Association study of MTHFD1 coding polymorphisms R134K and R653Q with migraine susceptibility.

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Abbreviations: IHS – International Headache Society, MA – migraine with aura, MO – migraine without aura, CSD – cortical spreading depression, FHM – Familial Hemiplegic Migraine, HCy – homocysteine, MTHFR – methylenetetrahydrofolate reductase , MTHF – 5,10-methylenetetrahydrofolate, SNP – single nucleotide polymorphism, NTD – neural tube defect, MTHFD1– MTHF dehydrogenase, MTHF cyclohydrolase, 10-formyltetrahydrofolate synthetase , RFLP – restriction-fragment length polymorphism, PCR – polymerase chain reaction, dNTPs – deoxynucleotide triphosphates, HRM – high resolution melt, χ^2 – chi-square, CI – confidence interval, LD – linkage disequilibrium, CEU (Utah residents with Northern and Western European ancestry)

ABSTRACT

Objective. – There is evidence that folate metabolism has a role in migraine pathophysiology, particularly in the migraine with aura subtype. In this study we investigate whether two non-synonymous single nucleotide polymorphisms (SNPs), rs1950902 (C401T; R134K) and rs2236225 (G1958A; R653Q), in MTHDF1 are associated with migraine in an Australian case-control population.

Background. – Increased plasma levels of homocysteine (HCy), one of the metabolites produced in the folate pathway, has been found to be a risk factor for migraine. There is also a genetic link, as a common polymorphism (C667T) that reduces the catalytic activity of MTHFR, the enzyme that catalyses the formation of HCy, is associated with an increase in risk of the migraine with aura (MA) subtype. MTHFD1 is a crucial multifunctional enzyme that catalyses three separate reactions of the folate pathway and therefore variants in MTHFD1 may also influence migraine susceptibility.

Methods. – The R134K and R653Q variants in MTHFD1 were genotyped in an Australian cohort of 520 unrelated migraineurs (162 were diagnosed with migraine without aura [MO] and 358 with MA) and 520 matched controls. Data were analysed for association with migraine and for interaction with the MTHFR C667T polymorphism.

Results. – We find no significant differences in genotype or allele frequencies for either SNP between migraineurs and controls, or when either MO or MA cases were compared to controls. In addition these MTHFD1 polymorphisms did not appear to influence the risk of MA conferred by the MTHFR 667T allele.

Conclusions – We find no evidence for association of the MTHFD1 R134K and R653Q polymorphisms with migraine in our Australian case-control population. However, as folate metabolism appears to be important in migraine, particularly with respect to the aura component, future studies using high throughput methods to expand the number of SNPs in folate-related genes genotyped and investigation of interactions between SNPs may be justified.

INTRODUCTION

Migraine is a common neurological disorder, affecting approximately 12% of the population [1]. Two main types of migraine have been classified by the International Headache Society, IHS [2]: migraine without aura (MO) or migraine with aura (MA), the latter distinguished by the presence of an aura preceding headache in the early stages of the attack. The aura can encompass various neurological disturbances, often visual, such as scintillating shapes, hallucinations or black spots. Cortical spreading depression (CSD), a slowly propagating wave of neuronal and glial depolarisation, is thought to underlie the aura and can activate trigeminal nociceptors [3, 4]. Migraine may be triggered by environmental influences, but the disorder also has a strong genetic component. Familial Hemiplegic Migraine (FHM) is a rare severe monogenic MA subtype in which the causal genes found thus far are channel proteins involved in ion or neurotransmitter transport in the brain. TWIK-related spinal cord potassium channel (TRESK, encoded by the KCNK18 gene) has also been shown to be causally involved in the more common form of MA [5]. Also for common migraine, variations in numerous genes involved in neuronal, hormonal and vascular functions have been suggested to contribute to susceptibility (reviewed in [6]) and genome wide association studies have now identified a number of susceptibility loci, particularly enriched near genes involved in synaptic or neuronal regulation [7].

