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Dizygotic twinning is not associated with methylenetetrahydrofolate reductase haplotypes

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BACKGROUND: Folate metabolism is critical to embryonic development, influencing neural tube defects (NTD) and recurrent early pregnancy loss. Polymorphisms in 5,10-methylenetetrahydrofolate reductase (MTHFR) have been associated with dizygotic (DZ) twinning through pregnancy loss. METHODS: The C677T and A1298C polymorphisms in MTHFR were genotyped in 258 Australasian families (1016 individuals) and 118 Dutch families (462 individuals) of mothers of DZ twins and a population sample of 462 adolescent twin families (1861 individuals). Haplotypes were constructed from the alleles, and transmission of the MTHFR haplotypes to mothers of DZ twins and from parents to twins in the adolescent twin families analysed. RESULTS: The C677T and A1298C were common in all three populations (frequencies > 0.29). There was strong linkage disequilibrium (D′ = 1) between the variants, showing that specific combinations of alleles (haplotypes) were transmitted together. Three haplotypes accounted for nearly all the variation. There was no evidence of any association between MTHFR genotype and twinning in mothers of twins, or of the loss of specific MTHFR genotypes during twin pregnancies. CONCLUSIONS: It is concluded that variation in twinning frequency is not associated with MTHFR genotype.

Key words: DZ twins/MTHFR/polymorphisms/twinning frequency

Introduction

Spontaneous dizygotic (DZ) twins are born following ovulation and fertilization of two ova and survival of both embryos. DZ twinning clusters within families and is under genetic control (Bulmer, 1970; Lewis *et al*., 1996; Meulemans *et al*., 1996). Taken together, the risk to first-degree female relatives is in excess of 2 (Bulmer, 1970; Lewis *et al*., 1996; Meulemans *et al*., 1996), and comparable with that for breast cancer (Claus *et al*., 1991). Variation in DZ twinning risk could result from variation in twin ovulation frequency and/or embryo survival.

Folate-dependent homocysteine metabolism is critical for female fertility and early embryonic development. Folic acid is essential for DNA replication and cellular methylation reactions. Abnormal metabolism of folate and homocysteine are associated with neural tube defects (NTDs) (Fleming, 2001) and recurrent abortions (Nelen *et al*., 1997). Several studies (Garabedian and Fraser, 1994; Kallen *et al*., 1994; Whiteman *et al*., 2000) have documented a positive association between NTD and the frequency of twins. Dietary supplementation around conception with folic acid alone, or in combination with other vitamins, reduces the incidence and recurrence of NTDs (Lumley *et al*., 2000; Fleming, 2001). Recent studies provide no support for suggestions of an increased frequency of twins following folate supplementation (Czeizel and Dudas, 1992; Lumley *et al*., 2000; Ericson *et al*., 2001; Li *et al*., 2003).

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism, and catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is essential for the subsequent conversion of homocysteine to methionine and functioning of the methylation cycle (Hobbs *et al*., 2000; van der Put *et al*., 2001). Decreased production of 5-methyltetrahydrofolate may lead to increased homocysteine concentrations and impaired methylation of proteins and DNA. During pregnancy, folate requirements increase, making folate deficiency more likely, and consequently MTHFR variants may have an effect on developing embryo(s).

Two common single nucleotide polymorphisms (SNPs) in MTHFR (C677T and A1298C) reduce enzyme activity (Frosst *et al*., 1995; van der Put *et al*., 1998; Weisberg *et al*., 1998) and are associated with both the twinning phenotype and variation in embryo survival. A case–control study investigated the C677T polymorphism in mothers with dichorionic twin pregnancies, and found a lower frequency of the 677T allele amongst mothers of twins compared with women who gave birth to singletons (Hasbargen *et al*., 2000). These authors

suggested that the 677T allele of *MTHFR* is protective against multiple pregnancies. The 677T allele is also associated with an increased risk of NTD (Botto and Yang, 2000). Analyses of *MTHFR* genotypes in spontaneous abortion reported decreased embryo viability in fetuses carrying the 677T and 1298C alleles (Isotalo *et al*., 2000; Volcik *et al*., 2001; Zetterberg *et al*., 2002). Carrying one or more variant alleles may be detrimental during early embryogenesis when folate requirements are high (Zetterberg *et al*., 2002). Any effect of the 677T allele to reduce multiple pregnancy in mothers of twins may act through reduced survival of twin embryos, or through some other mechanism. The aim of the present study was to investigate the role of *MTHFR* variants in twinning by studying transmission of *MTHFR* alleles in families of mothers of DZ twins and in a population sample of adolescent twins.

