ORIGINAL ARTICLE

# Thrombophilic Gene Mutations in Women with Repeated Spontaneous Miscarriage

Mohamed A. Mohamed,[1] Mahasen A. El Moaty,[2] Adel F. El Kholy,[2] Shuzan A. Mohamed,[2] and Amal I. Ali[2]

*Aim:* One of the main problems concerning repeated spontaneous miscarriage (RSM) is the etiological diagnosis. Thrombophilia and its relation to RSM is a matter of debate. In this case–control study, we determined the percentages of three thrombophilic mutations (factor V leiden, prothrombin, and methylenetetrahydrofolate reductase) amongst 20 cases with RSM and 20 control normal parous women. *Results:* There were high statistically significant increases in the number of cases with factor V, prothrombin, and methylenetetrahydrofolate reductase gene mutations compared with normal control and the percentage of multiple gene mutations was higher than single gene mutation. *Conclusion:* The prevalence of thrombophilic mutations is higher in cases of RSM than control.

## Introduction

**R**EPEATED SPONTANEOUS MISCARRIAGE (RSM) is usually defined as the loss of three or more consecutive pregnancies before 20 weeks of pregnancy (Bricker and Farquharson, 2002) and within this definition is a large and heterogeneous group of patients with many different causes of miscarriage. Although several causes of RSM have been established, >50% of cases remain unexplained. This is a challenging dilemma for both patients and physicians (Kovalevsky *et al.*, 2004).

Thrombophilia refers to any persistent hypercoagulable state that is associated with increased risk of thromboembolism. It may be genetically determined, acquired, or both (De-Sweet, 2002).

Thrombophilia, which involves interactions between inherited and acquired risk factors, has been suggested as a possible cause of RSM (Bauduer and Lacombe, 2005). The most common types of hereditary thrombophilia are factor V leiden (FVL), prothrombin (PTH), and methylenetetrahydrofolate reductase (MTHFR) genes mutations, but these are usually undiagnosed because most carriers are asymptomatic (Kovalevsky *et al.*, 2004).

Thrombosis in decidual vessels is believed to be one such cause, leading to intrauterine growth retardation, fetal death, and possibly RSM (Carp *et al.*, 2002).

Thrombophilia has been recently implicated in early pregnancy loss by impairing the initial vascularization process occurring at implantation, which is necessary for successful pregnancy (Azem *et al.*, 2004; Kujovich, 2004).

With the identification of genetic risk factors, there has been synergistic amplification of thrombotic risk when there is an abnormal mutant gene (e.g., FVL) plus an environmental issue (e.g., pregnancy). Current understanding indicates that a combination of risk factors, including multiple inherited thrombophilic defects associated with secondary hypercoagulable states, have a particularly strong association with adverse pregnancy outcome (Kutteh and Triplett, 2006).

The aim of our study was to clarify the association between mutations in thrombophilic factor genes (FVL, PTH, and MTHFR genes) and unexplained RSM.

## Materials and Methods

### Patients

Between January 2008 and June 2009, 20 consecutive women attending the Obstetrics and Gynecology Department, Benha University Hospital, complaining of history of spontaneous repeated miscarriage were recruited to this study. A control group of another 20 parous, age-matched women with no history of miscarriage were recruited from postnatal wards. Exclusion criteria were a previous history of thrombosis, pregnancy at the time of investigation, use of oral contraceptives, and any other known cause of miscarriage, such as abnormal parental karyotype, diabetes mellitus, abnormal thyroid function, abnormal hysterosalpingogram (anatomical abnormalities, intrauterine adhesions, and cervical incompetence), abnormal serum prolactin and plasma progesterone levels, luteal phase defect, toxoplasmosis, antinuclear factor, or antiphospholipid antibodies. All individuals gave informed consent to participate in this study.

### Methods

Venous blood (5 mL) was obtained from each subject and placed immediately into sterile vaccutainer tubes containing

Departments of [1]Obstetrics and Gynecology and [2]Medical Biochemistry, Benha Faculty of Medicine, Benha, Egypt.