There is evidence for co-morbidity of vascular conditions such as stroke and heart disease with migraine [8, 9], particularly the FHM subtype [10]. Stroke and heart attacks are correlated, and possibly causally related, with increased serum homocysteine (HCy) levels, which are modulated by the folate pathway [11]. Increased levels of HCy have also been observed in migraine: both in the plasma of MA sufferers compared to controls [12] and also in the cerebrospinal fluid of migraineurs, with particularly high levels in MA patients [13], suggesting that deregulation of the folate pathway may contribute to migraine pathophysiology. Folate metabolism is under the influence of numerous enzymes and cofactors such as vitamin B12, B2 and B9 (folic acid). Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism and catalyses the formation of 5-methylenetetrahydrofolate from 5,10-methylenetetrahydrofolate (MTHF), which is the predominant circulatory form of folate and the carbon donor for re-methylation of homocysteine to methionine. One relatively common functional single nucleotide polymorphism (SNP) in MTHFR, C677T, results in an alanine to valine substitution in the catalytic domain, reducing the enzyme activity by approximately 50%, and results in higher levels of plasma homocysteine in TT homozygotes [14]. The C677T polymorphism has been associated with a number of disorders including stroke and coronary artery disease [11], bipolar disorder,

schizophrenia and depression [15, 16] and neural tube defects (NTDs) [17]. Numerous studies have also investigated this polymorphism in migraine and found a significant association between the TT genotype and migraine, particularly the MA subtype. Although some studies have not replicated these results, meta-analyses in both European and Asian populations uphold an effect with respect to MA [18, 19].

Another important enzyme of the folate pathway is MTHF dehydrogenase, MTHF cyclohydrolase, 10-formyltetrahydrofolate synthetase (MTHFD1). MTHFD1 is a NADP-dependent trifunctional enzyme which, in three sequential reactions, provides one-carbon (1C) derivatives of tetrahydrofolate that are substrates for biosynthesis of thymidylate, purine nucleotides and methionine and thus is essential for DNA synthesis. MTHFD1 also utilizes MTHF and competes for this substrate with MTHFR so MTHFD1 variants may affect homocysteine plasma levels. Some relatively common non-synonymous SNPs in MTHFD1 have been identified: rs1950902 is a C to T transition at nucleotide 401, which results in an arginine to lysine substitution at amino acid 134 (C401T; R134K), and rs2236225 is a G to A transition at nucleotide 1958 resulting in an arginine to glutamate substitution at amino acid 653 (G1958A; R653Q). These have been tested for associations in a number of diseases or conditions which have been linked to either folate metabolism or homocysteine levels from epidemiological studies. In particular, the R653Q polymorphism has been found to increase risk of NTDs in offspring, spina bifida and heart defects [20-23], and may have a protective effect for acute lymphoblastic leukemia and other cancers [24], suggesting that this substitution can affect protein function. Both the R134K and R653Q polymorphisms have been reported to be associated with risk of gastric cancer in a Chinese population [25]. The observation of increased homocysteine levels and the increased risk of the MTHFR C667T allele with migraine, both particularly in relation to MA, led us to investigate the role of MTHFD1 SNPs rs1950902 and rs2236225 in an Australian migraine case-control population. This Australian cohort is larger and includes a higher proportion of MA sufferers, compared to other migraine cohorts in which MTHFD1 SNPs have been tested, and thus would give us more power to detect any associations.

METHODS

Study subjects

The study population consisted of 520 migraine cases and 520 age- and sex-matched controls that were recruited from South-Eastern Australia (Table 1). The subjects were adult Caucasians, having ancestors who emigrated within the last 160 years from various locations within the British Isles and other parts of Europe. Of the migraineurs, 162 were diagnosed with MO and 358 were diagnosed with MA. Migraineurs were diagnosed by a clinical neurologist as having either MO or MA based strictly on the widely accepted criteria specified by the IHS [2]. The study was originally approved by Griffith University, and subsequently by the Queensland University of Technology Ethics Committees for Experimentation on Human Subjects, and all individuals provided informed consent. A whole blood sample was collected from each participant and genomic DNA was extracted from white blood cells using a standard salting out method [26]. Samples used for the genotyping study were all from unrelated individuals and the control group consisted of individuals with no family history of migraine.

Genotyping

The genotype for rs1950902 was determined by restriction-fragment length polymorphism (RFLP) analysis. The primers 5'-GGCGTACAAGGAATGAAAC-3' and 5'-GGATGTGGATGGGTAAGTG-3' were used in polymerase chain reaction (PCR) with GoTaq® polymerase (Promega) to generate a 225 bp product using the following thermocycler conditions: 95°C for 10 min, 35 cycles of 95°C for 45 s, 48°C for 45s, 72°C for 45 s and 72°C for 7 min. PCR products were digested with *Bsm*AI (NEB) for 8h at 55°C resulting in two fragments of 180 and 45bp for the 401T allele and three fragments of 131, 49 and 45bp for the 401C allele which are distinguishable on 3% agarose gels.

