

Population- and Family-Based Studies Associate the *MTHFR* Gene with Idiopathic Autism in Simplex Families

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Published online: 12 November 2010
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Abstract Two methylenetetrahydrofolate reductase gene (*MTHFR*) functional polymorphisms were studied in 205 North American simplex (SPX) and 307 multiplex (MPX) families having one or more children with an autism spectrum disorder. Case–control comparisons revealed a significantly higher frequency of the low-activity 677T allele, higher prevalence of the 677TT genotype and higher frequencies of the 677T-1298A haplotype and double homozygous 677TT/1298AA genotype in affected individuals

relative to controls. Family-based association testing demonstrated significant preferential transmission of the 677T and 1298A alleles and the 677T-1298A haplotype to affected offspring. The results were not replicated in MPX families. The results associate the *MTHFR* gene with autism in SPX families only, suggesting that reduced *MTHFR* activity is a risk factor for autism in these families.

Keywords Autism spectrum disorders (ASDs) · Gene association · Methylenetetrahydrofolate reductase (*MTHFR*) · Functional polymorphism · Epigenetics · Methylation

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Introduction

Autism spectrum disorders (ASDs) are a group of debilitating neurodevelopmental conditions characterized by impairments in verbal and non-verbal communication, social interaction and the presence of repetitive behaviors and restricted interests (American Academy of Pediatrics 2001). These lifelong conditions, affecting about 1/110 individuals (Centers for Disease Control and Prevention (CDC) 2009), are generally thought to result from an interaction of genetic and environmental factors, and to be genetically heterogeneous (Risch et al. 1999; Bailey et al. 1996). Despite a general lack of success in identifying genes that are responsible for a majority of ASD cases, there are some striking observations that suggest that changes in both a wide variety of genes and epigenetic modifications (e.g. gene silencing or lack thereof) of genes important for normal brain development and growth, cognitive function and behavior are involved in the etiology of ASDs.

The term “epigenetics” refers to a heritable reversible change in gene expression that is mediated by mechanisms

other than alterations in the primary nucleotide sequence of a gene (Bird 2002) and involves methylation and histone modification. Epigenetic defects, including abnormal methylation patterns, have been implicated in idiopathic ASDs (Jiang et al. 2004; Schanen 2006) as well as ASD-associated syndromes such as the Prader-Willi/Angelman syndromes (PWS/AS) (Schanen 2006; Hogart et al. 2007; Bittel et al. 2007), Rett syndrome (Webb and Watkiss 1996; Krepischi et al. 1998; Ghosh et al. 2008) and Fragile-X syndrome (Tabolacci et al. 2005; Schanen 2006). Epigenetic modifications by methylation are particularly important to parent-of-origin specific methylation and gene expression patterns that are essential for brain development, brain growth, cognitive function, and behavior (Isles and Wilkinson 2000; Reik and Walter 2001; Goos and Silverman 2001; Davies et al. 2005).

MTHFR (5,10 methylenetetrahydrofolate reductase) is one of the key enzymes involved in DNA methylation. Two common functional polymorphisms in the *MTHFR* gene (677C → T in Exon 4 within the N-terminal catalytic domain and 1298 A → C in Exon 7 within the C-terminal regulatory domain) are associated with reduced enzyme activity (Frosst et al. 1995; van der Put et al. 1998). Individuals with the 677TT genotype have only 30% in vitro MTHFR enzyme activity compared to wild type whereas those with 677CT have 65 percent wild-type enzyme activity (Frosst et al. 1995). For A1298C, MTHFR enzyme activity is low in 1298CC, intermediate in 1298AC and significantly lower in 677CT/1298AC individuals (van der Put et al. 1998). Only the 677TT genotype is associated with thermolability (Frosst et al. 1995; van der Put et al. 1998); subjects with a 677TT genotype consistently share a 1298AA genotype (van der Put et al. 1998).

Individuals with the 677T and 1298C *MTHFR* alleles are predisposed to homocysteinemia (Frosst et al. 1995; van der Put et al. 1998), low plasma folate (Frosst et al. 1995; van der Put et al. 1998) and DNA hypomethylation (Friso et al. 2002; Castro et al. 2004); those with the 677TT/1298AA genotype manifest the highest homocysteine and lowest plasma folate levels (van der Put et al. 1998; Ulvik et al. 2007) and lowest DNA methylation (Friso et al. 2005). Children with autism have been found to have high plasma levels of homocysteine (Pasca et al. 2006) and a biochemical profile of reduced methylation capacity (James et al. 2006). A similar profile of DNA hypomethylation has also been noted in parents of children with autism (James et al. 2008).

To date there have been three case–control studies of the C677T and A1298C *MTHFR* functional polymorphisms in ASDs. Boris et al. (2004) found higher frequencies of the 677TT genotype, 1298AA genotype, and compound heterozygotes (677CT/1298AC) in Caucasian individuals with autism compared to controls. James et al. (2006) found an increased frequency of *MTHFR* compound heterozygotes

and, a slightly higher odds ratio for 677TT over 677CT in Caucasian individuals with idiopathic autism. Mohammad et al. (2009) found similar allele frequencies in their cohort of families with ASD from South India.

