), and MALDI TOFF mass spectrometry (Jurinke *et al.*, 2002; Jurinke *et al.*, 2004). Here we describe a multiplex single-base extension reaction assay that allows simultaneous analysis of 10 different mutations in thrombophilia- and folate-related genes in one reaction.

Materials and Methods

In total, 441 DNA samples were studied using a multiplex single-base extension reaction assay, 232 samples from women experiencing spontaneous abortions and 209 from their male partners who were used as controls. Of the women, 137 were of Slovenian, 55 of Albanian, and 40 of Macedonian origin, while of the men, 127 were of Slovenian, 51 of Albanian, and 31 of Macedonian origin. Couples of Slovenian origin were selected in the Institute of Medical Genetics, University Medical Center-Ljubljana, Ljubljana, Slovenia, while those of Macedonian and Albanian origin were selected in the Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov," Macedonian Academy of Sciences and Arts, Skopje, Macedonia. The study was approved by the Ethical Committee of the Macedonian Academy of Sciences and Arts, and all subjects gave informed consent for participation in the study.

Multiplex SNaPshot method for detection of 10 mutations in five thrombophilia- and three folate-related genes

All individuals were analyzed for the presence of 10 different mutations: *Factor V Leiden G1691A*, *Factor V H1299R*, *Factor II G20210A*, *Factor XIII V34L*, *PAI-I -675 4G/5G*, *FGB -455G/A*, *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G*, and *MTRR A66G*. We have designed a multiplex single-base extension method for simultaneous detection of the 10 mutations using the Multiplex SNaPshot kit (Multiplex SNaPshot™; Applied Biosystems, Warrington, WA) for the reaction, followed by capillary electrophoresis on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The PCR primers were designed to produce PCR fragments of 129–420 bp and to allow their separation by 6% polyacrylamide gel electrophoresis. Briefly the PCR amplification reaction contained 200 ng genomic DNA, 10 pmol of specific forward and reverse oligonucleotide primers (Table 1), 200 μL of each deoxyribonucleotide triphosphate (dNTP), 1.5 mM magnesium chloride, and 1 U Taq DNA polymerase (Ampli Tag Gold™; Applied Biosystems, Foster City, CA) in a total volume of 25 μL. The PCR conditions were 10 min initial

Table 1. Sequences of PCR Primers and Primers Used for Single-Base Extension Reaction

Mutation	*Primer*	*Sequence (5′-3′)*
FV Leiden	PCR For	TGA TGC CCA GTG CTT AAC AA
	PCR Rev	TCA CAC TGG TGC TAA AAA GGA
	SNaPshot	CCC CCC CCC CCC AGC AGA TCC CTG GAC AGG C
FV H1299R	PCR For	GTC AGA TGC CCA TTT CTC CA
	PCR Rev	AGA GGT TTG TCT GGC TGA GG
	SNaPshot	CCC CCC CCC CCC CCC CCC GCT GAA GTC TAG AGA AAG GGT TGT A
F II G20210A	PCR For	GAG AGT AGG GGG CCA CTC AT
	PCR Rev	CAT GAA TAG CAC TGG GAG CA
	SNaPshot	CCC CCC CCC CGT TCC CAA TAA AAG TGA CTC TCA GC
FIII V34L	PCR For	TTT GGA GGC AGA AGA GCA GT
	PCR Rev	CAA GGT CAG TAA GGG GCA GA
	SNaPshot	CAC AGT GGA GCT TCA GGG C
PAI-1 4G/5G	PCR For	GGC AGC TCG AAG AAG TGA AA
	PCR Rev	ACC TCC ATC AAA ACG TGG AA
	SNaPshot	CCC CCC CCC CCC CCC CCC ATG ATA CAC GGC TGA CTC CCC
FGB -455G/A	PCR For	GGG TCT TTC TGA TGT GTA TTT TTC A
	PCR Rev	TGA CCT ACT CAC AAG GCA ACC
	SNaPshot	ATT CTA TTT CAA AAG GGG C
MTHFR C677T	PCR For	CCA GTC CCT GTG GTC TCT TC
	PCR Rev	TCA CAA AGC GGA AGA ATG TG
	SNaPshot	CCC CCC CCC CCG CGG GAG CCG ATT TCA TCA T
MTHFR A1298C	PCR For	TTT GGG GAG CTG AAG GAC TA
	PCR Rev	GGA GGT CTC CCA ACT TAC CC
	SNaPshot	CCC CCC CCC CCC CCC CCC CCC CTG ACC AGT GAA GAA AGT GTC TTT GA
MTRR A66G	PCR For	TCC CCC ATT TTT CAG TTT CA
	PCR Rev	CCA TGT ACC ACA GCT TGC TC
	SNaPshot	AGG CCA TCG CAG AAG AAA T
MTR A2756G	PCR For	CCA AGC CCA CTG AGT TTA CC
	PCR Rev	TCC AAA GCC TTT TAC ACT CCT C
	SNaPshot	CCG AAT ATG AAG ATA TTA GAC AGG