Genotyping of rs2236225 was performed by high resolution melt (HRM) analysis. The primers 5'-CATTCCAATGTCTGCTCCAA-3'and 5'-GTTTCCACAGGGCACTCC-3' were used to amplify a 254 bp product by PCR using GoTaq® Hot Start polymerase (Promega) in a Rotor-Gene 6000 (Corbett Research) with the intercalating dye SYTO9 (Invitrogen) to detect fluorescence. After thermocycling: 95°C for 1 min and 45 cycles of 95°C for 5s and 60°C for 10s, melt curves were generated by increasing the temperature between 80 and 90°C, rising 0.1°C per 2s. Three distinct melting curves were detected and homozygous GG, heterozygous

GA and homozygous AA genotypes were confirmed by Sanger sequencing on a 3130 Genetic Analyzer (Applied Biosystems).

The MTHFR C667T polymorphism was genotyped by RFLP analysis as described previously [27].

Data Analysis

Hardy-Weinberg equilibrium was verified for observed genotype frequencies for each SNP to detect deviation from the normal genotype distribution in the population. Chi-square (χ^2) analysis was performed to test for significant differences in genotype and allele frequencies for each SNP in migraineurs, MO and MA subgroups versus controls to detect any association with migraine. Multivariate logistic regression analysis was performed to examine the possibility of combined effects of the MTHFR and MTHFD1 genes by incorporating genotype data for both MTHFD1 C401T and G1958A data in addition to the presence of absence of the MTHFR 667T allele. Risk magnitudes were estimated by calculating an odds ratios with 95% confidence intervals (CI). P-values of ≤ 0.05 were considered significant. The Statistical Package for Social Sciences (version 21) was used for statistical analyses. Power analysis indicated that if the polymorphisms were to confer a two-fold increase in risk of migraine, the case and control groups used in this study were of sufficient size to have approximately 80% power to detect an allelic association at the 0.05 level. Haploview was used to analyse the local linkage disequilibrium (LD) across the MTHFD1 locus [28] using HapMap data and analysis of LD between rs1950902 and s2236225 in this Australian migraine cohort was performed using PLINK (v1.07) [29].

RESULTS

The study population consisted of 520 migraine cases and 520 age- and sex-matched controls that were recruited from South-Eastern Australia. Clinical characteristics for the cohort are shown in Table 1. The female to male ratio is 2.6:1 in MO cases and 3.0 in MA cases, which corresponds to the increased prevalence of migraine observed in females [1]. However, although MA typically accounts for only a third of migraineurs, our migraine cohort has a higher proportion of MA cases compared to MO. This is probably the result of the more severe MA-sufferers being more likely to identify as migrainuers and/or volunteer.

MTHFD1 SNP rs1950902 (C401T; R134K) was genotyped by a RFLP assay. The distribution of genotypes was statistically consistent with Hardy-Weinberg expectations in both control and case populations (p=0.950 and p=0.624, respectively). Genetic and allelic distributions, shown in Table 2, were not significantly different between migraineurs and controls, or for either of the migraine subgroups, MO or MA, when compared to controls (Table 2). We obtained a minor allele frequency of 19.4% in controls and 19.3% in migraine cases for the 401T allele, which is similar to the reported frequencies in dbSNP of 19.1% and 20.4% from the 1000 Genomes and Hapmap-CEU populations, respectively.

MTHFD1 SNP rs2236225 (G1958A; R653Q) was genotyped using a HRM assay in the 520 control and 520 migraineur samples. Both controls and cases were in Hardy-Weinberg equilibrium (p=0.087 and p=0.495, respectively). Genotypic and allelic distributions did not differ significantly between total migraine cases and controls or when controls were compared with either the MO or MA subgroups (Table 2). We found a minor allele frequency of 43.7% in controls and 42.3% in migraineurs for the 1958A allele. Reported frequencies in dbSNP for rs2236225 are 34.6% and 42.0% for the 1000 Genomes and Hapmap-CEU populations, respectively.

The two SNPs rs1950902 and rs2236225 are separated by 26.5 kb on chromosome 14. Haploview analysis of linkage disequilibrium (LD) across the MTHFD1 locus using HapMap data suggests that the SNPs are in two separate LD blocks. Using genotyping data for rs1950902 and rs2236225 obtained from this study PLINK was used to determine whether the markers were independent of each other. This showed that the two SNPs are not highly correlated ($r^2 = 0.028$) or in high LD (D' = 0.39), but are not completely independent.

