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ORIGINAL ARTICLE

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Analysis of the MTHFR 1298A → C and 677C → T polymorphisms as risk factors for neural tube defects

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Abstract The thermolabile variant (677TT) of methylenetetrahydrofolate reductase (MTHFR) is a known risk factor for neural tube defects (NTDs). The relationship between a second MTHFR polymorphism (1298A → C) and NTD risk has been inconsistent between studies. We genotyped 276 complete NTD triads (mother, father and child affected with an NTD) and 256 controls for MTHFR 1298A → C. Our findings do not support a role for the 1298A → C polymorphism in NTDs (OR 0.85 (95% CI 0.49–1.47), $p = 0.55$), nor do we observe a combined effect with the 677C → T polymorphism.

Keywords MTHFR · A1298C · Neural tube defects · C677T · Linkage disequilibrium

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Introduction

The enzyme 5,10–methylenetetrahydrofolate reductase (MTHFR) plays an important role in folate metabolism by catalysing the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which acts as a methyl group donor for the remethylation of homocysteine to methionine. The thermolabile MTHFR 677C → T (A222V) variant is a risk factor for neural tube defects (NTDs) in some but not all populations (Botto and Yang 2000) and is associated with low folate and elevated homocysteine levels. However, the MTHFR 677TT genotype accounts for only 11.4% of the population attributable fraction in Ireland (Shields et al. 1999) and only partly explains low blood folates in NTD pregnancies (Molloy et al. 1998). This has led to the search for additional polymorphisms which influence folate and/or homocysteine levels in relation to neural tube defects, cardiovascular disease and cancer.

The identification of a second polymorphism within the *MTHFR* gene i.e., 1298A → C (E429A) (Viel et al. 1997; van der Put et al. 1998) and its apparent association with decreased enzyme activity (van der Put et al. 1998; Weisberg et al. 1998, 2001) has led to several association studies investigating the MTHFR 1298A → C polymorphism and NTDs. Results from these studies have been inconsistent and Yamada et al. (2001) found no difference in enzyme activity of 1298A → C variants. Some studies have reported an increased frequency of combined heterozygotes (*MTHFR* 677CT/1298AC) in NTD cases compared to controls (van der Put et al. 1998; Richter et al. 2001). Other studies, some with a relatively small sample size, show no association (Weisberg et al. 1998; Stegmann et al. 1999; Trembath et al. 1999; Barber et al. 2000; Volcik et al. 2000). Only one study to date has shown a direct association of the 1298C allele with NTDs (De Marco et al. 2002). Differences between studies may be due to small sample

sizes or genetic heterogeneity between populations. To resolve this controversy, we analysed the relationship between MTHFR 1298A → >C and NTDs in a large, genetically homogeneous population.

Subjects and methods

Study and control groups

The study group consisted of NTD-affected children plus their parents (triads) whom we recruited throughout Ireland from 1993 to date with the assistance of various branches of the Irish Association for Spina Bifida and Hydrocephalus. The NTD population comprised 276 complete triads and a small number of incomplete triads where DNA was not available from all three family members (three additional cases and one mother). The control population (256) was obtained from between 1986 and 1990 from 56,049 pregnant women attending the three main maternity hospitals in the Dublin area. Details of this collection have been described previously (Kirke et al. 1993). Informed consent and ethical approval were obtained for all samples collected.

Genetic analyses

Genomic DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (Qiagen, UK). Analysis of the MTHFR 677C → T polymorphism was performed by PCR–RFLP (restriction fragment length polymorphism) using *Hinf* I as previously described (Frosst et al. 1995). The MTHFR 1298A → C polymorphism was PCR amplified as described in van der Put et al. (1998) and genotyping was carried out via ASO (allele specific oligonucleotide) analysis. Genotype controls for the ASO analysis were sequenced using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, UK) and the ABI 377 automated sequencer. Verification of MTHFR 1298A → C genotyping was carried out on approximately 20% of samples by an independent PCR assay and analysed via Matrix Assisted Laser Desorption/Ionization–Time-of-Flight (MALDI–TOF) mass spectrophotometry (Sequenom) with >99% agreement.