EDTA and then the sample was divided into sterile Eppendorff tubes and stored at −80°C until molecular study of the thrombophilic gene mutations.

The detection of mutation in factor V (FV), PTH, and MTHFR genes was based on the reverse-hybridization principle. The assay covered the following three mutations: FV (G1691A), PTH (G20210A), and MTHFR (C677T) (HVD Strip Kit; HVD Life Science).

DNA isolation according to the protocol of Sambrook *et al.* (2001). The extracted DNA concentration was confirmed through measurement in a UV spectrophotometer, and the readings were taken at wavelengths of 260 and 280 nm. The readings at 260 nm allowed calculation of nucleic acid in the sample. An optical density of 1.00 corresponds to ∼50 μg/ mL for double-stranded DNA and 40 μg/mL for single-stranded DNA. The ratio between the readings at 260 and 280 nm ($OD_{260}/OD_{280}$) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have $OD_{260}/OD_{280}$ values of 1.7 and 2.0, respectively. If there is contamination with protein or phenol, the $OD_{260}/OD_{280}$ is significantly <1.7, and if there is contamination with RNA, the $OD_{260}/OD_{280}$ is significantly >2.0 (Haque *et al.*, 2003).

Polymerase chain reaction amplification according to the protocol of Sambrook *et al.* (2001), performed in a single multiplex reaction using biotinylated primers. All polymerase chain reaction reagents and DNA templates were kept refrigerated while all steps were performed until start of the thermal cycling program, using G-storm thermocycler.

Amplification products of FV, PTH, and MTHFR genes were also confirmed by gel electrophoresis (e.g., 3% agarose gel). The bands were suspected to be seen at 173, 202, and 223 bp.

Hybridization (Qublan *et al.*, 2005), performed at 45°C in a thermoshaker plate. In our study, hybridization of amplified products to a test strip that contained allele-specific oligonucleotide probes immobilized as an array of parallel lines was used, instead of using radioactive probes. The bound biotinylated sequences were detected using streptavidin–alkaline phosphatase and color substrates.

Color development (performed at room temperature). One milliliter of conjugate solution was added and incubated for 15 min at room temperature on a shaker. Then, 2 mL wash solution B was added and incubated for 5 min at room temperature on the shaker. After that, 1 mL color developer was added in each lane and incubated for 15 min at room temperature in the dark on the shaker. A purple color appeared upon positive reaction.

Interpretation of results.

- The genotype of a sample was determined using the enclosed collector sheet.
- A positive reaction of the uppermost control line indicated the correct function of conjugate solution and color developer. This line always stained positive.
- For each polymorphic position, one of the following staining patterns was obtained:
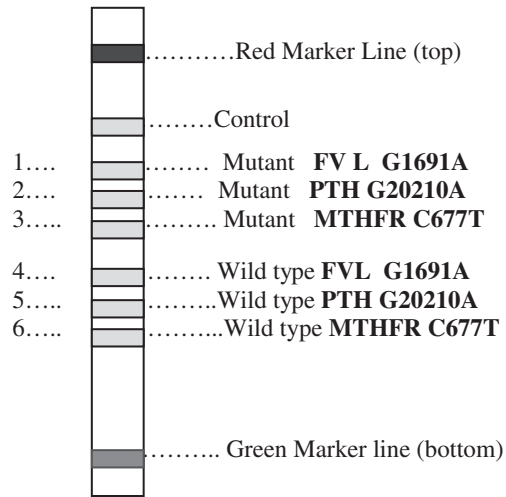


**FIG. 1.** Wild and mutant probes for each of the three genes on test strips.

(1) Wild-type probe only: normal genotype.
(2) Wild-type and mutant probe: heterozygous genotype (carrier person).
(3) Mutant probe only: homozygous mutant genotype (affected person).

Figure 1 shows the wild and mutant probes for each of the three thrombophilic genes on test strip design.