Materials and methods

Transmission of the C677T and A1298C polymorphisms in *MTHFR* was investigated in two populations of European descent. The SNPs were genotyped in 1016 individuals from 258 Australasian families and 462 individuals from 118 Dutch families in which two sisters had both given birth to spontaneous DZ twins (MODZT families). MODZT families were identified through records from various genetic epidemiological studies using twins and their families in Australia (Lewis *et al*., 1996), through organizations for mothers-of-twins in Australia and New Zealand (ANZ), and through appeals in the media in both countries. In the Netherlands, ascertainment was population-based through community records as part of a systematic recruitment to the Netherlands Twin Register (Meulemans *et al*., 1996; Boomsma *et al*., 2002). Mothers were explicitly asked about fertility treatments and all such cases were excluded.

In addition, the SNPs were typed in 1861 individuals from 462 families of DZ and monozygotic (MZ) adolescent twins recruited from schools in the Brisbane and surrounding areas of south-eastern Queensland (Zhu *et al*., 1999). Samples were collected from twins, siblings and their parents.

Study protocols were reviewed and approved by the Bancroft Centre Human Research Ethics Committee. Participation was voluntary and each patient provided their informed consent.

Genomic DNA was extracted (Miller *et al*., 1988) from peripheral venous blood samples. Zygosity of the twins was determined by differences in sex, eye colour or hair colour, and by typing nine independent microsatellite markers (AmpFLSTR® Profiler Plus™; Applied Biosystems, Foster City, CA, USA) in equivocal cases. The probability of dizygosity given concordance of all markers in the panel was $<10^{-3}$.

The C677T polymorphism (Val→Ala) in exon 4 of *MTHFR* (GenBank accession number rs1801133) was typed by PCR-RFLP in the MODZT families using a protocol and primers as described previously (Hasbargen *et al*., 2000). The C677T polymorphism in the adolescent twin families and the A1298C polymorphism (Glu→Ala) (GenBank accession number rs1801131) in both populations were genotyped using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR products of 114 bp and 115 bp for the C677T and A1298C polymorphisms were amplified using the primer pairs CCCGAAGCAAGGAGCTTTG, AAAGCGGAAGAATGTGT-CAGC and CCTGAAGAGCAAGTCCCCC, CCGGTTTGGTTCT-CCCG respectively. *Hin*fI or *Mbo*II restriction enzyme digestion and agarose gel electrophoresis were used to identify DNA controls required for each polymorphism in the Sequence Detection System (SDS) allelic discrimination assays. Using the standard protocol for the SDS assay, fluorescently labelled probes used for C677T polymorphism were: 5′-(VIC)-CTGCGGGAGCCGATTTCATCAT-6-carboxy-tetramethyl-rhodamine(TAMRA)-3′ and 5′-6-carboxy fluorescein-(FAM)-CTGCGGGAGTCGATTTCATCATCA-TAMRA-3′ to detect the C and T alleles respectively. Probes used for the A1298C polymorphism were 5′-(VIC)-CAAAGACACTTTCTTCACTGGT-CAGCTCC-TAMRA-5′ and 5′-FAM-CAAAGACACTTGCTTCA-CTGGTCAGCTC-TAMRA-3′ to detect the A and C alleles respectively. The final concentration of reagents in the PCR mix (reaction volume 15 µl) was 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol/l Primers, 175 nmol/l FAM probes and 200 nmol/l VIC probes. The reaction mix was added to 15 ng of genomic DNA that had been pre-dried in 96-well plates. Following optimization of each assay in the ABI Prism 7700 SDS, PCR reactions were amplified on ABI GeneAmp 9700 PCR machines for 2 min at 50°C, 10 min at 95°C, followed by 45 two-step cycles of 15 s at 95°C and 1 min at 62°C. End-point detection genotype analysis was performed on amplified samples using the ABI PRISM 7700 software using standard procedures.

The program Sib-pair was used to calculate allele and genotype frequencies, and perform two-parent, one-locus transmission/disequilibrium tests (TDT) to examine allele transmission to MODZT. The one-locus TDT was also used to examine paternal and maternal allele transmission to male and female offspring individually in the adolescent twin families. A two-locus TDT was performed using GENEHUNTER (Kruglyak *et al*., 1996) and TDTHAP (Clayton and Jones, 1999). The transmission of haplotypes was tested for the mother of a pair of DZ twins in MODZT families, and for a twin in the adolescent twin families. Segregation distortion and parent of origin effects were also tested for.