PCR, polymerase chain reaction.

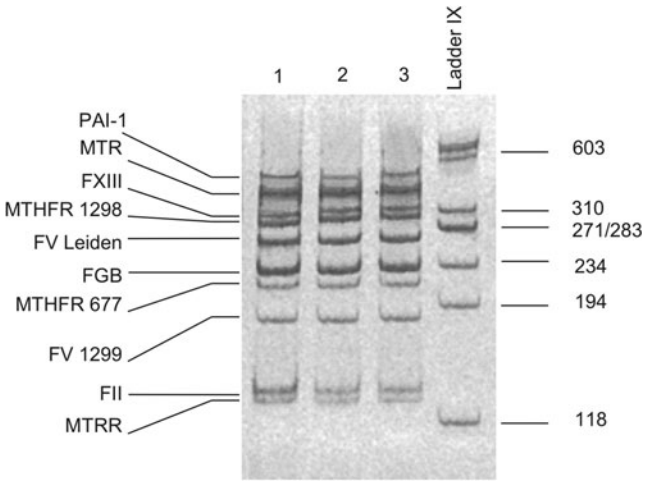

FIG. 1. Analysis of ten-plex polymerase chain reaction for detection of 10 thrombophilic mutations by 6% polyacrylamide gel electrophoresis.

denaturation at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 2.45 min at 72°C. A final extension was at 72°C for 30 min. After analysis of the PCR products on 6% polyacrylamide gels (Fig. 1), they were purified by the use of 1 μL of ExoSAP-IT® (USB, Cleveland, OH) per 5 μL of PCR product, with incubation at 37°C for 60 min and at 86°C for 15 min to deactivate the enzyme.

The purified PCR products formed the template for detection of the 10 mutations. In the following single-base extension reaction, the detection primers (SNaPshot primers) annealed adjacent to the single-nucleotide polymorphism position. Poly (dC) tails of different lengths were attached to the single-base extension primers (Table 1). The SNaPshot reaction contained 1 μL primer mix of all 10 single-base extension primers (final concentration of 1 pM each), 2 μL of purified PCR products, and 2 μL of SNaP-shot Multiplex kit in a final volume of 5 μL. The cycling profile consisted of 25 cycles of 95°C for 10 s, 55°C for 10 s, and 60°C for 30 s. After the reaction, 5′-phosphophoryl groups of unincorporated dideoxynucleotide triphosphates were removed by addition of 1.0 U of shrimp alkaline phosphatase (SAP; USB) and incubation at 37°C for 2 h and at 86°C for 20 min to inactivate the enzyme. Capillary electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer and the results were analyzed with Gene Mapper ID, Ver 4.0 (Applied Biosystems, Foster City, CA). Details of multiplex PCR and SNaPshot reaction products are given in Table 2. Due to the influence of the dye, size, and nucleotide composition on the mobility shift of the DNA fragments, the reported sizes differ by a few bases from the actual sizes, and this is particularly true with the shorter fragments as the relative contribution of the dye is greater. A representative electrophoreogram from a patient heterozygous for seven thrombophilic mutations is shown on Figure 2.

