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Genetic thrombophilic mutations among couples with recurrent miscarriage

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BACKGROUND: Some cases of recurrent first trimester miscarriage (RM)—the loss of three or more consecutive pregnancies at <12 weeks' gestation—have a thrombotic aetiology. METHODS: We determined (i) the prevalence of three thrombophilic mutations [factor V Leiden (FVL), prothrombin G20210A (PTG) and methylenetetrahydrofolate reductase (MTHFR) C677T] amongst 357 Caucasian couples with RM and 68 parous Caucasian couples with no history of miscarriage and (ii) the prospective outcome of untreated pregnancies amongst couples with RM in which either partner carried a thrombophilic mutation. RESULTS: The allele frequencies of FVL (2%), PTG (2%) and MTHFR C677T (31%) were similar between cases and controls. The prevalence of multiple thrombophilic mutations (greater than one mutation) was also similar between cases and controls. Amongst couples in whom either partner carried greater than one thrombophilic allele, the relative risk of miscarriage in a future untreated pregnancy was 1.9 (95% confidence interval, 1.3–2.8) compared with those couples who carried no thrombophilic mutation. CONCLUSION: The prevalence of thrombophilic mutations is similar in couples with RM and parous controls. In couples with RM, multiple genetic thrombophilic mutations in either partner significantly increases the risk of miscarriage in a subsequent pregnancy.

Key words: factor V Leiden/first trimester miscarriage/methylenetetrahydrofolate reductase C677T/prothrombin G20210A/thrombophilic mutation

Introduction

Pregnancy is a hypercoagulable state secondary to (i) an increase in coagulation factors, (ii) a reduction in naturally occurring anticoagulants and (iii) an impairment of fibrinolysis (Stirling *et al*., 1984; Clark *et al*., 1998). The evolutionary advantage of these changes is thought to be stabilization of haemochorial placentation and reduction in post-partum blood loss.

Some cases of recurrent first trimester miscarriage (RM)—the loss of three or more consecutive pregnancies at less than 12 weeks' gestation—may have a thrombotic aetiology. The evidence for this comes from histological studies reporting microthrombi to be a common finding in the placental vasculature of pregnancies amongst women with RM (Rushton, 1988; Out *et al*., 1991; Rai *et al*., 1996) and studies reporting an increased prevalence of thrombophilic mutations [factor V Leiden (FVL), prothrombin G20210A (PTG) and methylenetetrahydrofolate reductase (MTHFR) C677T] amongst women with RM (Rey *et al*., 2003). In addition, antiphospholipid antibodies (lupus anticoagulant and anticardiolipin antibodies), an acquired thrombophilic defect, are now recognized as an important treatable cause of RM (Kutteh, 1996; Rai *et al*., 1997).

We have reported that whilst the prevalence of FVL is similar amongst those with RM compared with controls, women with RM who carry the FVL mutation have a significantly higher miscarriage rate in future untreated pregnancies compared with those who have a normal factor V genotype (Rai *et al*., 2002). It has been reported that the fetal thrombophilia genotype, which will of course be determined by the parental thrombophilia genotype, is important in governing pregnancy outcome (Dizon-Townson *et al*., 1997). We therefore determined (i) the prevalence of the three thrombophilia mutations in both partners of couples with RM and (ii) the prospective outcome of untreated pregnancies amongst couples in whom either partner carried a thrombophilia mutation.

Subjects and methods

This study was approved by the local research ethics committee of St Mary's Hospital NHS Trust, London.

Thrombophilic mutation prevalence study

Subjects

Between October 2000 and February 2003, 357 consecutive Caucasian couples attending the Recurrent Miscarriage Clinic at St Mary's Hospital, London, were recruited to this study. A control group of 68 parous, age-matched Caucasian couples with no history of miscarriage were recruited from post-natal wards. No participant in this study had a personal or family history of venous thromboembolic disease. The demographic details and outcomes of previous pregnancies of these women are summarized in Table I. All individuals gave signed informed consent to participate in this study.