Results

Frequencies for the 677T allele (MODZT = 0.342 ± 0.018; adolescent twins = 0.345 ± 0.012) and 1298C allele (MODZT = 0.295 ± 0.015; adolescent twins = 0.289 ± 0.011) were similar in Australian samples (Table I). The frequency of the 677T and 1298C alleles in the MODZT families from the Netherlands were 0.310 and 0.305 respectively (Table I). Both variants were in Hardy-Weinberg equilibrium in all three groups.

Table I. Allele and haplotype frequencies for the MTHFR C677T and A1298C SNPs in affected sister pair families with DZ twins (MODZT families) and Australian adolescent twin families

Families	MODZT		Adolescent twins
	Netherlands	ANZ[a]	
Alleles			
677T	0.310 (0.022)	0.342 (0.018)	0.345 (0.012)
1298C	0.305 (0.022)	0.295 (0.015)	0.289 (0.011)
Haplotypes[b]			
677C/1298A	0.400 (0.023)	0.362 (0.018)	0.366 (0.012)
677C/1298C	0.296 (0.021)	0.295 (0.015)	0.288 (0.011)
677T/1298A	0.293 (0.021)	0.342 (0.018)	0.345 (0.012)
677T/1298C	0.011 (0.005)	0.000 (0.000)	0.000 (0.000)

Values in parentheses are SEM.
[a]Australian and New Zealand families.
[b]Differences in haplotype frequencies between the ANZ and Netherlands families were significant ($P < 0.0003$).

There was strong linkage disequilibrium (LD) between the two variants (D′ = 1), showing that specific combinations of alleles (haplotypes) are transmitted together. Three haplotypes were common (Table I). The 677T/1298C allele was observed so the variants can occur in *cis*. However, the haplotype was rare and only observed in Netherlands families. The PCR products from individuals carrying this haplotype were sequenced to confirm the SNP typing results (data not shown). The haplotype frequencies in the samples from Australia and New Zealand (ANZ) were similar, but there were small yet significant differences in haplotype frequencies between the ANZ and Netherlands families ($P < 0.0003$; Table I). The genotype counts (Table II) were consistent with the effects of strong LD observed between the variants, which are separated by only 1902 bp in the genomic sequence.

The frequencies of transmitted and non-transmitted haplotypes to MODZT in 135 families (where parents were informative) were similar and did not differ significantly ($P > 0.9$; Table III). The allele frequency for the 677T allele in mothers of twins ($n = 416$) was 0.35, which was not significantly different from the allele frequency for the 677T allele in fathers of twins (0.36, $n = 323$) from the adolescent MZ and DZ twin families ($\chi^2_2 = 1.3$, $P = 0.53$).

The transmission of *MTHFR* alleles to 102 MZ twins and 371 DZ twins from informative families was also compared to obtain evidence of effects of *MTHFR* variants on the survival of twins (Table III). There was no excess sharing of haplotypes among either MZ or DZ twins that would be expected if *MTHFR* variants contributed to variation in twin survival. No evidence was observed of any parent-of-origin effects (data not shown).

Table II. MTHFR genotype counts for C677T and A1298C in families of mothers of twins (MODZT families) from Australasia and the Netherlands and from Australian adolescent twin families

Genotype C677T	CC	CT	TT
A1298C			
ANZ[a] mothers of twins			
AA	79	172	90
AC	183	132	0
CC	58	0	0
Netherlands mothers of twins			
AA	63	102	30
AC	76	64	1
CC	37	2	0
Adolescent twin families			
AA	194	429	189
AC	331	329	0
CC	126	0	0

[a]Australian and New Zealand families.

Discussion

The frequencies of *MTHFR* genotypes in the present sample were similar to other published studies for populations of European descent (Wilcken *et al*, 1996; Stegmann *et al*., 1999; Botto and Yang, 2000; Volcik *et al*., 2001; Rosenberg *et al*., 2002; Zetterberg *et al*., 2002). The present sample of twins from Australia was predominantly of Anglo-Celtic descent, with 95% of grandparents having reported ancestry from the British Isles. The frequency of the 677CC/1298AC genotype was lower than reported for a Canadian population of predominantly European (Celtic) origin (Isotalo and Donnelly, 2002).

In some previous studies, individuals with 677TT/1298CC or 677CT/1298CC genotypes were not observed, and the 677TT/1298AC genotype was rare (van der Put *et al*., 1998; Weisberg *et al*., 1998; Zetterberg *et al*., 2002). These groups concluded that the alleles only occur in the *trans* configuration. In the present study, there was strong LD between the two variants consistent with the short distance (1902 bp) between the variable bases in the *MTHFR* DNA sequence. The 677T/1298C allele was observed, but the haplotype was rare and found in only a small number of families from the Netherlands.