*Statistical analysis*

The results are presented as means ± standard deviation and percentages. Comparisons of categorical variables were made between cases and controls using chi-square test.

The Statistics Package for Social Sciences (SPSS) and Microsoft Office Excel were used for data processing and analysis. Differences will be considered as statistically significant for a *p*-value of <0.05.

**Results**

Our study was performed on 20 women who had RSMs and 20 healthy women as a normal control group for detection of mutation in the three thrombophilic genes, FV, PTH, and MTHFR.

Table 1 contains a description of the clinical data of the RSM cases and the normal control group. These data include maternal age, previous live births, and family history of RSM.

TABLE 1. CLINICAL PROFILE OF REPEATED SPONTANEOUS MISCARRIAGE CASES AND NORMAL CONTROL GROUP

| Parameters | Normal control (n = 20) | RSM cases (n = 20) |
|---|---|---|
| Maternal age (years) | 29.0 ± 4.80 (20–39) | 31.4 ± 6.82 (22–45) |
| Previous live births, *n* (%) | 20/20 (100%) | 5/20 (25%) |
| Family history of RSM, *n* (%) | – | 4 (20%) |

RSM, repeated spontaneous miscarriage.

TABLE 2. PERCENTAGES OF ALLELE FREQUENCIES OF FACTOR V, PROTHROMBIN, AND METHYLENETETRAHYDROFOLATE REDUCTASE GENES MUTATIONS AMONG REPEATED SPONTANEOUS MISCARRIAGE CASES AND NORMAL CONTROL GROUP

| Parameter | | | Normal control (n = 20) | RSM cases (n = 20) | Total (n = 40) | $X^2$ | p |
|---|---|---|---|---|---|---|---|
| FV gene | Normal | No. (%) | 19 (95.0%) | 6 (30.0%) | 25 (62.5%) | 18.03 | <0.001[a] |
| | Mutant | No. (%) | 1 (5.0%) | 14 (70.0%) | 15 (37.5%) | | |
| PTH gene | Normal | No. (%) | 19 (95.0%) | 7 (35.0%) | 26 (65%) | 15.8 | <0.001[a] |
| | Mutant | No. (%) | 1 (5.0%) | 13 (65.0%) | 14 (35%) | | |
| MTHFR gene | Normal | No. (%) | 20 (100.0%) | 6 (30.0%) | 26 (65.0%) | 18.6 | <0.001[a] |
| | Mutant | No. (%) | – | 14 (70.0%) | 14 (35.0%) | | |

[a]$p < 0.001$, high significant.
FV, factor V; PTH, prothrombin; MTHFR, methylenetetrahydrofolate reductase.

Table 2 shows that there are statistically significant increases in the percentages of cases with FV, PTH, and MTHFR gene mutations compared with the normal control group (70%, 65%, and 70%, respectively; $p < 0.001$ for all).

Table 3 shows that there are statistically significant increases in the percentages of cases with heterozygous and homozygous mutations in FV gene (heterozygous 60% and homozygous 10%), PTH gene (heterozygous 35% and homozygous 30%), and MTHFR gene (heterozygous 45% and homozygous 25%), when compared with normal controls ($p < 0.001$ for all).

Table 4 shows that there are high statistically significant increases in the percentages of cases with single (20%) and multiple gene mutations (65%) when compared with normal controls ($p < 0.001$ for all).

## Discussion

Although several medical causes have been established, up to 50% of cases of RSM remain unexplained after standard gynecological, hormonal, and karyotypic investigations (Lissalde-Lavigne et al., 2005). Acquired and inherited thrombophilia factors are possible causes of RSM (D'Uva et al., 2008).

Single-nucleotide polymorphisms of the genes coding for coagulation factors are the causes of congenital thrombophilia, which might lead to early RSM and fetal loss in advanced pregnancy. The most frequent reasons of thrombophilia are gene mutations in FVL (G1691 A), PTH (G20210A), and 5, 10-MTHFR (C677T) (Agnieszka et al., 2008).