All couples with RM were investigated according to our protocol (Rai *et al*., 2001). In particular, all women were screened for antiphospholipid antibodies and both partners had peripheral blood karyotyping performed. Genetic testing for three thrombophilic mutations—FVL, PTG and MTHFR C677T—was performed on genomic DNA extracted from leukocytes from whole blood, using a standard Qiagen kit.

Factor V Leiden

PCR using known primers was used to amplify exon 10 of the *factor V* gene, which contains the mutation G→A at nucleotide position 1691 (Bertina *et al*., 1994). The amplified segment of DNA was then digested overnight using the enzyme *Mnl*I (New England BioLabs) at 37°C. Digested segments of DNA were separated by electrophoresis in a 1.8% agarose gel stained with ethidium bromide at a potential difference of 100 V. Digested bands of DNA were examined under UV light. PCR amplification using specific primers generates a 267-bp fragment spanning the mutation site. The G1691A mutation on the *factor V* gene destroys a *Mnl*I cleavage site. Digestion of the amplified fragment with *Mnl*I generates three fragments—37, 67 and 163 bp in length—in the presence of normal factor V genotype. In the presence of the affected allele, digestion with *Mnl*I generates two fragments—67 and 200 bp in length. A heterozygous sample would therefore generate four bands—37, 67, 163 and 200 bp in length—and a homozygous sample would generate only two bands—67 and 200 bp in length.

Prothrombin G20210A

PCR using specific primers was used to amplify a 345-bp segment, followed by overnight digestion with the restriction endonuclease *Hind*III at 37°C, followed by gel electrophoresis as described above (Poort *et al*., 1996). The G20210A mutation in the 3′ untranslated region of the prothrombin gene does not disrupt a natural recognition site for any restriction endonuclease. The primers used are designed to introduce a *Hind*III cleavage site only if the mutant allele is present. Amplification by PCR followed by subsequent digestion with *Hind*III yields one fragment of 345 bp in a normal individual, two fragments of 322 and 23 bp in a homozygous individual and three fragments of 345, 322 and 23 bp in a heterozygous individual.

Table I. Demographic details of the case–control prevalence study population

	Recurrent early miscarriages (N = 357)	Controls (N = 68)
Age (years) [median (range)]	34 (19–46)	33 (18–42)
Number of previous miscarriages [median (range)]	3 (3–17)	0
Number with previous live birth (%)	135 (38)	68 (100)

Methylenetetrahydrofolate reductase C677T

PCR using specific primers was used to amplify a 198-bp segment, followed by overnight digestion with the restriction endonuclease *Hinf*I at 37°C, followed by gel electrophoresis as described above (Frosst *et al*., 1995). The MTHFR C677T mutation creates a recognition site for the *Hinf*I restriction endonuclease. Amplification of the *MTHFR* gene using specific primers generates a 198-bp PCR product. Subsequent digestion with *Hinf*I will generate two fragments of 175 and 23 bp in length in the presence of an affected allele and in a homozygous individual. A heterozygous individual will give three fragments—198, 175 and 23 bp in length.

Prospective pregnancy study

Study population

The outcome of the next pregnancy of those participating in the prevalence study was determined. No woman took any medication during pregnancy apart from folic acid (400 µg/day) as prophylaxis against neural tube defects. Women with antiphospholipid syndrome were excluded, as were those who took aspirin (Figure 1). Both the patients and clinicians were blinded to the results of thrombophilic mutation status.

Women were followed from the time of a positive urine pregnancy test and had fortnightly serial pelvic ultrasound scans at our clinic until 12 weeks' gestation. After this time, the remainder of their antenatal care was provided at St Mary's Hospital.

