European Journal of Human Genetics (2006) 14, 768–772

www.nature.com/ejhg

ARTICLE

Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population

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The risk of neural tube defects (NTDs) is known to have a significant genetic component that could act through either the NTD patient and/or maternal genotype. The success of folic acid supplementation in NTD prevention has focused attention on polymorphisms within folate-related genes. We previously identified the 1958G>A (R653Q) polymorphism of the trifunctional enzyme MTHFD1 (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase; often referred to as 'C1 synthase') as a maternal risk for NTDs, but this association remains to be verified in a separate study to rule out a chance finding. To exclude this possibility, we genotyped an independent sample of mothers with a history of an NTD-affected pregnancy derived from the same Irish population. In this sample there was a significant excess of 1958AA homozygote mothers of NTD cases ($n=245$) compared to controls ($n=770$). The direction and magnitude of risk (odds ratio 1.49 (1.07–2.09), $P=0.019$) is consistent with our earlier finding. Sequencing of the *MTHFD1* gene revealed that this association is not being driven by another common variant within the coding region. We have established that the *MTHFD1* 1958G>A polymorphism has a significant role in influencing a mother's risk of having an NTD-affected pregnancy in the Irish population.
European Journal of Human Genetics (2006) **14,** 768–772. doi:10.1038/sj.ejhg.5201603; published online 22 March 2006

Keywords: folate; NTD; spina bifida; MTHFD1; C1 synthase; Irish

Introduction

Neural tube defects (NTDs) are a common human birth defect that present as a number of different types, including spina bifida, anencephaly and encephalocele. Maternal consumption of a folic acid supplement in the weeks prior to and after conception can prevent up to 70% of NTDs,[1] suggesting that variants in genes involved in folate metabolism may contribute to NTD risk. In the Irish population, two genetic variants have been identified as risk factors for NTDs to date. These include the case risk variant 677C>T (A222V; dbSNP rs1801133) within the MTHFR enzyme (5,10-methylenetetrahydrofolate reductase)[2] and the more recently identified maternal risk variant 1958G>A (R653Q; dbSNP rs2236225) within the

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Received 27 September 2005; revised 4 January 2006; accepted 20 January 2006; published online 22 March 2006

MTHFD1 enzyme (methylenetetrahydrofolate-dehydrogenase/methenyltetrahydrofolate-cyclohydrolase/formyltetrahydrofolate synthetase; often referred to as 'C1 synthase').[3] Although the association between the MTHFR 677C>T polymorphism and risk of NTDs has been demonstrated in a number of populations,[4] the MTHFD1 1958G>A association requires replication. Although association studies are a plausible way to identify the common genetic variants that impact on common disease, the problem of false positive associations is significant. More than half the associations in the literature have not been repeated by independent studies and, apart from population-specific effects, the failure to replicate an association is often due to the limited power of studies with small sample sizes.[5] Since the publication of our previous study, we have completed our collection of Irish NTD samples and wished to test the MTHFD1 1958G>A polymorphism in samples that had not been tested previously, that is, a confirmatory cohort.

Subjects and methods

Subjects

Our confirmatory NTD group consisted of 245 mothers of NTD cases, 127 fathers of NTD cases and 176 NTD cases, and were not part of a syndrome. These included 91 complete NTD triads, that is, mother, father and affected case, all from the same family, and 37 NTD case and mother pairs from the same family. The remaining NTD samples were from the same or different family, as outlined in Table 1. The majority (98%) of our NTD cases and, therefore, the majority of our NTD triads were NTD cases affected with isolated spina bifida aperta (173/176). The remaining cases were affected with isolated encephalocele (<2%). Among the parents of NTD cases within our sample, <8% of their NTD offspring were affected by isolated encephalocele, isolated anencephaly, spina bifida aperta, and encephalocele or spina bifida and anencephaly.

Table 1 Details of NTD families

NTD family members	*No. of families (%)*	*No. of samples (%)*
Mother, father and case[a]	91 (31)	273 (50)
Mother and case[b]	37 (13)	74 (14)
Father and case	2 (<1)	4 (<1)
Father and mother	31 (10)	46 (8)
Case only	46 (16)	62 (11)
Mother only	86 (29)	86 (16)
Father only	3 (1)	3 (<1)
Total	296	548

[a]Complete NTD triads ($n=91$) were used in the TDT test. A total of 65 of the triads were informative.
[b]The log-linear analysis included the complete NTD triads and 37 mother and case pairs.

Information on NTD case type was not available for 83/245 of our mothers of NTD cases, but their pregnancies were affected with spina bifida aperta, anencephaly or encephalocele. All families affected with spina bifida occulta were excluded. Our confirmatory control sample, $n=770$, was derived from a collection of blood samples from 56 049 pregnant women with no history of an NTD-affected pregnancy as detailed previously.[6] Controls used in this study do not overlap with those used in our initial analysis.[3] All NTD and control samples were derived from the Irish population, who may be described as a North-Western European population. The low level of immigration into Ireland during the last century means that population stratification is unlikely to confound genetic analyses.