Our data showed significant increases of allele frequency as well as homozygosity and heterozygosity of FVL gene in women with unexplained RSMs when compared with normal controls. Many reports in the literature (Foka et al., 2000; Zammiti et al., 2006; Ivanov et al., 2009) have been in agreement with our study. On the other hand, Murphy et al. (2000) and Jivraj et al. (2006) showed a nonsignificant difference in FVL allele frequency between women with unexplained RSM and controls. Our finding may be explained by Dizon-Townson et al. (1997), who reported that microthrombi in the placental vasculature of future pregnancies were found to be a common finding among women with RSM. Also, they found that fetal carriage of FVL was associated with histologically proven placental infarction. Moreover, Burton et al. (1999) suggested that, before 8 weeks gestation, the maternal arterial connections with the intervillous space are restricted to tortuous networks, following which direct channels of communication are established, which are initially of small caliber but later become delineated by the end of first trimester. This suggests that thrombosis of the placental vasculature may lead to first-trimester RSM.

After that, Kujovich (2004) reported that mutation in the FV gene increases thrombin generation, with a four- to eightfold increased risk of thrombosis in heterozygous mutation and an 80-fold increased risk in homozygous mutation, which leads to vascular placental insufficiency that has been suggested as a potential cause of early RSM.

Another possible prothrombotic risk factor is PTH mutation. In the case of PTH, our study showed a significant increase in allele frequency as well as homozygosity and

TABLE 3. PERCENTAGES OF GENOTYPE FREQUENCIES OF FACTOR V, PROTHROMBIN, AND METHYLENETETRAHYDROFOLATE REDUCTASE GENE MUTATIONS AMONG REPEATED SPONTANEOUS MISCARRIAGE CASES AND NORMAL CONTROL GROUP

| Parameter | | Normal control (n = 20) % | RSM cases (n = 20) % | Total (n = 40) % | $X^2$ | p |
|---|---|---|---|---|---|---|
| Genotype of FV | Normal (−/−) | 19 (95.0%) | 6 (30.0%) | 25 (62.5%) | 18.1 | <0.001[a] |
| | Heterozygous (+/−) | 1 (5.0%) | 12 (60.0%) | 13 (32.5%) | | |
| | Homozygous (+/+) | – | 2 (10.0%) | 2 (5.0%) | | |
| Genotype of PTH | Normal (−/−) | 19 (95.0%) | 7 (35.0%) | 26 (65.0%) | 16.1 | <0.001[a] |
| | Heterozygous (+/−) | – | 7 (35.0%) | 7 (17.5%) | | |
| | Homozygous (+/+) | 1 (5.0%) | 6 (30.0%) | 7 (17.5%) | | |
| Genotype of MTHFR | Normal (−/−) | 20 (100.0%) | 6 (30.0%) | 26 (65.0%) | 21.5 | <0.001[a] |
| | Heterozygous (+/−) | – | 9 (45.0%) | 9 (22.5%) | | |
| | Homozygous (+/+) | – | 5 (25.0%) | 5 (12.5%) | | |

[a]$p < 0.001$, highly significant.

TABLE 4. PERCENTAGES OF SINGLE AND MULTIPLE GENE MUTATIONS AMONG REPEATED SPONTANEOUS
MISCARRIAGE CASES AND NORMAL CONTROL GROUP

| Parameter | | Normal control (n = 20) | RSM cases (n = 20) | Total (n = 40) | $X^2$ | p |
|---|---|---|---|---|---|---|
| No gene mutation (normal gene) | No. (%) | 18 (90%) | 3 (15.0%) | 21 (52.5%) | 26.4 | <0.001[a] |
| Single gene mutation | No. (%) | 2 (10%) | 4 (20.0%) | 6 (15.0%) | | |
| Multiple gene mutations | No. (%) | – | 13 (65.0%) | 13 (32.5%) | | |

[a]$p < 0.001$, highly significant.

heterozygosity of PTH gene mutation in women with unexplained RSM when compared with normal controls. Our results concurred with those of Foka et al. (2000), Pihusch et al. (2001), and Ivanov et al. (2009). Our results may be explained by the fact that the PTH G20210A mutation was associated with a higher PTH clotting activity and a two- to sevenfold increase in risk of venous thrombosis, which may lead to miscarriage (Ercan et al., 2008).