Ten DNA samples with different alleles of the 10 studied thrombophilic mutations, determined previously by DNA sequencing, were used to validate the multiplex SNaPshot method. Further, the SNaPshot genotyping results from 50 selected samples were confirmed by PCR-RFLP for *FV Leiden*, *MTHFR C677T*, and *MTR A66G* using the enzymes *Mnl*I (New England BioLabs, Beverly, MA) (Bertina *et al.*, 1994), *Hinf*I, and *Hind*III (New England BioLabs) (Frosst *et al.*, 1995), respectively. Digested segments of DNA were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide, and examined under ultraviolet light. For the other mutations, confirmation was done by sequencing of the specific PCR product using ABI Prism® BigDye terminator cycle sequencing ready reaction kit v1.1 (Applied Biosystems, Foster City, CA) and forward or reverse primer (Table 1) according to the manufacturer's protocol. The reactions were run on an ABI Prism 3130 Genetic Analyzer and the results were analyzed with Sequencing Analysis Software 5.3.1.

Statistical analysis

Statistical analysis was performed using SISA (www.home.clara.net/sisa) statistics. Discrete variables were analyzed using Fisher's exact test or the χ^2 test. *p*-Values less than 0.05 were taken as statistically significant. Relative risk and 95% confidence intervals were calculated where appropriate.

Results

The multiplex SNaPshot method for the testing of 10 thrombophilic mutations was validated by initial analysis of 10 DNA samples genotyped previously by DNA sequencing and representing normal, heterozygotes, and homozygotes for each studied mutation. Fifty samples representing normal and/or mutant alleles of the studied mutations, obtained by SNaPshot analysis, were genotyped by PCR-RFLP (for *FV Leiden*, *MTHFR C677T*, and *MTR A66G* mutations) and sequencing for evaluating the method. The positive and

Table 2. Details of Multiplex PCR and SNaPshot Reaction Products

Mutation	*Nucleotide change*	*PCR fragment (bp)*	*SNaPshot primer orientation*	*SNaPshot result* (N/M)	*SNaPshot fragment* N *(bp)*	*SNaPshot fragment* M *(bp)*
FXIII V34L	G/T	331	R	C/A	21	22
MTRR A66G	A/G	129	F	A/G	22.6	24.5
FGB -455G/A	G/A	222	R	C/T	24.4	26.6
MTHFR C677T	C/T	213	F	C/T	27	37
MTR A2756G	A/G	383	F	A/G	29	26
FV Leiden	G/A	265	F	G/A	33	34.5
FII G20210A	G/A	133	F	G/A	36	38
PAI-1 4G/5G	T/G	420	R	A/C	41	40
FV H1299R	A/G	177	R	T/C	46	45
MTHFR A1298C	A/C	314	F	A/C	49	48

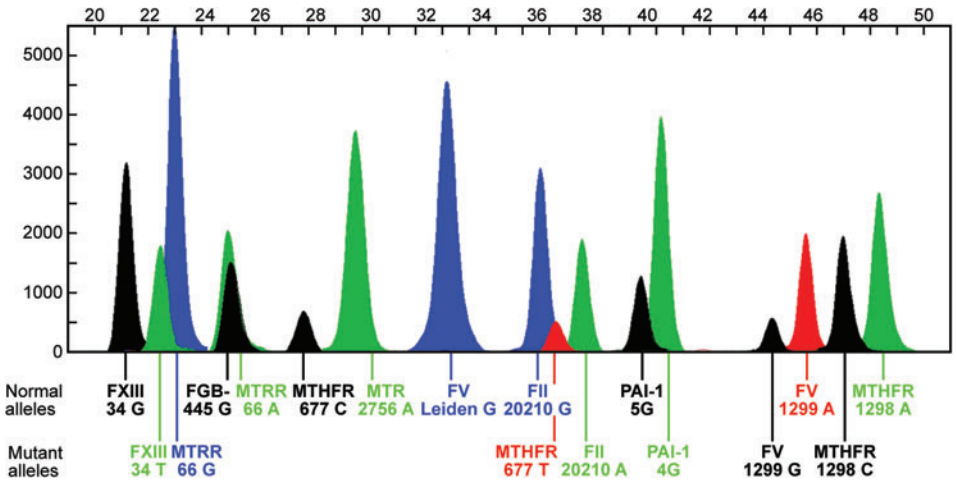

FIG. 2. Electrophoreogram from Gene Mapper ID, Ver 4.0, analysis of 10 mutations in five thrombophilia- and three folate-related genes in a patient with seven mutations in heterozygous state. Normal and mutant alleles are indicated on the left. Color images available online at www.liebertonline.com/gtmb

negative predictive values for the new multiplex SNaPshot method were 100%.