Statistical analysis

Statistical analysis was performed using SPSS version 12 (SPSS, Chicago, IL, USA). Discrete variables were analysed using Fisher's

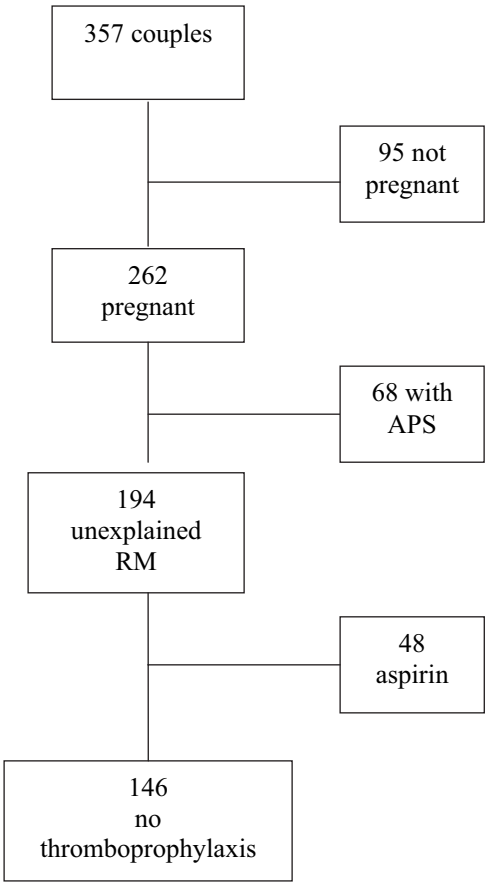

Figure 1. Flow chart of couples recruited and subsequent follow-up. APS, antiphospholipid syndrome.

exact test or the χ^2 test, and continuous variables were analysed using the Mann–Whitney *U*-test. *P*-values less than 0.05 were taken as statistically significant. Relative Risk and 95% confidence intervals were calculated where appropriate.

Results

The frequencies of FVL, PTG and MTHFR C677T alleles were similar between both individuals and couples with RM compared with controls (Table II). The allele frequencies for all three mutations were similar to those previously reported (Holmes *et al*., 1999; Pickering *et al*., 2001; Rai *et al*., 2001; Alonso *et al*., 2002; Carp *et al*., 2002b; Pauer *et al*., 2003). The prevalence of multiple thrombophilic mutations (greater than one mutation in the same individual) was also similar among (i) individuals with RM and controls and (ii) couples with RM and controls (Table III).

The live birth rate in the next pregnancy in which no thromboprophylaxis was administered was significantly lower amongst couples in whom either partner carried multiple (greater than one) mutations [2 of 12 (17%)] compared with couples in whom neither partner carried a mutation [28 of 50 (56%); $P < 0.03$] (Table IV). A breakdown of thrombophilic genotypes for each partner with multiple mutations, together with gestational ages at which the next pregnancy ended, is summarized in Table V. Women in the group with multiple mutations were of similar age (median, 34 years; range, 25–46) and had a similar number of previous miscarriages (median, 3; range, 3–5) compared with women in the group with no mutations (median age, 33 years; range, 25–46; $P = 0.15$; median number of previous miscarriages, 3; range 3–5; $P = 0.73$). Of note, 50% of miscarriages occurred at ≥8 weeks' gestation. This is higher than the 2% that has been previously reported (Brigham *et al*., 1999).

The live birth rate amongst women (i) with no thrombophilic mutations was 56% (28 of 50) and (ii) with one thrombophilic mutation (FVL, PTG or homozygous MTHFR) 55% (11 of 20) and (iii) amongst those with greater than one mutation 25% (1 of 4).

The relative contribution of the paternal as compared with the maternal genotype in determining pregnancy is summarized in Tables IV and V. Of note, the miscarriage rate amongst couples in whom the male partner carried greater than 1 thrombophilic mutation was 87.5% (7 of 8) versus 75% (3 of 4) when the female partner carried greater than one thrombophilic mutation.

Discussion

This is the first study to report the prevalence of three thrombophilic mutations (FVL, PTG and MTHFR) in both the female and male partners of couples with RM. The allele frequencies of the three thrombophilic mutations examined were similar in couples with RM compared with parous controls with no history of miscarriage. However, the prospective pregnancy study reported that pregnancies amongst couples in whom either partner carried more than one thrombophilic mutation were at significantly increased risk of future miscarriage compared with couples in whom neither partner carried a mutation.