Results

Case-control comparisons

A total of 99.3% of our samples (1,081/1,089) were successfully genotyped for the MTHFR 1298A → C polymorphism and 99.8% (1,087/1,089) for the MTHFR 677C → T polymorphism. The genotyping success did not differ between groups. Allele frequencies for the MTHFR 1298A → C polymorphism were similar in NTD triad and control groups with an approximate allele frequency of 0.70 and 0.30 for A and C alleles, respectively (Tables 1 and 2). Comparisons of genotype frequencies between NTD triad groups and controls did not reveal any statistically significant differences (Tables 1 and 2: case-controls, $p = 0.55$). Lack of association was also confirmed by analysis of case-parent-triad data using a log linear model (Weinberg et al. 1998). As we reported previously, (Whitehead et al. 1995; Shields et al. 1999), the 677TT genotype is a risk factor for NTD cases (Tables 1 and 2, $p = 0.01$).

Table 1 MTHFR1298A → C genotype frequencies in NTD triads and controls

1298A → C	Controls $n = 256$	NTD cases $n = 277$	NTD mothers $n = 274$	NTD fathers $n = 274$
AA	0.52[a]	0.54	0.48	0.52
AC	0.37	0.36	0.41	0.38
CC	0.11	0.10	0.11	0.10
AC/AA vs CC[b]		p[c] = 0.55[d]	$p = 0.78$[e]	$p = 0.58$[f]
A	0.70	0.72	0.69	0.71
C	0.30	0.28	0.31	0.29

[a]Genotype frequencies: due to rounding, all columns may not sum to one
[b]Comparison of MTHFR 1298A → C genotypes (AC/AA vs CC) in NTD study groups compared to controls
[c]Statistical significance was assessed by using χ^2 analysis
[d]Odds ratio 0.85 (0.49 – 1.47, 95% confidence interval)
[e]Odds ratio 0.93 (0.54 – 1.60, 95% confidence interval)
[f]Odds ratio 0.86 (0.49 – 1.49, 95% confidence interval)

Table 2 677C → T genotype frequencies in NTD triads and controls

677 C –> T	Controls $n = 255$	NTD cases $n = 279$	NTD mothers $n = 277$	NTD fathers $n = 276$
CC	0.49	0.39	0.38	0.39
CT	0.40	0.43	0.50	0.50
TT	0.10	0.19	0.13	0.10
CT/CC vs TT[a]		$p = 0.01$[b]	$p = 0.38$[c]	$p = 0.91$[d]
C	0.70	0.60	0.62	0.64
T	0.30	0.40	0.38	0.36

[a]Comparison of MTHFR 677C → T genotypes (CT/CC vs TT) in NTD study groups compared to controls
[b]Odds ratio 2.02 (1.22–3.34, 95% confidence interval)
[c]Odds ratio 1.27 (0.74–2.18, 95% confidence interval)
[d]Odds ratio 1.03 (0.59–1.81, 95% confidence interval)

MTHFR genotype combinations

Combined genotype frequencies of the MTHFR 1298A → C/677C → T polymorphisms are shown in Table 3. Linkage disequilibrium (LD) is evidenced by the deviation of the observed joint frequencies from expected assuming independence (Table 3). Thus, the 677T allele is nearly always found in *trans* with the 1298A allele and the 1298C allele with the 677C allele. The presence of some individuals with 1298AC/677TT and 1298CC/677CT genotypes shows that they are not in complete linkage disequilibrium and that a historical recombination event(s) has separated these alleles.

An effect of combined genotypes on NTD risk was tested by analysing the joint distributions of cases and controls using a model for the gamete frequencies of the four possible combinations of alleles (Weir 1996). The model assumes that the two loci are in Hardy–Weinberg equilibrium, although not necessarily in linkage equilibrium. Maximum likelihood estimates of the parameters and their standard errors are shown in Table 4. The model provided a satisfactory fit to the observed joint

Table 3 MTHFR 1298A → C/ 677C → T genotype frequencies in NTD triads and controls

677C → T	CC		CT		TT	
1298A → C	Obs[a] (*n*)	Exp[b]	Obs (*n*)	Exp	Obs (*n*)	Exp
Controls						
AA	0.15 (39)	0.25	0.27 (69)	0.21	0.09 (24)	0.05
AC	0.23 (58)	0.18	0.13 (34)	0.15	0.007 (2)	0.04
CC	0.11 (29)	0.05	NO[c]	0.04	NO	0.01
NTD cases						
AA	0.12 (34)	0.21	0.23 (64)	0.23	0.19 (52)	0.10
AC	0.17 (46)	0.14	0.19 (53)	0.15	NO	0.06
CC	0.10 (27)	0.04	NO	0.04	NO	0.02
NTD mothers						
AA	0.09 (26)	0.18	0.27 (74)	0.24	0.12 (33)	0.06
AC	0.18 (48)	0.16	0.23 (63)	0.21	0.004 (1)	0.05
CC	0.10 (28)	0.04	0.004 (1)	0.06	NO	0.01
NTD fathers						
AA	0.15 (40)	0.20	0.28 (76)	0.27	0.09 (26)	0.05
AC	0.15 (41)	0.14	0.22 (61)	0.19	0.01 (3)	0.04
CC	0.09 (26)	0.04	0.004 (1)	0.06	NO	0.01