Allele and genotype frequencies were obtained for 232 female partners who had experienced spontaneous abortions and their male partners ($n=209$) using this multiplex single-base extension reaction assay. In all the studied groups, no significant deviation from the Hardy–Weinberg equilibrium was found (data not shown).

The allele frequencies of the mutations are shown in Table 3. Those of *Factor II G20210A, A* allele and *Factor V H1299R (A→G), G* allele were significantly higher in the female partners than in the male partners, 2.4% and 0.7% ($p=0.0499$), and 9.3% and 5.7% ($p=0.0485$), respectively. Those of *Factor V Leiden* mutation and *PAI-1 5G/4G, 4G* allele were slightly higher in the female partners (2.8% and 56.5%, respectively) than in the male partners (2.4% and 52.9%, respectively), but the difference was not statistically significant. There was a reverse trend for the alleles of *Factor XIII V34L* and *FGB -455G/A*, which were more frequent in the male partners (28.9% and 28.5%, respectively) than in the female partners (25% and 25.2%, respectively). Frequencies of *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G*, and *MTRR A66G* mutations were similar in both genders.

Frequencies of *Factor V Leiden*, *Factor II G20210A*, and *MTHFR C677T* mutations in Slovenian couples were slightly lower than in those of Macedonian or Albanian ethnicity (Table 3). In the Slovenian couples, there was a significantly higher frequency of *Factor V H1299R* mutation in the women than in

Table 3. Distribution of Allele Frequencies of 10 Mutations in Thrombophilia- and Folate-Related Genes, Among Couples of Slovenian, Macedonian, and Albanian Ethnicity

		Slovenian couples			*Macedonian couples*			*Albanian couples*			*Total*		
Mutation	*Allele*	*Female partner* n=*137*	*Male partner* n=*127*	p	*Female partner* n=*40*	*Male partner* n=*31*	p	*Female partner* n=*55*	*Male partner* n=*51*	p	*Female partner* n=*232*	*Male partner* n=*209*	p
Factor V Leiden	*G*	0.974	0.976	0.8875	0.962	0.952	0.7494	0.973	0.990	0.3504	0.972	0.976	0.7034
	A	0.026	0.024		0.038	0.048		0.027	0.010		0.028	0.024	
Prothrombin G20210A	*G*	0.985	0.996	0.2063	0.962	0.984	0.4451	0.964	0.990	0.2030	0.976	0.993	**0.0499**
	A	0.015	0.004		0.038	0.016		0.036	0.010		0.024	0.007	
Factor V H1299R	*A*	0.905	0.957	**0.0203**	0.938	0.952	0.7174	0.891	0.902	0.8602	0.907	0.943	**0.0485**
	G	0.095	0.043		0.062	0.048		0.109	0.098		0.093	0.057	
Factor XIII V34L	*G*	0.737	0.693	0.2592	0.812	0.758	0.4307	0.736	0.725	0.8580	0.750	0.711	0.1866
	T	0.263	0.307		0.188	0.242		0.264	0.275		0.250	0.289	
FGB -455G/A	*G*	0.745	0.717	0.4687	0.750	0.661	0.2474	0.755	0.745	0.8744	0.748	0.715	0.2757
	A	0.255	0.283		0.250	0.339		0.245	0.255		0.252	0.285	
PAI-1 5G/4G	*5G*	0.562	0.587	0.5686	0.575	0.452	0.1443	0.564	0.618	0.4244	0.435	0.471	0.3025
	4G	0.438	0.413		0.425	0.548		0.436	0.382		0.565	0.529	
MTHFR C677T	*C*	0.617	0.634	0.6855	0.538	0.565	0.7483	0.555	0.471	0.2218	0.588	0.584	0.8904
	T	0.383	0.366		0.462	0.435		0.445	0.529		0.412	0.416	
MTHFR A1298C	*A*	0.686	0.681	0.9025	0.750	0.774	0.7379	0.736	0.794	0.3222	0.709	0.722	0.6588
	C	0.314	0.319		0.250	0.226		0.264	0.206		0.291	0.278	
MTR A2756G	*A*	0.770	0.756	0.7024	0.762	0.742	0.7773	0.845	0.794	0.3302	0.787	0.763	0.4041
	G	0.230	0.244		0.238	0.258		0.155	0.206		0.213	0.237	
MTRR A66G	*A*	0.431	0.417	0.7567	0.450	0.532	0.3307	0.400	0.441	0.5441	0.427	0.440	0.6873
	G	0.569	0.583		0.550	0.468		0.600	0.559		0.573	0.560	