In the study on MTHFR, our results showed a significant increase of allele frequency as well as homozygosity and heterozygosity of the MTHFR gene in women with unexplained RSM when compared with normal controls.

These findings were in agreement with those of Nelen et al. (2000), Ogasawara et al. (2000), and Unfried et al. (2002). The finding regarding MTHER may be explained by Berry et al. (1995), who mentioned that MTHFR mutation homozygosity might predispose to a thermolabile variant of the MTHFR enzyme, with 50% of normal activity, which in turn explains hyperhomocysteinemia, which may lead to structural and neurological effects on the fetus, causing fetal loss.

In the case of multiple gene mutations, our results showed a significantly increased prevalence of multiple thrombophilic gene mutations (of FVL, PTH, and MTHFR C677T) in women with RSM compared with the normal control group.

The concept of multiple gene mutations as a risk factor for RSM has been supported by many authors (Coulam et al., 2006; D'Uva et al., 2008; Vora et al., 2008), but disagreed by others (Carp et al., 2002; Jaslow et al., 2010).

The role of multiple gene mutations may be explained by uncontrolled and enhanced coagulation in the intervillous spaces of the placenta, which may induce fibrin deposition within the fetal circulation that could lead to fetal stem vessel thrombosis, placental infarction, and miscarriage (Lissalde-Lavigne et al., 2005).

Moreover, as several factors interact to create thrombosis, the action of thrombophilic factors during placentation may become amplified, thereby giving rise to modifications in placental circulation and subsequent RSM. The bottom line for the study is that multiple thrombophilic gene mutations and/or single gene mutations are risk factors for recurrent miscarriage.

These results emphasize the role of thrombophilia as a cause of RSM, which remains a common complication of pregnancy, in order to establish a possible protocol that supports the etiological diagnosis of RSM associated with thromboprophylaxis of these patients.

However, prospective longitudinal study of acquired thrombophilia is recommended. Study of other congenital thrombophilic factors is also recommended.

## Disclosure Statement

## References

Agnieszka SM, Krzysztof D, Piotr P, et al. (2008) [Inherited thrombophilia in women with recurrent miscarriages and pregnancy loss in anamnesis—own experience]. Ginekol Pol 79:630–634.

Azem F, Many A, Yovel I, et al. (2004) Increased rates of thrombophilia in women with repeated IVF failure. Hum Reprod 19:368–370.

Bauduer F, Lacombe D (2005) Factor V Leiden, prothrombin 20210A, methylenetetrahydrofolate reductase C677T, and population genetics. Mol Genet Metab 86:91–99.

Berry CW, Bramabati B, Eskes TKAB, et al. (1995) The Euro-Team Early Pregnancy (ETEP) protocol for recurrent miscarriage. Hum Reprod 10:1516–1520.

Bricker L, Farquharson RG (2002) Types of pregnancy loss in recurrent miscarriage: implications for research and clinical practice. Hum Reprod 17:1345–1350.

Burton GJ, Jauniaux E, Watson AL (1999) Maternal arterial connection to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. Am J Obstet Gynecol 181:718–724.

Carp H, Dolizky M, Tur-Kaspa I, et al. (2002) Hereditary thrombophilias are not associated with a decreased live birth rate in women with recurrent miscarriage. Fertil Steril 78:58–62.

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.

De-Sweet M (2002) Thromboembolism. In: De-Sweet M, Medical Disorders in Obstetric Practice, 4th edition. Blackwell Science, UK, pp 100–124.