Genotyping

Extraction of genomic DNA was carried out using the QIAamp DNA Blood Mini Kit, Qiagen, UK. Genotyping of the MTHFD1 1958G>A polymorphism was performed using a PCR–RFLP assay as described previously.[3] Appropriate controls were included in all assays and at least 10% of samples were repeat genotyped with >99% agreement. Verification of the MTHFD1 1958G>A PCR–RFLP assay also included genotyping a subset of samples using two independent assays. These assays were carried out on different platforms (one gel-based, one mass spectrophotometer-based) and share no common primers or reagents.

Sequencing

All 28 exons of the *MTHFD1* gene including the 3′ untranslated region were PCR amplified and directly sequenced on both strands using the BigDye® Terminator Kit Version 1.1 and the ABI Prism 377 DNA Sequencer (Applied Biosystems, UK) in 10 individuals ($5\times$ 1958AA, $5\times$ 1958GG). The primers and conditions of all assays are available upon request.

Statistical methods

A log-linear model was employed to compare each NTD group, that is, NTD cases, NTD fathers and NTD mothers, to controls[7] using SAS PROC NLMIXED statistical software. Statistical significance was tested using likelihood ratio χ^2 tests. The transmission of alleles from parents to affected offspring was analysed using the transmission disequilibrium test (TDT) and significance assessed using the McNemar χ^2 test.[8] The NTD triad data were also analysed using log-linear analysis and modelled as described by Weinberg *et al*.[9] Linkage disequilibrium was estimated by D' and r^2 using the program Haploview 3.2 (http://www.broad.mit.edu/mpg/haploview/).

Results

The allele and genotype frequencies within each NTD group and controls are shown in Table 2. Similar to our original analysis,[3] comparison of mothers of NTD cases to controls showed statistically significant differences between the two groups (Table 3). Again, the maternal MTHFD1 1958AA genotype confers the risk association, 1.49 (1.07–2.09), $P=0.019$ (two-sided *P*-value). Analysis of maternal risk by NTD type could be performed for mothers of spina bifida cases only, owing to the very small numbers in the other categories or the unknown status of NTD type in 33% (83/245) of mothers of NTD cases. The 1958AA genotype was also a significant maternal risk factor for spina bifida cases only compared to controls, odds ratio 1.56 (1.05–2.33), $P=0.027$. The availability of NTD triads also allowed us to examine the transmission of alleles from parents to NTD-affected offspring and the possible interaction of NTD case and maternal effects using both the TDT test[8] and log-linear models.[9] There were 91 NTD triads and 37 incomplete (NTD case and mother) triads. This is considerably fewer than the number available to us in our original paper,[3] but we still observed a similar trend. A total of 65/91 complete triads were informative for the TDT analysis and this analysis showed a slightly skewed transmission of the G allele (G: 54%, $n=45$; A: 46%, $n=39$; $P=0.51$). The log-linear model of the NTD triads gave similar odds ratio values for the homozygous AA maternal effect, odds ratio 1.58 (1.05–2.38), $P=0.028$ (data not shown). These estimates are very similar to that obtained in our first study and therefore provide an additional degree of confirmation. As before, our control samples showed slightly more heterozygotes than expected (Hardy–Weinberg equilibrium test $P=0.04$). Our MTHFD1 1958G>A genotyping assay has been verified as already outlined; thus, systematic genotyping error is an unlikely reason for this deviation from Hardy–Weinberg equilibrium. We have observed this phenomenon in other control groups from the Irish population[3,10,11]and in the published genotype frequencies of other populations, including Dutch,[12] Turkish[13] and Mexican.[14] This observation gives weight to the concept that transmission of the 1958A allele is possibly associated with fetal inviability as we suggested originally,[3] or that heterozygosity has conferred a recent selective advantage.

The MTHFD1 1958G>A polymorphism may be the 'disease-causing' variant or it may be in linkage disequilibrium with another variant, thus acting as a marker. To explore this further, we searched for additional variants within the coding region of *MTHFD1* by sequencing homozygotes for this polymorphism, that is, 5 × 1958AA and 5 × 1958GG. Potential novel variants that were found associated with one genotype as opposed to the other would suggest linkage disequilibrium. The only other common polymorphism found in the coding region was the previously identified variant R134K (rs1803950). This variant is not in strong linkage disequilibrium with R653Q based on the sequencing results and analysis of frequencies within control samples (D' 0.402 (CI 0.2–0.57), LOD 2.55; r^2 0.024). An intronic SNP (rs3818240) was also identified between exons 16 and 17, but did not appear to be in

Table 2 Allele and genotype frequencies of MTHFD1 1958G>A in confirmatory NTD groups and control individuals