Several studies have reported effects of *MTHFR* genotypes on fetal viability (Isotalo *et al*., 2000; Volcik *et al*., 2001; Zetterberg *et al*., 2002). It is not clear whether specific alleles at individual SNPs in *MTHFR* or the combined presence of one or more variant alleles at the C677T and A1298C SNPs predispose to embryo or fetal loss during pregnancy (Isotalo *et al*., 2000; Volcik *et al*., 2001; Zetterberg *et al*., 2002). MTHFR is a

Table III. Transmitted (T) and non-transmitted (NT) haplotypes in affected sister pair families with DZ twins (transmission to mothers of twins, MODZT families,) and Australian adolescent twin families (transmission from parents to twins). In adolescent twin families, haplotype transmission to MZ and DZ twins are given separately

Families	MODZT		Adolescent twins				
	Female		MZ		DZ		Transmitted alleles[c]
	T[a]	NT[b]	T	NT	T	NT	
Haplotype							
677C/1298A	53	52	38	41	128	132	0.503 (0.461–0.544)
677C/1298C	40	40	21	33	121	116	0.470 (0.425–0.515)
677T/1298A	42	43	43	28	122	123	0.527 (0.483–0.570)
677T/1298C	0	0	0	0	0	0	0.000 (0.000–0.000)

[a]Transmitted haplotypes.
[b]Non-transmitted haplotypes.
[c]Mean transmission frequency with 95% confidence limits for haplotype transmission for the combined sample.

homodimer and may be subject to complex regulation (Goyette *et al*., 1998). Recent biochemical studies of MTHFR compared the wild-type enzyme with purified variant forms of the C677T (Ala222Val) and A1298C (Glu429Ala) variants (Yamada *et al*., 2001), and activities of the wild-type or A1298C variant enzymes were indistinguishable. The C677T variant protein dissociated more easily into monomers and lost its flavin adenine dinucleotide (FAD) cofactor on dilution, leading to the loss of enzyme activity (Yamada *et al*., 2001). The results of these studies supported the association of the C677T variant with increased risk of disease. Association with A1298C may result from direct effects of the variant or through LD with the C677T and/or other variants.

The C677T variant is associated with a moderate risk of NTD (Botto and Yang, 2000), giving an increased odds ratio of 1.6 for infants homozygous for the 677T allele. Higher frequencies of variant *MTHFR* alleles were observed in fetal samples and spontaneous abortions compared with control samples (Isotalo *et al*., 2000; Zetterberg *et al*., 2002), although the pattern of allele differences was not the same in each study. Twins may be at higher risk for embryo loss, and mothers with dichorionic twin pregnancies had a lower frequency of the 677T allele compared with women who gave birth to singletons (Hasbargen *et al*., 2000). In the present larger study, the transmission disequilibrium of haplotypes was tested (given the high LD between the polymorphisms in *MTHFR*), and no evidence was observed for an association between haplotypes at *MTHFR* and twinning phenotype in MODZT. The allele frequency for the 677T allele in the mothers of twins (0.35) was similar to that in the mothers of singletons in a previous study (Hasbargen *et al*., 2000). The allele frequency was not significantly different from that in fathers of MZ and DZ twins (0.36) from the adolescent twin families. Population studies do not show evidence for fathers contributing to variation in either DZ or MZ twins (Lewis *et al*., 1996). The present study had good power to detect association between MTHFR and twinning. The odds ratio for transmission of the 677T variant to mothers of twins was 0.87, with a tight confidence interval (0.70–1.07).

Haplotype transmission was also considered to MZ or DZ twins, but no evidence was seen for any effects of *MTHFR* genotype on the survival of twin embryos. The present sample comprised individuals who survived the entire pregnancy. Calculations based on the data of others (Zetterberg *et al*., 2002) suggested that large deviations in adult haplotype frequencies caused by embryo loss from abortion would not be expected. No difference was observed in haplotype transmission that might have been caused by embryo or fetal loss.

Periconceptional folate supplementation has been strongly recommended to reduce the incidence of NTDs, but a meta-analysis found that the reported increase in the frequency of twins following folate supplementation was not significant (Lumley *et al*., 2000). Recent results from a large population-based cohort study in China found no evidence for effects of folic acid supplementation on twinning frequency (Li *et al*., 2003), though this population generally has a low frequency of twins. Based on the results of the present studies, it can be concluded that variation in twinning frequency is not associated with genotype at *MTHFR* in mothers of twins, or with the loss of specific *MTHFR* genotypes during twin pregnancies.

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