A polymorphism in the *MTHFD1* gene increases a mother's risk of having an unexplained second trimester pregnancy loss

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Low maternal folate or vitamin B_{12} status has been implicated in numerous pregnancy complications including spontaneous abortion. The primary aim of this study was to test a polymorphism within the trifunctional folate enzyme MTHFD1 (5,10-methylene-tetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, 10-formyltetrahydrofolate synthetase) for an association with a mother's risk of having an unexplained second trimester pregnancy loss. We genotyped 125 women who had at least one unexplained spontaneous abortion or intrauterine fetal death between 13 and 26 weeks gestation and 625 control women with no history of prior pregnancy loss. Our study is the first to identify an association between the MTHFD1 $1958G \rightarrow A$ (R653Q) polymorphism and the maternal risk of having an unexplained second trimester pregnancy loss. Women who are MTHFD1 1958AA homozygous have a 1.64-fold increased risk of having an unexplained second trimester loss compared to women who are MTHFD1 1958AG or 1958GG [OR 1.64 (1.05–2.57), $P = 0.03$]. It has been reported that polymorphisms in 5,10-methylenetetrahydrofolate reductase (MTHFR), $677C \rightarrow T$ (A222V), transcobalamin II (TCII), $776C \rightarrow G$ (P259R), are associated with pregnancy loss. Both variants were tested in this study. Neither showed evidence of significantly affecting the maternal risk of having a second trimester pregnancy loss. In conclusion, the MTHFD1 1958AA genotype may be an important maternal risk factor to consider during pregnancy.

Key words: abortion/fetal death/second trimester/spontaneous/unexplained

Introduction

A substantial proportion (15–50%) of second trimester pregnancy losses remain unexplained (Gaillard *et al*., 1993; Drakeley *et al*., 1998; Incerpi *et al*., 1998; Faye-Peterson *et al*., 1999). Although placental insufficiency is a common finding in these cases (Faye-Petersen *et al*., 1999), its etiology is often unknown.

Maternal hyperhomocysteinemia has been associated with early pregnancy loss and a number of other adverse pregnancy outcomes associated with placental insufficiency, such as intrauterine growth restriction, preeclampsia, abruptio placentae and fetal death (Ray and Laskin, 1999; Nelen *et al*., 2000a,b; Cotter *et al*., 2001; De la Calle *et al*., 2003), although not in all studies (Coumans *et al*., 1999; Hogg *et al*., 2000; Alfirevic *et al*., 2001; Hietala *et al*., 2001). Reduced dietary intake of B vitamins such as folic acid, B_6 and B_{12} often leads to elevated plasma homocysteine levels (Refsum *et al*., 2004), prompting investigation of genetic variants involved in folate and vitamin B_{12} metabolism as potential genetic risk factors for pregnancy-related complications.

We have recently showed that a polymorphism [$1958G \rightarrow A$ (R653Q, dbSNP rs2236225] within the gene encoding the trifunctional enzyme MTHFD1 (5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, 10-formyltetrahydrofolate synthetase) is a maternal risk for severe placental abruption (Parle-McDermott *et al*., 2005), a maternal risk for having a pregnancy affected by a neural tube defect (NTD) and is possibly involved in fetal inviability (Brody *et al*., 2002). This enzyme plays a central role in folate metabolism and provides carbon-1 units for DNA synthesis (Hum *et al*., 1988). Homozygosity for the $677C \rightarrow T$ (A222V, dbSNP rs1801133) variant of 5,10-methylenetetrahydrofolate reductase (MTHFR), known to be associated with elevated homocysteine levels, has been implicated as both a maternal genetic risk for recurrent pregnancy loss (Nelen *et al*., 1997; Lissak *et al*., 1999; Unfried *et al*., 2002; Kumar *et al*., 2003) and a fetal genetic

risk for spontaneous abortion (Isotalo *et al.*, 2000; Zetterberg *et al.*, 2002a). However, some studies failed to find the 677TT genotype as a risk factor for recurrent pregnancy loss (reviewed in Zetterberg, 2004). The transcobalamin II (TCII) 776C→G (P259R, dbSNP rs1801198) variant has been reported to confer an increased fetal genetic risk of early spontaneous abortion (Zetterberg *et al.*, 2002b) and influence levels of circulating vitamin B_{12} bound to TCII (Afman *et al.*, 2002; Miller *et al.*, 2002). It has also been suggested that the TCII 776C→G polymorphism interacts with the MTHFR 677TT genotype to confer an even higher fetal genetic risk of spontaneous abortion than either polymorphism separately (Zetterberg *et al.*, 2003).