Values given in bold have statistical significance ($p<0.05$).

their male partners ($p = 0.0203$). A similar trend was obvious in the other ethnic groups, but their small number has restricted the power of the statistical tests.

Discussion

The SNaPshot genotyping method that we have designed detects 10 different mutations in thrombophilia- and folate-related genes that may be implicated in etiopathogenesis of spontaneous abortions and other vascular pathologies. This method efficiently and simultaneously detects the 10 mutations. Compared to other genotyping methods such as PCR-RFLP, reverse dot blot hybridization, sequencing, or mass spectrometry, this method is straightforward, accurate, easy to perform, and cost efficient.

With its use, we have estimated the prevalence of *Factor V Leiden*, *prothrombin G20210A*, *Factor V H1299R*, *FXIII V34L*, *PAI-I -675 4G/5G*, *MTHFR C677T and A1298C*, *MTR A2756G*, and *MTRR A66G* mutations in couples with spontaneous abortions of Slovenian, Macedonian, or Albanian ethnicity. We used the men as the control group, since thrombophilia is a maternal risk factor that leads to adverse pregnancy outcome. Several studies of couples with spontaneous abortions and fertile couples have found no difference in prevalence of thrombophilic mutations between studied groups (Jivraj *et al.*, 2006; Jauniaux *et al.*, 2006; Rodger *et al.*, 2010).

The *prothrombin G20210A* and *Factor V H1299R* mutations in our study had a significantly higher prevalence among women than men. This implicates their possible involvement in pathogenesis of spontaneous abortions. These mutations were found only in the heterozygous state in both genders. We found no association between thrombophilia due to *factor V Leiden*, *FXIII V34L*, *PAI-I −675 4G/5G*, *MTHFR C677T* and *A1298C*, *MTR A2756G* and *MTRR A66G* and spontaneous abortions.

Prothrombin G20210A mutation, which results in increased levels of prothrombin and is a potential risk for thrombosis, has been identified as a risk factor for pregnancy loss in several studies and has been associated mainly with early spontaneous abortions (Finan *et al.*, 2002). However, *Factor V H1299R* is associated with inherited mild-activated protein C resistance but mainly in homozygous state and predisposing to thrombosis. *Factor V Leiden*, responsible for more than 75% of inherited activated protein C resistance, is a common inherited thrombotic risk factor and there are conflicting results about but its association with spontaneous abortions especially early in pregnancy is controversial (Brenner, 1999; Brenner *et al.*, 1999; Hatzis *et al.*, 1999; Tranquilli *et al.*, 2004; Coulam *et al.*, 2006; Rodger *et al.*, 2010). Its prevalence in our study was higher in women than in men (2.8% and 2.4%), but not statistically significant. Our results are in concordance with the one mentioned above and do not exclude the relevance of this mutation in second trimester abortions and late pregnancy complications.

Based on the association of the deficiency of coagulation factor XIII with high frequency of fetal wastage in mice, the polymorphic site within the coding region of coagulation *factor XIII* A subunit gene *V34L* that lowers plasma factor XIII–specific activity could be associated with spontaneous abortion (Koseki-Kuno *et al.*, 2003). Mutation in *-455 G→A* promoter position of *β-fibrinogen* gene is associated with higher plasma β-fibrinogen level and increased clot formation. We found that the mutant genotypes of *Factor XIII V34L (G/T + T/T)* and *FGB -455G/A (G/A + A/A)* to be more frequent in the men than in the women (50.24% and 43.57% for *F XIII V34L*, and 48.8% and 42.66% for *FGB -455G/A*).