Oterino et al. have previously reported that the pathogenic role of the MTHFR 667T allele in migraine is modulated by the functional polymorphisms in other folate pathway enzymes, including thymidylate synthase (TS) and MTHFD1 [30]. We previously found a positive association of the MTHFR 667T allele with the MA subtype of migraine in a subset of this cohort [31]. To be able to test for gene-gene interactions between MTHFR and MTHFD1 in this population, we genotyped the remainder of the population for MTHFR C667T. Using this larger cohort, in conjunction with the data obtained for MTHFD1, we performed a logistic regression analysis in which MTHFR genotype was dichotomized into non C667T allele or C667T allele carriers and the risk of migraine with this marker was considered both separately and in a model which also included the variant alleles for both MTHFD1 rs1950902 and rs2236225 markers (Table 3). As reported previously we found that the MTHFR 667T allele conferred an increased a risk of migraine (OR = 1.5, CI = 1.01-2.23, p = 0.043), and in particular the MA subtype (OR = 1.83, 95%, CI = 1.01-2.23, p = 0.004). The odds ratio for MTHFR in MA increased only slightly increased (OR = 1.87, 95% CI = 1.18-2.98, p = 0.008) when MTHFD1 alleles were taken into account, suggesting that polymorphism at MTHFD1 has a minimal effect on MA risk conferred by MTHFR 667T in this cohort. A formal interaction analysis was performed for the total migraine cohort with regression models including MTHFR and both MTHFD1 variants (considered separately). This analysis did not reveal any statistically significant main or interaction terms (results not shown).

DISCUSSION

In relation to migraine, most emphasis to date has been on the role of MTHFR polymorphisms, in particular the C667T variant which is both common (minor allele frequency is 32.5% in 1000 Genome dataset), and results in a substantial reduction in enzyme activity. However, polymorphisms in other folate pathway enzyme genes have also been investigated for association with migraine, including 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), methionine synthase reductase (MTRR), thymidylate synthase (TYMS), nicotinamide N-methyltransferase (NNMT), serine hydroxymethyltransferase 1 (SHMT1) and MTHFD1 [30, 32, 33]. MTHFD1 is a crucial enzyme of the folate pathway with multiple functions. In mice homozygous disruption of the Mthfd1 gene is early embryonic lethal, while mice heterozygous for the disruption appear healthy, but have impaired folate-mediated one carbon metabolism [34].

This study evaluated whether two non-synonymous SNPs in MTHFD1, rs1950902 (C401T; R134K) and rs2236225 (G1958A; R653Q) are associated with the risk of migraine. No significant association was found for either SNP with migraine, or when analysis was performed in either the MO or MA sub-groups of migraine (Table 2). This is the first study of the R134K polymorphism in relation to migraine. The MTHFD1 R134K polymorphism results in an arginine to lysine change in the dehydrogenase/cyclohydrolase domain of the protein, although there are no studies to date of its functional consequences. The MTHFD1 R653Q polymorphism is located within the 10-formyltetrahydrofolate synthetase domain and may affect biosynthesis of thymidylate, purine nucleotides and methionine. Biochemical analysis of the R653Q protein has shown that is more thermolabile than the wild type protein and results in reduced MTHFD1 metabolic activity in transfected cells [23]. A previous study in a Spanish population has also investigated MTHFD1 R653Q with respect to migraine risk, and found, in agreement with this study, no significant differences in genotypic and allelic distributions between controls and migraineurs, or when analysis was performed with either MO or MA patients and controls [30]. However, when they analysed the MTHFD1 R653Q genotype in conjunction with MTHFR C667T genotype and found that the Q653 allele modulated the risk conferred by the MTHFR T667 allele, while we did not find a large effect in this study (Table 3). Oterino et al. also found that a tandem repeat polymorphism in the TMYS promoter also interacted with MTHFR T667 to greatly increase the likelihood of experiencing aura in migraineurs [30]. MTRR and MTR have also been investigated with respect to migraine

and a haplotype of MTRR was associated with a reduced risk for MA [33]. Therefore interactions between SNPs within a gene, and between different genes of the folate pathway, may influence migraine susceptibility. Many polymorphisms in folate pathway genes have been identified, including non-synonymous SNPs, which in particular may have functional consequences. We were unable to test whether MTHFD1 polymorphisms influenced HCy plasma levels in this study as this information was not assayed with sample collection. Another study of migraineurs where this data was available found that the number of MTHFR C667T alleles was the best genetic predictor of HCy levels, but by using a multi-dimensionality reduction algorithm could show that other folate pathway enzyme polymorphisms, including MTHFD1 R653Q, predicted higher HCy plasma levels and the MA phenotype [32]. However, how HCy levels and the risk of suffering MA are connected is still unclear as a study by Scher et al. found the risk conferred by MTHFR C667T genotype to be independent of HCy levels [35].