	Members of families with NTD			*Control group*
	Fathers (n = 127)	*Children (n = 176)*	*Mothers (n = 245)*	*Control (n = 770)*
Genotype				
GG	33 (0.26)[a]	50 (0.28)	62 (0.25)	209 (0.27)
GA	63 (0.50)	84 (0.48)	118 (0.48)	411 (0.53)
AA	31 (0.24)	42 (0.24)	65 (0.27)	150 (0.19)
Allele				
G	129 (0.51)	184 (0.52)	242 (0.49)	829 (0.54)
A	125 (0.49)	168 (0.48)	248 (0.51)	711 (0.46)

[a]Data in parentheses are genotype frequencies and may not sum to 1 in some columns due to rounding.

Table 3 Odds ratio estimates of MTHFD1 1958G>A genotype comparisons between NTD groups and controls

Comparisons (AA vs GA/GG)	*OR*	*OR (LL)*	*OR (UL)*	P-*value**
Mothers of all NTD cases/controls	1.49	1.07	2.09	0.02
Mothers of SB cases only/controls	1.56	1.05	2.33	0.03
Fathers of all NTD cases/controls	1.33	0.86	2.08	0.20
All NTD cases/controls	1.30	0.88	1.91	0.19

*Statistical significance was tested using likelihood ratio χ^2 analysis. OR = odds ratio; OR (LL) = 95% confidence interval, lower limit; OR (UL) = 95% confidence interval, upper limit; SB = Spina Bifida Aperta cases only.

linkage disequilibrium with R653Q based on the sequencing data. We conclude that there are no common variants within the coding region of the *MTHFD1* gene that are in strong linkage disequilibrium with the R653Q (1958G > A) polymorphism.

Discussion

The importance of a mother's nutritional status in foetal growth and development is well recognised,[15] but how maternal nutrition interplays with a mother's genetic makeup during pregnancy requires further exploration. Our analysis of a second set of samples from the Irish population has confirmed that the 1958AA genotype of *MTHFD1* is a maternal risk for NTDs. Observing the same association in two separate samples from the same population is highly unlikely to have occurred by chance. Although rather speculative, we also propose that transmission of the 1958A allele increases the chance of foetal inviability owing to a more severe NTD phenotype or other complications. It is noteworthy that we have recently identified this polymorphism as a maternal risk for severe abruptio placentae and unexplained second trimester loss.[10,11] Thus, this variant within the MTHFD1 enzyme appears to have a significant effect during pregnancy, but a functional effect has not been demonstrated to date. The enzyme activities of MTHFD1 play a central role in carbon-1 metabolism by providing folate cofactors for DNA synthesis and for cellular methylation reactions. Our previous analysis showed no association between the R653Q polymorphism and levels of plasma folate, red cell folate or plasma homocysteine levels.[3] Amino acid position 653 occurs within the 10-formyltetrahydrofolate synthetase domain of the MTHFD1 trifunctional enzyme and, therefore, it is possible that the R653Q polymorphism affects the supply of 10-formyltetrahydrofolate required for purine synthesis. Thus, any potential impact on the rate of DNA synthesis and consequently the rate of cell doubling is likely to have a major impact on pregnancy and embryonic development. It is also possible that the R653Q variant is acting as a marker for an unknown variant within the genomic region of the *MTHFD1* gene. We have resequenced all 28 exons of *MTHFD1* in 10 individuals (on both strands) and can state that the effect of the R653Q polymorphism is not being driven by another common variant within the coding region of *MTHFD1*. The HapMap assembly for samples of European ancestry reveals that the R653Q variant lies in a block of linkage disequilibrium over a ~30 kb region. The majority (96%) of 1958A alleles ('Q') lie within a single haplotype, suggesting that this region of the genome might have been subjected to selection pressure in the past. All other known variants in this block are within intronic regions that are unlikely to contain regulatory sequences and, therefore, are not obvious candidates for a disease association. A higher-resolution haplotype map of this region will be required to tease out whether R653Q is the 'disease-causing' variant and/or another variant is driving this association.

In conclusion, we have confirmed the MTHFD1 1958G > A polymorphism as a maternal risk for having an NTD-affected pregnancy by showing the same association in two separate samples from the Irish population. This association is another demonstration of the importance of common genetic differences between individuals and how they can particularly impact on women during the crucial period of pregnancy.

Acknowledgements

We wish to thank the Irish Association for Spina Bifida and Hydrocephalus and the Public Health Nurses for assistance with subject recruitment and the following Dublin maternity hospitals for control recruitment: National Maternity Hospital Holles Street, the Coombe Womens' Hospital and the Rotunda Hospital. This research was supported by the Intramural Research Program of the NICHD, NIH. The Health Research Board of Ireland provided support with the provision of a DNA sequencer. We also thank Riona Nolan and Tracey Claxton for their technical assistance.

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