Factors that influence the fibrinolysis, particularly PAI-1, are active at implantation and placentation. They modify trophoblast migration and prevent additional hemorrhage at placentation. The *-675 5G/4G* polymorphism results in elevated endothelial expression of *PAI-1* and reduced fibrinolytic activity. The difference between prevalence of mutant *4G* allele in female and male partners (56.5% and 52.9%, respectively) that we have found was not statistically significant.

The lack of association between thrombophilia due to *factor V Leiden*, *FXIII V34L*, *FGB -455G/A*, and *PAI-I -675 4G/5G* mutations and spontaneous abortions that we have found accords with the published data of Brenner *et al*. (1999), Tranquilli *et al.* (2004), Coulam *et al.* (2006), and Rodger *et al.* (2010).

Mutations in enzymes involved in one-carbon metabolism lead to elevation of blood levels of homocysteine as a thrombotic risk factor. The *MTHFR 677C/T* mutation is the most prominent one giving reduced activity of *MTHFR* from 35% in heterozygous to 70% in homozygous state. The prevalence of *MTHFR C677T* and *A1298C*, *MTR A2756G*, and *MTRR A66G* mutations (Table 3) did not show statistical difference between the female and male partner groups. Hyperhomocysteinemia is a rare finding in pregnant woman today because of high vitamin supplementation during pregnancy. There are still conflicting results from meta-analysis elucidating the role of mutations in folate-related genes in adverse pregnancy outcome, and our results support those that did not find association (Zetterberg *et al.*, 2002; Powers *et al.*, 2003; Kujovich, 2004; Rodger *et al.*, 2010).

We recognize the limitations of our study with respect to defining the association of thrombophilic mutations with spontaneous abortions and these are population-specific genotype effects, unequal representation of different ethnic groups, and lack of fertile couples as literally concordant controls. For valid conclusions about the role of the studied thrombophilic mutations in the Macedonian, Albanian, and Slovenian populations, analysis of ethnically matched couples with normal pregnancies as controls are warranted.

We conclude that this multiplex single-base extension reaction assay for simultaneous analysis of 10 different mutations in thrombophilia- and folate-related genes is an easy-to-perform and cost-effective genotyping technique. The mutations included in this assay have a substantial role in the pathogenesis of spontaneous abortion, which needs to be fully elucidated.

Acknowledgment

This study was supported by the grant 14-2414/1 from Ministry of Education and Science of the Republic of Macedonia (to D. Plaseska-Karanfilska).

Disclosure Statement

No competing financial interests exist.

References

Bertina RM, Koeleman BP, Koster T, *et al.* (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 369:64–67.

Brenner B (1999) Inherited thrombophilia and pregnancy loss. Thromb Haemost 82:634–640.

Brenner B, Sarig G, Weiner Z, *et al.* (1999) Thrombophilic polymorphisms are common in women with fetal loss without apparent cause. Thromb Haemost 82:6–9.

Coulam CB, Jeyendran RS, Fishel LA, *et al.* (2006) Multiple thrombophilic gene mutations rather than specific gene mutations are risk factors for recurrent miscarriage. Am J Reprod Immunol 55:360–368.

Finan RR, Tamim H, Ameen G, *et al.* (2002) Prevalence of factor V G1691A (factor V-Leiden) and prothrombin G20210A gene mutations in a recurrent miscarriage population. Am J Hematol 71:300–305.

Ford HB, Schust DJ (2009) Recurrent pregnancy loss: etiology, diagnosis, and therapy. Rev Obstet Gynecol 2:76–83.

Frosst P, Blom HJ, Milos R, *et al.* (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111–113.

Hassold T, Chen N, Funkhouser J, *et al.* (1980) A cytogenetic study of 1000 spontaneous abortions. Ann Hum Genet 44:151–178.

Hatzis T, Cardamakis E, Drivalas E, *et al.* (1999) Increased resistance to activated protein C and factor V Leiden in recurrent abortions. Review of other hypercoagulability factors. Eur J Contracept Reprod Health Care 4:135–144.

