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ORIGINAL ARTICLE

Methylenetetrahydrofolate reductase gene polymorphisms and cerebral palsy in Chinese infants

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Genetic polymorphisms of methylenetetrahydrofolate reductase (*MTHFR*) have been suggested as being associated with cerebral palsy (CP) but the evidence is uncertain. The purpose of this study was to investigate whether *MTHFR* gene polymorphisms contribute to the development of CP in Chinese infants. For this study, 169 health controls and 159 infants with CP including 43 cases also suffering from mental retardation (MR) were recruited. Genomic DNA was prepared from venous blood and all five single nucleotide polymorphisms in *MTHFR* (rs4846049, rs1476413, rs1801131, rs1801133 and rs9651118) were genotyped using TaqMan technology. There were no significant differences in allele or genotype frequencies between the CP patients and controls at any of the five genetic polymorphisms. Subgroup analysis found statistically significant difference in allele and genotype frequencies between cases with both CP and MR (CP + MR) compared with both CP-only cases and controls at rs4846049, rs1476413 and rs1801131. The frequencies of the T alleles of rs4846049, rs1476413 and the G allele of rs1801131 were greater in the CP + MR patients than in the CP-only patients and controls. This study provides the first evidence pointing to a *MTHFR* gene polymorphism as a potential risk factor for CP combined with MR.
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Keywords: cerebral palsy; gene polymorphism; mental retardation; MTHFR

INTRODUCTION

Cerebral palsy (CP) covers a group of non-progressive chronic disorders of motor function and posture caused by lesions of the developing fetal or infant brain.[1,2] The characteristic signs of CP are spasticity, movement disorders, muscle weakness, ataxia and rigidity. The clinical classification of CP is based on motor deficit and other combined symptoms; such as mental retardation (MR), epilepsy, optic and hearing loss.[3] CP is the most common cause of severe physical disability in childhood, occurring in 1–2/1000 live births.[4] Its prevalence rises dramatically with decreasing gestational age at birth.[5] The possible risk factors for CP are abundant with each making only a small contribution, many cases are multifactorial in origin and exhibit marked etiologic heterogeneity. Risk factors for CP can be categorized as prenatally, perinatally and postnatally acquired of which about 70–80% are acquired prenatally.[6] A mounting body of recent evidence points to genetic influences on the occurrence of CP. This evidence includes familial data,[7] twin studies[8] and specific genetic factors,[9–12] and indicate that CP may be related to genomic factors, as well as to environmental incursions during brain development.

Methylenetetrahydrofolate reductase (MTHFR) catalyses irreversibly the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl donor for methionine synthesis from homocysteine and a key single carbon donor that participates in S-adenosylmethionine synthesis and the methylation of DNA. MTHFR also has an important role in the modulation of plasma homocysteine status by converting it into methionine. Studies showed that *MTHFR* gene polymorphisms are associated with inherited thrombophilias,[13–15] which can result in adverse pregnancy outcomes such as CP.[16] However, the evidence on association between *MTHFR* and CP is somewhat contradictory.[13,17,18] We concluded that there was a need for a large case–control study to investigate the association of *MTHFR* polymorphisms and CP, particularly the Chinese context. The present study therefore focuses on the potential linkage between *MTHFR* polymorphisms and CP in infants in central China.

SUBJECTS AND METHODS

Subjects

The study population consisted of 159 CP patients (50 girls 31.4%, 109 boys 68.6%, mean age ± s.d.: 16.9 ± 14.4 months) chosen from centers for CP

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rehabilitation in the Third Affiliated Hospital of Zhengzhou University, Zhengzhou Children's Hospital and the First Affiliated Hospital of the Henan Traditional Chinese Medical College from 1 May 2008 to 31 Oct 2009. Of the total 159 cases, 43 cases also suffered from MR. The 169 healthy control subjects included in the study were chosen from the Child Healthcare Department at the same hospital during the same period and were matched for age, sex and ethnicity (64 girls 37.9%, 105 boys 62.1%, mean age ± s.d.: 16.4 ± 13.8 months). All subjects including CP patients and healthy controls were Han Chinese from Henan province and had been informed consent of this study. CP patients were diagnosed by a child neurologist either by clinical examination or by using medical records. MR was identified by a specialist from the Department of Child Development based on the Bayley Scales measurement of mental developmental index (<70).[19] Children with hypotonia, ataxia, myopathy, genetic syndrome or chromosomal anomaly were excluded. Approval for the study was obtained from the ethics committee of Zhengzhou University in accordance with the Helsinki declaration.