We considered the MTHFD1 1958G→A polymorphism as a prime candidate for association with maternal risk of second trimester pregnancy loss based on the association of low folate and hyperhomocysteinemia with early pregnancy loss and with disorders of placental vascularity and the maternal association of this polymorphism with increased risk of other pregnancy complications. We also investigated MTHFR 677C→T and TCII 667C→G polymorphisms as candidates for second trimester loss based on their prior reported association with pregnancy loss.

Subjects and methods

Subjects

Cases and controls were drawn from a bank of blood samples of 56,049 pregnant women. Samples were obtained during their first clinical visit at the three main Dublin maternity hospitals between 1986 and 1990. These hospitals deliver approximately 90% of births within the Dublin area (Kirke *et al.*, 1993). This bank of samples is representative of a homogeneous population, and due to the low level of immigration into Ireland during the collection period, population stratification is unlikely to confound our genetic analyses. Women with a history of at least one unexplained second trimester pregnancy loss ($n = 125$) during a previous pregnancy were identified retrospectively from the computerized records of the Coombe Women's Hospital. Individual chart reviews were then performed to confirm the details of each case. Cases were women with a previous history of spontaneous abortion or *in utero* fetal demise occurring spontaneously between 13 and 26 weeks gestation. Women in whom a clinical explanation for the spontaneous abortion or fetal death was apparent were excluded. Thus, women with incompetent cervix, preterm premature rupture of membranes, preterm labor, placental abruption, maternal medical disease or fetal malformations were not included. The control group ($n = 625$) consisted of a random sample of women from the same bank. Data on parity and maternal age when the blood sample was collected was available for all cases except one and for 118/625 of the controls. All links to personal identifiers were removed from the samples before genetic testing. Appropriate ethical approval was obtained for all samples collected.

Genotyping methods

Genomic DNA was extracted from cases and controls using the QIAamp DNA Blood Mini Kit from Qiagen, West Sussex, UK. Genotyping of the MTHFR 677C→T and MTHFD1 1958G→A polymorphisms was performed using restriction fragment length polymorphism (PCR–RFLP) using *Hinf*I and *Msp*I, respectively, as previously described (Frosst *et al.*, 1995; Hol *et al.*, 1998; Brody *et al.*, 2002). The TCII 776C→G polymorphism was genotyped using an allele-specific primer extension assay and scored by matrix-assisted laser desorption/ionization-time of flight (MALDI–TOF) mass spectrometry (Sequenom, San Diego, CA, USA). Appropriate controls were included in all assays and genotyping consistency was tested by analysing between 10 and 15% of samples in duplicate, resulting in 100% agreement. In addition, the MTHFD1 1958G→A PCR-RFLP assay was verified by repeat genotyping subsets of our samples with two independent genotyping assays. These assays are carried out on different platforms (one gel based, one mass-spec based) and share no common primers or reagents. The primer sequences and assay conditions for all assays are available upon request.

Statistical analysis

The association between case–control status and genotype was examined using a number of standard odds ratios. To have a common approach for all analyses, a log linear model was employed. The statistical software (SAS PROC NLMIXED) allows estimation of non-linear functions of the parameters of the model and provides standard errors calculated using the delta method (Agresti, 1990). The parameterization of the model can easily be modified for the computation of different odds ratios. This approach enabled us to estimate log odds ratios and their standard errors for the computation of confidence intervals, as well as to check the goodness of fit of different models. Potential gene–gene interaction effects were also examined. Tests of interactive dominant or recessive effects of specific combined genotypes were performed using a series of non-hierarchical logistic regression models (Piegorsch *et al.*, 1994). Statistical significance was assessed using likelihood ratio chi-square tests.

Results

Most of the cases (116/125) had experienced just one second trimester pregnancy loss. The remaining cases experienced two ($n = 7$) or three ($n = 2$) second trimester pregnancy losses. The average age of our cases was 30 ± 5.23 and controls were 26.3 ± 5.09 (data on just 118/ 625 controls). Among the case group 12% of women had a parity of 0, and 88% had a parity of 1. Among the control group where data was available, 43% had a parity of 0, and 57% had a parity of 1.