Hefler L, Jirecek S, Heim K, *et al.* (2004) Genetic polymorphisms associated with thrombophilia and vascular disease in women with unexplained late intrauterine fetal death: a multicenter study. J Soc Gynecol Investig 11:42–44.

Hogge WA, Byrnes AL, Lanasa MC, *et al.* (2003) The clinical use of karyotyping spontaneous abortions. Am J Obstet Gynecol 189:397–400; discussion 400–392.

Jauniaux E, Farquharson RG, Christiansen OB, *et al.* (2006) Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. Hum Reprod 21:2216–2222.

Jivraj S, Rai R, Underwood J, *et al.* (2006) Genetic thrombophilic mutations among couples with recurrent miscarriage. Hum Reprod 21:1161–1165.

Jurinke C, Oeth P, van den Boom D (2004) MALDI-TOF mass spectrometry: a versatile tool for high-performance DNA analysis. Mol Biotechnol 26:147–164.

Jurinke C, van den Boom D, Cantor CR, *et al.* (2002) The use of MassARRAY technology for high throughput genotyping. Adv Biochem Eng Biotechnol 77:57–74.

Koseki-Kuno S, Yamakawa M, Dickneite G, *et al.* (2003) Factor XIII A subunit-deficient mice developed severe uterine bleeding events and subsequent spontaneous miscarriages. Blood 102:4410–4412.

Kujovich JL (2004) Thrombophilia and pregnancy complications. Am J Obstet Gynecol 191:412–424.

Pickering W, Marriott K, Regan L (2001) G20210A prothrombin gene mutation: prevalence in a recurrent miscarriage population. Clin Appl Thromb Hemost 7:25–28.

Powers RW, Dunbar MS, Gallaher MJ, *et al.* (2003) The 677 C-T methylenetetrahydrofolate reductase mutation does not predict increased maternal homocysteine during pregnancy. Obstet Gynecol 101:762–766.

Rai R, Shlebak A, Cohen H, *et al.* (2001) Factor V leiden and acquired activated protein C resistance among 1000 women with recurrent miscarriage. Hum Reprod 16:961–965.

Rodger MA, Betancourt MT, Clark P, *et al.* (2010) The association of factor V leiden and prothrombin gene mutation and placenta-mediated pregnancy complications: a systematic review and meta-analysis of prospective cohort studies. PLoS Med 7:e1000292.

Stephenson MD, Awartani KA, Robinson WP (2002) Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. Hum Reprod 17:446–451.

Strom CM, Ginsberg N, Applebaum M, *et al.* (1992) Analyses of 95 first-trimester spontaneous abortions by chorionic villus sampling and karyotype. J Assist Reprod Genet 9:458–461.

Tranquilli AL, Giannubilo SR, Dell'Uomo B, *et al.* (2004) Adverse pregnancy outcomes are associated with multiple maternal thrombophilic factors. Eur J Obstet Gynecol Reprod Biol 117: 144–147.

Ulvik A, Ueland PM (2001) Single nucleotide polymorphism (SNP) genotyping in unprocessed whole blood and serum by real-time PCR: application to SNPs affecting homocysteine and folate metabolism. Clin Chem 47:2050–2053.

Yenicesu GI, Cetin M, Ozdemir O, *et al.* (2010) A prospective case-control study analyzes 12 thrombophilic gene mutations in Turkish couples with recurrent pregnancy loss. Am J Reprod Immunol 63:126–136.

Yilmaz S, Bayan K, Tuzun Y, *et al.* (2006) A comprehensive analysis of 12 thrombophilic mutations and related parameters in patients with inflammatory bowel disease: data from Turkey. J Thromb Thrombolysis 22:205–212.

Zetterberg H, Coppola A, D'Angelo A, *et al.* (2002) No association between the MTHFR A1298C and transcobalamin C776G genetic polymorphisms and hyperhomocysteinemia in thrombotic disease. Thromb Res 108:127–131.

Address correspondence to:
Dijana Plaseska-Karanfilska, M.D., Ph.D.
Bul. Krste Misirkov 2
1000 Skopje
Republic of Macedonia

E-mail: dijana@manu.edu.mk