Genotyping

A total of five single nucleotide polymorphisms (SNPs), whose minor allele frequencies in the Chinese Han population were more than 0.1, were selected from the dbSNP database (www.ncbi.nlm.nih.gov/SNP) and the hapmap human SNP database (www.hapmap.org). The five SNPs are rs4846049 in 3′UTR, rs1476413 in intron 10, rs1801131 in exon 7, rs1801133 in exon 5 and rs9651118 in intron 2, respectively. Of these, rs1801131 (A1298C) and rs1801133 (C677T) are known functional variants of MTHFR. Genomic DNA was prepared from venous blood using AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Union city, CA, USA) according to the recommended procedure. SNPs were genotyped on the ABI 7900 DNA detection system (Applied Biosystems, Foster City, CA, USA) using TaqMan technology and the probes were designed by the Applied Biosystems service. A standard 5 μl PCR reaction was carried out using TaqMan Universal PCR Master Mix reagent kits under the guidelines provided. The person who analyzed the genotype results was blind to the clinical data.

Statistical analysis

We conducted Hardy–Weinberg equilibrium tests, allele and genotype frequency analysis online on the SHESIS software platform (http://analysis.bio-x.cn/). Linkage disequilibrium was measured using standardized D′, and the discrepancies in allele and genotype frequencies on single loci between patients and controls were compared using a Monte Carlo simulation strategy. The program SNPSpD (http://gump.qimr.edu.au/general/daleN/matSpD/), which takes marker linkage disequilibrium information into consideration, was used to correct for multiple testing performed on each individual SNP. The numbers of observations for each haplotype were compared using χ^2 tests. Bonferroni correction was applied for haplotype analysis. All reported *P*-values were two-tailed and statistical significance was set at $P<0.05$.

RESULTS

The frequencies of alleles/genotypes of the five investigated SNPs are listed in Table 1. The χ^2 goodness-of-fit test showed that the genotypic distribution of the five SNPs in both CP patients and controls was in Hardy–Weinberg equilibrium. For the totality of the subjects, there were no significant differences of allele or genotype frequencies between CP patients and controls at any of the five genetic polymorphisms. Subgroup analysis was done according to gender, gestation age, birth weight, birth asphyxia and combined CP + MR in CP patients. Significant differences in allele and genotype frequencies were observed between CP + MR patients and controls at rs4846049 (P=0.031 after SNPSpD correction), rs1476413 (P=0.028 after SNPSpD correction) and rs1801131 (P=0.014 after SNPSpD correction) (Table 1), and a similar differential pattern for these three SNP allele/genotype frequencies was observed between the CP + MR and CP-only patients (rs4846049, P=0.016; rs1476413, P=0.027; rs1801131, P=0.004 after SNPSpD correction) (Table 2). The frequencies of the T allele of rs4846049, the T allele of rs1476413 and the G allele of rs1801131 (A1298C) were greater in CP + MR patients

Table 1 Allele and genotype frequencies of SNPs in *MTHFR*

Group	*Allele frequency*		P	*Genotype frequency*			P	*H–W*
rs4846049	G	T		G/G	G/T	T/T		
CP+MR	65 (0.756)	21 (0.244)	0.014	25 (0.581)	15 (0.349)	3 (0.070)	0.009	0.719
CP	271 (0.852)	47 (0.148)	0.668	115 (0.723)	41 (0.258)	3 (0.019)	0.565	0.766
Control	292 (0.864)	46 (0.136)		124 (0.734)	44 (0.260)	1 (0.006)		0.163
rs1476413	C	T		C/C	C/T	T/T		
CP+MR	65 (0.756)	21 (0.244)	0.011	25 (0.581)	15 (0.349)	3 (0.070)	0.008	0.719
CP	270 (0.849)	48 (0.151)	0.513	114 (0.717)	42 (0.264)	3 (0.019)	0.545	0.700
Control	293 (0.867)	45 (0.133)		125 (0.740)	43 (0.254)	1 (0.006)		0.183
rs1801131	G	T		G/G	G/T	T/T		
CP+MR	21 (0.244)	65 (0.756)	0.004	3 (0.070)	15 (0.349)	25 (0.581)	0.004	0.719
CP	44 (0.138)	274 (0.862)	0.515	3 (0.019)	38 (0.239)	118 (0.742	0.549	0.977
Control	41 (0.121)	297 (0.879)		1 (0.006)	39 (0.231)	129 (0.763)		0.283
rs1801133	A	G		A/A	A/G	G/G		
CP+MR	51 (0.593)	35 (0.407)	0.667	15 (0.349)	21 (0.488)	7 (0.163)	0.800	0.939
CP	208 (0.654)	110 (0.346)	0.162	69 (0.434)	70 (0.440)	20 (0.126)	0.358	0.733
Control	209 (0.618)	129 (0.382)		61 (0.361)	87 (0.515)	21 (0.124)		0.238
rs9651118	C	T		C/C	C/T	T/T		
CP+MR	13 (0.151)	73 (0.849)	0.097	1 (0.023)	11 (0.256)	31 (0.721)	0.262	0.983
CP	62 (0.195)	256 (0.805)	0.227	9 (0.057)	44 (0.277)	106 (0.667)	0.348	0.135
Control	79 (0.234)	259 (0.766)		10 (0.059)	59 (0.349)	100 (0.592)		0.742

Abbreviations: CP+MR, cerebral palsy combined with mental retardation; H–W, Hardy–Weinberg; MTHFR, methylenetetrahydrofolate reductase; SNP, single nucleotide polymorphism.