Dizon-Townson DS, Meline L, Nelson LM, et al. (1997) Fetal carriers of the factor V Leiden mutation are prone to miscarriage and placental infarction. Am J Obstet Gynecol 177:402–405.

D'Uva M, Di-Micco P, Strina I, et al. (2008) Etiology of hypercoagulable state in women with recurrent fetal loss without other causes of miscarriage from Southern Italy: new clinical target for antithrombotic therapy. Biologics 2:897–902.

Ercan B, Tamer L, Sucu N, et al. (2008) Factor V Leiden and prothrombin G20210A Gene Polymorphism in patients with Coronary Artery Disease. Yonsei Med J 49:237–243.

Foka ZJ, Lambropoulos AF, Saravelos H, et al. (2000) Factor V Leiden and prothrombin G20210A mutations, but not methylenetetrahydrofolate reductase C677T, are associated with recurrent miscarriages. Hum Reprod 15:458–462.

Haque HA, Pfeiffer RM, Beerman MP, et al. (2003) Performance of high throughput DNA quantification methods. BMC Biotechnol 3:20–24.

Ivanov PD, Komsa-Penkova RS, Konova EI, *et al*. (2009) Association of inherited thrombophilia with embryonic and post-embryonic recurrent pregnancy loss. Blood Coagul Fibrinolysis 20:134–140.

Jaslow CR, Carney JL, Kutteh WH (2010) Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril 3:1234–1243.

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

Kovalevsky G, Clarisa R, Gracia CR, *et al*. (2004) Evaluation of the association between hereditary thrombophilias and recurrent pregnancy loss. Arch Intern Med 164:558–563.

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

Kutteh WH, Triplett DA (2006) Thrombophilias and recurrent pregnancy loss. Semin Reprod Med 24:54–66.

Lissalde-Lavigne G, Cochery-Nouvellon E, Mercier E, *et al.* (2005) The association between hereditary thrombophilias and pregnancy loss. Haematologica 90:1223–1230.

Murphy RP, Donoghue C, Nallen RJ, *et al*. (2000) Prospective evaluation of the risk conferred by factor V Leiden and thermolabile methylenetetrahydrofolate reductase polymorphisms in pregnancy. Arterioscler Thromb Vasc Biol 20:266–270.

Nelen WLDM, Blom HK, Steegers EAP, *et al*. (2000) Hyperhomocysteinemia and recurrent early pregnancy loss: a meta-analysis. Fertil Steril 74:1196–1199.

Ogasawara M, Aoki K, Okada S, *et al*. (2000) Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril 73:300–304.

Pihusch R, Buchholz T, Lohse P, *et al*. (2001) Thrombophilic gene mutations and recurrent spontaneous abortion: prothrombin mutation increases the risk in the first trimester. Am J Reprod Immunol 46:124–131.

Qublan Hs, Malkawi HY, Tahat YA, *et al*. (2005) *In-vitro* fertilization treatment: factors affecting its results and outcome. J Obstet Gynecol 25:689–693.

Sambrook J, Fritsch EF, Maniatt S (2001) Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Unfried G, Griesmacher A, Weismuller W, *et al*. (2002) The C677T polymorphism of the methylene-tetrahydrofolate reductase gene and idiopathic recurrent miscarriage. Obstet Gynecol 99:614–619.

Vora S, Shetty S, Ghosh K (2008) Thrombophilic dimension of recurrent fetal loss in Indian patients. Blood Coagul Fibrinolysis 19:581–584.

Zammiti W, Mtiraoui N, Mercier E, *et al*. (2006) Association of factor V gene polymorphisms (Leiden; Cambridge; Hong Kong and HR2 haplotype) with recurrent idiopathic pregnancy loss in Tunisia. A case–control study. Thromb Haemostasis 95:612–617.

Address correspondence to:
*Mohamed A. Mohamed, M.D.*
*Departments of Obstetrics and Gynecology*
*Benha Faculty of Medicine*
*Benha 8*
*Egypt*

*E-mail:* mahamadsalam@hotmail.com