Three polymorphisms were genotyped in our second trimester pregnancy loss case ($n = 125$) and control ($n = 625$) groups with 98.9% of all subjects successfully genotyped for MTHFD1 1958G→A, 98.4% for MTHFR 677C→T and 97.8% for TCII 776C→G. Comparison of allele and genotype frequencies between cases and controls is shown in Table I.

The MTHFD1 1958AA genotype is clearly enriched in the second trimester pregnancy loss case group compared to controls. MTHFD1 1958AA women have a significantly increased risk of having an unexplained second trimester pregnancy loss than women who are MTHFD1 1958AG or 1958GG [odds ratio 1.64 (1.05–2.57) $P = 0.03$]. The control group shows deviation from Hardy–Weinberg equilibrium with slightly more MTHFD1 1958AG heterozygotes than expected ($P = 0.03$). We have observed this in other control groups from our population (A.Parle-McDermott, unpublished data). Published frequencies from other populations including Dutch (Hol *et al.*, 1998), Turkish (Akar *et al.*, 2001), Italian (De Marco *et al.*, 2004) and Mexican (Shi *et al.*, 2003) are also skewed towards heterozygote excess, although the deviations from Hardy–Weinberg equilibrium in these smaller samples did not reach statistical significance.

We observed increased frequencies of the TCII 776G allele (48 versus 45%) and the 776GG genotype (24 versus 20%) in cases compared to controls (Table I). Although this difference was not statistically significant, the TCII 776C→G polymorphism cannot be completely ruled out as a risk factor for second trimester loss. Comparison of the allele and genotype frequencies of the MTHFR 677C→T polymorphism showed no difference between cases and controls. Thus, the MTHFR 677C→T polymorphism is not a significant maternal risk factor for unexplained second trimester pregnancy loss in the Irish population.

We also examined our data for the possibility of combined genetic factors having an additive effect on risk of second trimester loss. We tested the following genotype combinations for the possibility of an interactive effect: MTHFD1 1958AA and MTHFR 677TT (OR 1.25, $P = 0.75$), MTHFD1 1958AA and TCII 776GG (OR 1.20, $P = 0.75$) or 776CG/GG (OR 1.16, $P = 0.77$), and MTHFR 677TT and TCII 776GG (OR 0.81, $P = 0.78$) or 776CG/GG (OR 0.70, $P = 0.59$). No significant genotype interactive effects on the risk of second trimester pregnancy loss were observed.

Table I. Comparison of MTHFD1 1958G→A, MTHFR 677C→T and TCII 776C→G polymorphisms in mothers with a history of second trimester pregnancy loss and control mothers

	Genotypes			Alleles	
	GG	AG	AA	G	A
MTHFD1 1958G→A					
Case mothers	32 (0.26)	58 (0.47)	33 (0.27)	122 (0.50)	124 (0.50)
Control mothers	173 (0.28)	333 (0.54)	113 (0.18)	679 (0.55)	559 (0.45)
A verus G	Odds ratio 1.23 (95% CI 0.93–1.63), $P = 0.14$*				
AA verus AG/GG	Odds ratio 1.64 (95% CI 1.05–2.57), $P = 0.03$†				
MTHFR 677C→T	CC	CT	TT	C	T
Case mothers	55 (0.44)	55 (0.44)	14 (0.11)	165 (0.67)	83 (0.33)
Control mothers	271 (0.44)	270 (0.44)	73 (0.12)	812 (0.66)	416 (0.34)
T versus C	Odds ratio 0.98 (95% CI 0.73–1.31), $P = 0.90$				
TT versus CT/CC	Odds ratio 0.94 (95% CI 0.51–1.73), $P = 0.85$‡	CG			
TCII 776C→G	CC	CG	GG	C	G
Case mothers	33 (0.27)	61 (0.50)	29 (0.24)	127 (0.52)	119 (0.48)
Control mothers	184 (0.30)	306 (0.50)	121 (0.20)	674 (0.55)	548 (0.45)
C versus G	Odds ratio 1.15 (95% CI 0.88–1.52), $P = 0.31$				
GG versus CC/CG	Odds ratio 1.25 (95% CI 0.79–1.98), $P = 0.34$§				

Data in parentheses are allele or genotype frequencies.
*χ^2 analysis.
†Goodness of fit statistic G^2, $P = 0.80$.
‡Goodness of fit statistic G^2, $P = 0.99$.
§Goodness of fit statistic G^2, $P = 0.65$.