Table 2 Distribution of *MTHFR* alleles and genotypes in CP cases with and without MR

Group	*Allele frequency*		P	*Genotype frequency*			P	*H–W*
rs4846049	G	T		G/G	G/T	T/T		
CP+MR	65 (0.756)	21 (0.244)	0.005	25 (0.581)	15 (0.349)	3 (0.070)	0.006	0.719
CP*	174 (0.888)	22 (0.112)		76 (0.776)	22 (0.224)	0 (0.000)		0.211
rs1476413	C	T		C/C	C/T	T/T		
CP+MR	65 (0.756)	21 (0.244)	0.007	25 (0.581)	15 (0.349)	3 (0.070)	0.008	0.719
CP*	173 (0.883)	23 (0.117)		75 (0.765)	23 (0.235)	0 (0.000)		0.188
rs1801131	G	T		G/G	G/T	T/T		
CP+MR	21 (0.244)	65 (0.756)	0.001	3 (0.070)	15 (0.349)	25 (0.581)	0.003	0.719
CP*	19 (0.097)	177 (0.903)		0 (0.000)	19 (0.194)	79 (0.806)		0.288
rs1801133	A	G		A/A	A/G	G/G		
CP+MR	51 (0.593)	35 (0.407)	0.165	15 (0.349)	21 (0.488)	7 (0.163)	0.351	0.939
CP*	133 (0.679)	63 (0.321)		47 (0.480)	39 (0.398)	12 (0.122)		0.385
rs9651118	C	T		C/C	C/T	T/T		
CP+MR	13 (0.151)	73 (0.849)	0.186	1 (0.023)	11 (0.256)	31 (0.721)	0.418	0.983
CP*	43 (0.219)	153 (0.781)		7 (0.071)	29 (0.296)	62 (0.633)		0.178

Abbreviations: CP+MR, cerebral palsy combined with mental retardation; CP*, CP without MR; MTHFR, methylenetetrahydrofolate reductase.

Table 3 The linkage disequilibrium among the SNPs

D′/r²	*rs4846049*	*rs1476413*	*rs1801131*	*rs1801133*	*rs9651118*
rs4846049		0.962	0.972	0.862	0.997
rs1476413	0.926		0.972	0.862	0.997
rs1801131	0.852	0.852		0.975	0.997
rs1801133	0.214	0.214	0.247		0.960
rs9651118	0.045	0.045	0.041	0.441	

Abbreviation: SNP, single nucleotide polymorphism.
The standardized D′ values are shown above the diagonal, and the r^2 values are shown below the diagonal.

Table 4 Estimated *MTHFR* haplotype frequencies

	Frequencies			
Haplotype	*CP with MR*	*Controls*	P-*value*	*OR (95% CI)*
G C T A T	51.00 (0.593)	201.15 (0.595)	0.562	0.866 (0.533 ~ 1.409)
G C T G C	13.00 (0.151)	69.91 (0.207)	0.172	0.639 (0.335 ~ 1.220)
G C T G T	1.00 (0.012)	15.34 (0.045)	0.129	0.234 (0.031 ~ 1.796)
T T G G T	21.00 (0.244)	34.33 (0.102)	0.005*	2.695 (1.470 ~ 4.941)
Global			0.021*	

Abbreviations: CI, confidence interval; CP, cerebral palsy; MR, mental retardation; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio.
Loci chosen for haplotype analysis: rs4846049, rs1476413, rs1801131, rs1801133, rs9651118. Haplotypes with a frequency <0.01 in both control & case have been dropped.
**P*-values after Bonferroni correction (×5).

than in controls or CP-only patients. No significant differences were detected in either allele or genotype frequencies of the other two SNPs, rs1801133 (C677T) and rs9651118. There was no difference in the allele or genotype frequencies in the other CP subgroups classified by gender, gestation age, birth weight and birth asphyxia (data not shown).