As the folate-metabolising proteins may act synergistically or antagonistically, variants in the various genes may augment or compensate each other. Thus future studies would benefit by expanding the number of genes analysed in individuals and explore how they interact. For example, in a study investigating folate-related gene and risks of spina bifida and conotruncal heart defects, 118 SNPs in 14 different folate-related genes were genotyped [22]. Furthermore, analysis of the role of folate-related genes in migraine may be complicated by the nutritional status of the individuals, similar to that seen for determining stroke risk [36]. Folate supplementation in early pregnancy has been robustly shown to reduce the risk of NTDs [37], and there is evidence from clinical trials in migraine patients that folate and vitamin B treatment helps to ameliorate migraine symptoms [38-40]. MTHFR C667T genotype, as well as MTRR A66G, were found to have an effect on this [40], however, other folate gene SNPs, including the MTHFD1 variants reported here were not tested. Thus environmental factors, such as the folate status of the individual may also play a role in whether a particular SNP in a folate pathway enzyme is important to migraine risk, e.g. it may only increase migraine risk in an individual with low folate or vitamin B intake such as observed for risk of NTDs in offspring [21].

In conclusion, while the MTHFD1 R134K and R653Q have been found to be associated with other disorders in which folate metabolism is known to be involved, we find no evidence that on their own they increase migraine susceptibility in an Australian case-control population. The R653Q results confirm previous studies in other

populations, but this is the first study of the R134K polymorphism in migraine. However, as folate metabolism appears to be important in migraine, particularly with respect to the aura component, future studies using high throughput methods to expand the number of SNPs in folate-related genes genotyped and explore interactions between SNPs are warranted.

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Table 1. Characteristics of Migraine Patients According to MO and MA Subgroups and Controls

Parameter		MO	MA	Controls
Number		162	358	520
Age: Mean (range)		47.8 (18-67)	42.3 (18-65)	43.8 (18-63)
Sex	Female	117	270	387
	Male	45	88	133
Migraine Frequency	<1/month	28.1%	31.8%	N/A
	1-2/month	30.0%	31.2%	
	3-4/month	15.1%	14.7%	
	>4/month	26.8%	22.4%	

MO – migraine with aura, MA – migraine without aura
N/A – not applicable

Table 2. Genotype and allele frequencies of MTHFD SNPs rs1950902 and rs2236225 in a migraine case-control population.

SNP	Group	Genotypes			P	Alleles		P
rs1950902 C401T		CC (%)	CT (%)	TT (%)		C (%)	T (%)	
	Control	326 (65.1)	156 (31.1)	19 (3.8)		808 (80.6)	194 (19.4)	
	Migraine	307 (64.8)	151 (31.9)	16 (3.4)	0.923	765 (80.7)	183 (19.3)	0.974
	MO	90 (63.4)	45 (31.7)	7 (4.9)	0.813	225 (79.2)	59 (20.8)	0.597
	MA	217 (65.4)	106 (31.9)	9 (2.7)	0.692	540 (81.3)	124 (18.7)	0.727
		GG (%)	GA (%)	AA (%)		G (%)	A (%)	
rs2236225 G1958A	Control	150 (29.8)	267 (53.0)	87 (17.3)		567 (56.3)	441 (43.7)	
	Migraine	164 (34.0)	228 (47.3)	90 (18.7)	0.196	556 (57.7)	408 (42.3)	0.523
	MO	58 (38.7)	70 (46.7)	22 (14.7)	0.120	186 (62.0)	114 (38.0)	0.077
	MA	106 (31.9)	158 (47.6)	68 (20.5)	0.277	370 (55.7)	294 (44.3)	0.832

MO - Migraine without aura, MA - Migraine with aura
P-values were calculated by χ^2-analysis, significance is taken at $P \leq 0.05$

Table 3. Logistic regression analysis of MTHFR C667T, and MTHFD1 C401T and G1958A, to explore gene-gene interactions.

Group	Variables in the model	Odds Ratio	95% CI	Significance (P)
All Migraine	667T	1.50	1.01-2.23	0.043*
	667T + 401T +1958A	1.50	0.96-2.33	0.074
MO	667T	0.80	0.40-1.57	0.509
	667T + 401T +1958A	0.71	0.32-1.56	0.393
MA	667T	1.83	1.21-2.76	0.004*
	667T + 401T +1958A	1.87	1.18-2.98	0.008*

MO - Migraine without aura, MA - Migraine with aura
667T - MTHFR 667T allele, 401T - MTHFD1 401T allele, 1958A - MTHFD1 1958A allele
P-values were calculated by regression analysis, significance was taken at $P \leq 0.05$ (*).