Discussion

The etiology of second trimester pregnancy loss is often unclear. It is frequently characterized by placental vascular pathology; however, the underlying mechanisms of placental dysfunction are not well understood, and many second trimester losses are unexplained. Suboptimal folate or B_{12} metabolism due to either a deficient diet or a genetic predisposition appears to increase the risk of a number of pregnancy complications including spontaneous abortion.

Ours is the first study to show that the MTHFD1 1958G→A polymorphism increases a woman's risk of having an unexplained pregnancy loss. Similarly to our NTD (Brody et al., 2002) and severe abruptio placentae (Parle-McDermott et al., 2005) studies, the risk appears to be associated with the MTHFD1 1958AA homozygote genotype [OR 1.64 (1.05–2.57), $P = 0.03$]. We found no evidence of an interactive effect on risk between the MTHFD1 1958AA genotype and the following: MTHFR 677TT, TCII 776GG or TCII 776CG/GG. The phenotypic effect of this variant is not currently known, but it possibly influences the rate of DNA synthesis and subsequently the rate of cell division, the timing of which is critical during pregnancy and fetal development. Our observations of significantly more 1958AG heterozygotes in the general population than expected, and the apparent selection against transmission of the 1958A allele in our NTD study suggest that the 1958AA genotype, when carried by the developing fetus, may also contribute to loss. Although beyond the scope of our study, it would be interesting to genotype unexplained spontaneously aborted embryos/fetuses for the MTHFD1 1958G→A polymorphism with the tentative prediction that more than expected would be homozygous for 1958AA. This allele appears to have detrimental effects in terms of pregnancy and development, but its high frequency in the population suggests that the extent of this effect is not severe or that heterozygotes have some selective advantage; this may explain the deviation from Hardy–Weinberg equilibrium in our controls.

Our results indicate that maternal MTHFR 677C→T or TCII 776C→G genotypes do not independently contribute to risk of second trimester pregnancy loss. An interactive effect between TCII 776CG

or 776GG and MTHFR 677TT on early fetal loss has been reported (Zetterberg et al., 2003). We applied logistic regression analysis to this data (reconstructed to the best of our abilities from Zetterberg et al., 2002a,b, 2003) and found that the interaction between MTHFR 677TT and TCII 776CG/GG was not significant ($P = 0.77$). Similarly, we found no evidence in our study of second trimester pregnancy loss cases and controls for interactive effects between the MTHFR 677TT and TCII 776GG ($P = 0.78$) or TCII 776CG/GG ($P = 0.59$).

Our study has a number of limitations. We were unable to collect information on a number of maternal risk factors, such as tobacco or alcohol use, that contribute to fetal loss. Prenatal diagnosis and routine ultrasound were not available at the time these samples were collected. However, we were able to consider maternal age and the mean age among cases was 30 years, well under the threshold (35+ years) at which substantially increased complications related to maternal age are expected (Cunningham and Leveno, 1995). Our cases were identified from mothers whose pregnancies occurred before evaluation for maternal clotting disorders became a common clinical practice; as such, the presence of inherited or acquired thrombophilia among cases cannot be ruled out. If undiagnosed thrombophilias were present more or less randomly in the study population, they would reduce our ability to show an effect for vitamin and homocysteine-related genetic factors. Another limitation of our study is that we have previously shown that the MTHFD1 1958AA genotype is a maternal risk for NTDs (Brody et al., 2002), and although all losses with fetal malformations were excluded, we cannot completely rule out that unrecognized NTDs caused some of the pregnancy losses. The rate of NTD-associated pregnancy losses is 1/50. If unrecognized NTDs form part of our case group, it is unlikely that they would have a significant impact on our analyses.

In conclusion, we have identified the MTHFD1 1958AA genotype as an independent maternal risk factor for unexplained pregnancy loss during the second trimester of pregnancy. The data presented here requires replication in another population to substantiate the role of the MTHFD1 1958G→A polymorphism in second trimester pregnancy

loss. These results should also prompt the testing of our prediction that fetuses with the MTHFD1 1958AA genotype exhibit reduced viability.

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