The discordance between the prevalence data and the pregnancy outcome data clearly suggests that there is an additional factor other than parental thrombophilic mutation status that determines pregnancy outcome. In this respect, we are currently investigating the role of the fetal thrombophilia genotype.

This study has certain potential limitations. Whilst it should be acknowledged that heterozygosity for the MTHFR mutation is not thought to be an independent risk factor for systemic venous thrombosis, we have included those heterozygous for

Table II. Allele frequencies of factor V Leiden (FVL), prothrombin G20210A (PTG) and methylenetetrahydrofolate reductase (MTHFR) C677T

	Allele frequency (%)						
	Recurrent miscarriage			Controls			Significance
	Female (*n* = 714)	Male (*n* = 714)	Couples (*n* = 1428)	Female (*n* = 136)	Male (*n* = 136)	Couples (*n* = 272)	
FVL	2	2	2	2	2	2	NS
PTG	2	2	2	4	2	3	NS
MTHFR	32	30	31	32	37	35	NS

Couples, either female or male partner carries mutation; NS, not significant.

Table III. Results showing the prevalence of multiple (greater than one) thrombophilic defects within the same individual

	Prevalence (%)						
	Recurrent miscarriage			Controls			Significance
	Female (*n* = 357)	Male (*n* = 357)	Couples (*n* = 357)	Female (*n* = 68)	Male (*n* = 68)	Couples (*n* = 68)	
Multiple defects	4	3	7	6	4	10	NS

Couples, either female or male partner carries greater than one thrombophilic mutation; NS, not significant.

Table IV. Pregnancy outcome in couples with mutations

Mutation	Live birth rate (%)	*P*-value	RR	95% CI
No mutation in either partner	56 (28/50)		1	
Multiple mutations in either partner	17 (2/12)	0.02	1.9	1.3–2.8
FVL in either partner	25 (2/8)	0.14	1.7	1.0–2.8
FVL in female	0 (0/1)	0.89	1.7	0.7–4.0
FVL in male	29 (2/7)	0.34	1.6	0.9–2.9
PTG in either partner	50 (3/6)	0.88	1.1	0.5–2.7
PTG in female	50 (1/2)	0.58	1.1	0.3–4.7
PTG in male	50 (2/4)	0.77	1.1	0.4–3.2
MTHFR (HO) in either partner	50 (18/36)	0.66	1.1	0.7–1.8
MTHFR (HO) in both partners	50 (1/2)	1	1.1	0.3–4.7
MTHFR (HE or HO) in both partners	55 (21/38)	1	1.0	0.6–1.6
MTHFR (HO) in female	59 (10/17)	1	0.9	0.5–1.8
MTHFR (HE) in female	54 (27/50)	1	1.0	0.7–1.6
MTHFR (HO) in male	42 (8/19)	0.42	1.3	0.8–2.2
MTHFR (HE) in male	60 (28/47)	0.84	0.9	0.6–1.5

FVL, factor V Leiden; PTG, prothrombin G20210A; MTHFR, methylenetetrahydrofolate reductase; HO, homozygous state; HE, heterozygous state; RR, relative risk of a miscarriage in a subsequent pregnancy; 95% CI, 95% confidence intervals.
Live birth rate in a prospective untreated pregnancy when mutation present in either partner or both partners compared with couples without mutations.

Table V. Genetic make-up of couples affected with multiple (greater than one) mutations and the gestational age at which the pregnancy ended

Maternal genotype			Paternal genotype			Gestation at which pregnancy ended (weeks)
FVL	PTG	MTHFR	FVL	PTG	MTHFR	
–/–	–/–	–/+	–/–	–/+	–/+	5
–/–	–/–	–/+	–/+	–/+	–/+	5
–/–	–/+	–/+	–/–	–/–	–/+	5
–/–	–/–	–/–	–/+	–/–	–/+	5
–/–	–/–	+/+	–/+	–/–	–/+	6
–/–	–/–	+/+	–/+	–/–	+/+	8
–/–	–/–	–/+	–/+	–/–	+/+	8
–/+	–/–	–/+	–/–	–/–	–/+	9
–/–	–/+	–/+	–/–	–/–	–/+	12
–/–	–/–	+/+	–/+	–/–	–/+	12
–/–	–/–	–/+	–/+	–/–	+/+	40
–/–	–/+	+/+	–/–	–/–	–/–	41