[a]Obs = Observed frequencies
[b]Exp = Expected frequencies derived by multiplying the genotype frequencies for each allele within each group
[c]NO = Genotype not observed

Table 4 Analysis of genotype frequencies using models based on Hardy–Weinberg equilibrium. Estimated (by maximum likelihood) allele frequencies for marginal models and gamete frequencies for joint models

	Cases	Controls
Allele frequency (SE)		
677T	.3978 (.021)	.3039 (.020)
1298C	.2832 (.019)	.2980 (.020)
Gamete frequency (SE)		
677C – 1298A	.3190 (.020)	.4036 (.022)
677C – 1298C	.2832 (.019)	.2924 (.020)
677T – 1298A	.3978 (.021)	.2983 (.020)
677T – 1298C	0	.0056 (.004)

G^2 (likelihood ratio goodness of fit statistic) = 6.54, df = 3, p = 0.088 for cases and G^2 = 9.43, df = 5, p = 0.093 for controls

genotype data for both cases and controls. The estimated gamete frequencies confirm that the two alleles are in linkage disequilibrium. The difference between cases and controls is due to an increase of the 677T allele in the cases compared to controls, and unrelated to 1298.

Transmission disequilibrium test (TDT)

The transmission disequilibrium test was performed on informative MTHFR 1298 heterozygotes (n = 157) (Spielman et al. 1993). This analysis showed slight preferential transmission of the wildtype 1298A allele but was not statistically significant (A: 57%, n = 90; C: 43%, n = 67; p = 0.08, McNemar chi square 3.08, Odds Ratio 1.34 (0.97–1.87)). Previously, we analysed 218 triads for the MTHFR 677C → T TDT and this showed evidence of increased T allele transmission but failed to reach statistical significance (p = 0.10; Shields et al. 1999). Re–analysis of this data set with additional triads (58 additional triads) also revealed preferential transmission of the 677T allele and statistical significance was reached in this instance (C: 41%, n = 87; T: 59%, n = 125; p = 0.011, McNemar chi square 6.46, Odds Ratio 1.44 (1.08–1.91)). The increased transmission of the 1298A allele is most likely explained by the linkage disequilibrium between 677T and 1298A.

Discussion

Our study group includes one of the largest NTD triad sets worldwide and probably one of the most genetically homogenous NTD populations examined to date. The frequency we observed for the MTHFR 1298C allele (0.30) is similar to that reported for other Caucasian populations. Our case-control comparisons and log linear analysis do not find an association between 1298A → C and NTDs. This is in contrast to De Marco et al. (2002), the only group to date to find a direct association of the MTHFR 1298C allele and increased risk of NTDs.

Consistent with our previous work, our expanded sample set showed an increased frequency of MTHFR 677 TT homozygotes in cases compared to controls (Whitehead et al. 1995; Shields et al. 1999). Ours, like most studies, also found evidence of linkage disequilibrium between the MTHFR 1298A → C and the 677C → T alleles (van der Put et al. 1998; Stegmann et al. 1999; Barber et al. 2000; Richter et al. 2001). Consistent with previous reports (van der Put et al. 1998; Richter et al. 2001), we also observed increased frequency of combined MTHFR heterozygotes in NTD cases compared to controls, but further analysis did not reveal any evidence of combined MTHFR genotype effects in relation to NTD risk that could not be simply explained as a by-product of linkage disequilibrium. TDT analysis of MTHFR 1298A → C triads shows a non–significant trend of the wildtype 1298A allele being passed preferentially to the NTD case. Our larger study population showed significant preferential transmission

of the 677T allele ($p = 0.01$) and it is this transmission that is probably driving the slight preferential transmission of the 1298A allele due to the linkage disequilibrium between these two alleles.

In conclusion, analysis of our large genetically homogeneous population does not support a role of the MTHFR 1298A → C polymorphism in neural tube defects.

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