The estimation of linkage disequilibrium for all pairs of SNP markers showed strong linkage disequilibrium (D′ > 0.8) (Table 3). We then constructed haplotypes of all SNPs and analyzed only the most common ones (those with a frequency <0.01 were excluded from the analysis). Haplotype analysis revealed a significant global *P*-value (*P*=0.021 after Bonferroni correction, Table 4) and haplotype frequency discrepancies. The haplotype *TTGGT* (rs4846049, rs1476413, rs1801131, rs1801133 and rs9651118) was observed to be strongly associated with CP + MR (*P*=0.001, odds ratio=2.695, 95% confidence interval=1.470 ~ 4.941, *P*=0.005 after Bonferroni correction).

DISCUSSION

CP covers a range of neurological conditions that share common disorders of motor function and posture. The underlying cause of CP remains obscure in most cases and efforts to reduce the incidence of perinatal brain injury in term infants have failed despite the improvement in obstetric and neonatal care. The genetic influences affecting CP have been the subject of much attention in recent years.[10–12,20,21] Several genes have been identified as risk factors, and have been categorized as thrombophilic, cytokine, apolipoprotein E and a group related to cardiovascular physiology and the functioning of the immune system.[21] Among the candidate genes, several SNPs of the *MTHFR* genes have been investigated either in normal populations or CP patients.[11,13,22] A case–control study from Australia found that *MTHFR* C677T was associated with an increased risk of developing any type of CP and was also associated with diplegia for all gestation periods.[13] Other studies have produced contrary results and have failed to confirm an association between *MTHFR* C677T (rs1801133) and CP.[11,23,24] In the present study of Chinese infants, there were no significant differences of allele or genotype frequencies between CP patients and controls at any of the five *MTHFR* gene polymorphisms. These contradictory findings might be owing to ethnic or racial-ethnic differences of folate in the diet or metabolism or to other characteristics of CP cases. The largest population study for geographical and

ethnic variation of the *MTHFR* 677C>T allele demonstrated ethnic and geographical variation.[25] The frequency of the homozygous TT genotype is particularly high in northern China (20%), southern Italy (26%) and Mexico (32%). The incidence of neural tube defect is high in Mexico and northern China, but not in southern Italy.[22] The impact of such marked geographical and ethnic distribution of the *MTHFR* 677C>T allele on the distribution of the disease is still unclear. There is no evidence to show that geographical and ethnic variations in the *MTHFR* 677C>T allele is associated with CP.

The prevalence of severe MR is about 3.0/1000 live births and in most cases the causes are unknown. MR is also an important condition in Down's syndrome, hypothyroidism or CP. The prevalence of MR varies with the type of CP and increases significantly when epilepsy is present and is common in severely disabled CP patients. In this study, 27% of the cases suffered from both CP + MR, and this proportion is in line with other similar studies.[26] An association between *MTHFR* polymorphisms and intelligence quotient in Down's syndrome patients has been reported,[27] but not in MR-only patients.[28] In the cohort for this study, we observed a significant difference in allele and genotype frequencies between CP + MR patients and controls at rs4846049, rs1476413 and rs1801131 and there was a statistically significant difference in the frequencies of the three SNPs between CP + MR and CP-only cases. Furthermore, the haplotype TTGGT (rs4846049, rs1476413, rs1801131, rs1801133 and rs9651118) was statistically associated with MR. A1298C (rs1801131), which changes a glutamate into an alanine residue, destroys an *Mbo*II recognition site and results in decreased MTHFR activity, has been associated with lower blood folate and higher homocysteine levels in some studies. These results suggest that folate metabolic gene polymorphisms are associated with CP + MR and that the genes susceptible to CP differ somewhat according to the clinical CP subtype although such genes are not the only possible causes of this clinical syndrome.

Gender related pathophysiological features are thought to be related to CP.[29,30] We therefore, checked the gender differences in genetic susceptibility to CP, but found no association between the five *MTHFR* gene polymorphisms and CP. Prematurity is another important contributor to CP. The overall reported incidence of CP is 1.2/1000 live birth in term infants compared with 86/1000 live birth in extremely premature infants.[20] Studies have identified specific gene polymorphisms that specifically predispose to prematurity.[11,31] In this study, we compared gestation age differences with genetic susceptibility to CP, but, no difference was observed with respect to the *MTHFR* gene polymorphisms, which is in accordance with a previous report.[11]

In conclusion, our results support the conclusion that none of the five SNPs in *MTHFR* contribute to the occurrence of CP in Chinese infants. However, this is the first report to our knowledge to demonstrate that *MTHFR* genetic polymorphisms are associated with CP combined with MR. It adds to the existing evidence that certain gene variants may in some way contribute to CP. Additional investigations are needed to test the linkage of genetic factors with different types of CP. However, it should be emphasized that our findings are still preliminary, given the small number of subjects in each group and further replication studies with a larger number of subjects will be necessary to provide a more definitive answer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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