–/–, wild type; –/+, heterozygous; +/+, homozygous; FVL, factor V Leiden; MTHFR, methylenetetrahydrofolate reductase C677T; PTG, prothrombin G20210A.

this mutation as we are examining (i) the allele frequencies of the three thrombophilic mutations and (ii) the contribution of the MTHFR allele in combination with either the FVL allele or the PTG allele to pregnancy outcome. Folic acid supplementation could also lower plasma homocysteine levels, which are elevated amongst homozygous carriers of MTHFR, which would potentially reduce the likelihood of this being an important risk factor for miscarriage. However, on direct questioning, all women in this study took only a low dose of folic acid (400 μg) as prophylaxis against neural tube defects.

The placenta has a dual blood supply—one maternal and one fetal. The maternal blood, originating from uterine spiral arteries, circulates in the intervillous space, coming into contact with the syncytiotrophoblast and subsequently draining back through decidual veins. The fetal blood supply within the placenta takes origin from the umbilical arteries. Blood circulates in the fetal villi within stem vessels and subsequently drains back via the umbilical vein. A hypercoagulable state within the fetal circulation could lead to fetal stem vessel thrombosis, placental infarction in the distribution of fetal vessels and subsequent miscarriage. In support of this, fetal carriage of FVL has been reported in association with histologically proven placental infarction (Dizon-Townson *et al.*, 1997).

This large prevalence study addresses many of the deficiencies in the existing data on the prevalence of genetic thrombophilias amongst women with RM. Published studies have been plagued by (i) the small size of individual studies leading to a lack of power (Wramsby *et al.*, 2000; Younis *et al.*, 2000; Carp *et al.*, 2002b; Finan *et al.*, 2002; Rey *et al.*, 2003), (ii) stratification bias in which there is an underestimation of the prevalence amongst controls and a spurious increase in prevalence amongst cases and (iii) admixture bias in which there has been poor matching of cases and controls because of racial heterogeneity (Carp *et al.*, 2002b).

Data reporting the prospective outcome of untreated pregnancies amongst women with RM and a genetic thrombophilia are sparse. There are two prospective studies of the pregnancy outcome of women with RM in whom a genetic thrombophilic defect has been identified (Carp *et al.*, 2002a; Rai *et al.*, 2002). The study from our unit (Rai *et al.*, 2002) reported a significantly lower live birth rate (38%) amongst 16 women who carried the FVL mutation compared with those who have a normal factor V genotype (69%). The second study reported the outcome of one patient with FVL (miscarried), three with the prothrombin gene mutation (all live births) and 16 homozygous for the MTHFR mutation (25% live birth rate).

Despite the lack of prospective randomized placebo-controlled studies, women with RM who carry a thrombophilic defect are being offered thromboprophylaxis with heparin during pregnancy. Heparin does not cross the placenta and would therefore not have an anticoagulant effect on the fetal side of the placental vasculature. As this study demonstrates that the paternal thrombophilic genotype and, by inference, the fetal genotype contribute to determining pregnancy outcome, the role of thromboprophylactic agents that cross the placenta needs to be investigated. Of these agents, warfarin is thought to be teratogenic in the first trimester (Ageno *et al.*, 2004), but aspirin is not associated with fetal development abnormalities (Kozer *et al.*, 2003).

In conclusion, our study demonstrates that the prevalence of multiple thrombophilic mutations is similar in couples with recurrent miscarriage and parous controls. In couples with recurrent early miscarriage, multiple genetic thrombophilic mutations in either partner significantly increase the risk of miscarriage in a subsequent pregnancy.

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