Increasingly, DNA methylation defects are being recognized in association with ASDs, and *MTHFR* and its role in folate metabolism may contribute to epigenetic mechanisms that modify complex gene expression causing autism. Here we present our findings from population- and family-based studies on the *MTHFR* C677T and A1298C alleles in 205 SPX family trios and a follow-up analysis in 307 MPX families.

Methods

Participants

Based on the assumption that *MTHFR* is likely to be relevant in ASDs, we first tested 205 Caucasian SPX family trios (simplex ASD subjects and their biological parents) recruited by the Autism Spectrum Disorders-Canadian American Research Consortium (ASD-CARC; www.autismresearch.com). In the majority of families, family history information was available for a minimum of three generations and did not reveal further cases of ASDs. All probands met clinical criteria for an ASD found in the absence of a known etiology (i.e., excluding karyotype-detectable chromosomal anomalies and Fragile X syndrome) and was confirmed via standardized tests including the Autism Diagnostic Observation Schedule (ADOS) (Lord et al. 1989), Autism Diagnostic Interview-Revised (ADI-R) (Lord et al. 1994) and/or the PDD Behavior Inventory (PDDBI) (Cohen and Sudhalter 2005; Cohen et al. 2010). Among 205 affected individuals, 166 were assessed with the ADI-R, 136 of whom also had the completed PDDBI and five of which also have ADOS data; 39 individuals were assessed with the PDDBI only. All affected individuals in the SPX families met diagnostic criteria for autism or ASD.

This was followed by a study of 307 MPX families. DNA samples for 138 families were purchased from the Autism Genetics Resource Exchange (AGRE; www.agre.org), and the remaining 169 families were recruited by ASD-CARC. Family selection criteria for the MPX families have been described previously (Liu et al. 2009). All affected individuals in the MPX families had a clinical standard diagnosis of ASD by no less than the ADI-R and ADOS and standardization according to DSM-IV criteria.

Samples for population controls consisted of 384 heel prick blood spots from 192 male and 192 female anonymous neonates, obtained through the Ontario Ministry of Health, taken for the purpose of newborn PKU (phenylketonuria) testing.

DNA Extraction and Genotyping

Blood, saliva or buccal samples were obtained from probands and their parents. Genomic DNA was extracted according to standard protocols. For samples with limited genomic DNA, whole genome amplification was carried out using Qiagen's Repli-G Kit.

The two intragenic SNPs in the *MTHFR* gene, C677T (rs1801133) and A1298C (rs1801131), were genotyped according to published procedures as described by Yi et al. (2002) and Frosst et al. (1995). Genotyping of the two SNPs for MPX families was carried out using validated custom TaqMan SNP Genotyping Assays (<http://www.appliedbiosystems.com>) on an ABI Prism 7900HT. Genotypes were automatically scored with the SDS 2.2.2 software using standard parameters. In a sample of 100 individuals studied using both methods, there was 100% concordance between the results.

Statistical Analyses

Prior to data analysis, each polymorphism was assessed in parents and affected cases using χ^2 for deviations from Hardy–Weinberg equilibrium with the HWE program (Ott 1999). Linkage disequilibrium (LD) of the two SNPs was assessed using the 2LD program (Zhao 2004). The haplotype frequencies in the populations were estimated using the HAP program (Zhao 2004). Comparisons of allele, genotype and haplotype distributions between the controls and autistic individuals were carried out with SPSS v14.0. To perform family-based single-marker and multi-locus tests of association, the FBAT program (v. 1.5.1) was used (Laird et al. 2000). Family-based association tests were performed under an additive model. Results were considered significant at $p < 0.05$. For MPX families, one

affected individual was randomly selected from each family and included in the case–control comparisons.

Results

SPX Families

Allele and genotype frequencies in individuals with an ASD and their parents from SPX families versus the general population comparison group are summarized in Table 1 for C677T and in Table 2 for A1298C.

These distributions did not deviate from Hardy–Weinberg equilibrium except in SPX parents for A1298C. The allele and genotype distributions of C677T polymorphisms in the probands differed significantly from those in the comparison group, with the T allele being more frequent in children with an ASD (42.9%) compared to controls (32.3%) ($p = 0.0004$). This difference was primarily due to the higher prevalence of the T/T genotype in autistic individuals compared to controls (19.0% vs. 10.7%; $p = 0.0016$). The frequency of the heterozygous 677CT genotype in the ASD group (47.8%) did not differ from that in the controls (43.2%). The results for the A1298C polymorphism were not significant. We next examined genotype combinations of the two SNPs in affected individuals and controls. Homozygosity for both the 677T and 1298A alleles, but not heterozygosity (i.e. 677CT and 1298AC), was significantly higher in affected individuals than in controls (19.1% vs. 10.7%; $p = 0.007$) (Table 3).

FBAT for single SNPs in SPX families revealed excess transmission of the two risk alleles suggested by case–control analysis from parents to affected offspring ($p = 6.5 \times 10^{-5}$ for 677T and $p = 0.015$ for 1298A) (Table 4).

Table 1 Allele and genotype distributions of *MTHFR* C677T in children with autism (probands), their parents and the comparison group

	Allele					Genotype					HWE	
	N	C	T	χ^2 ^a	p ^b ($df = 1$)	N	C/C	C/T	T/T	χ^2 ^a	p ^b ($df = 2$)	p ^c ($df = 1$)
Comparison group	768	520 (67.7%)	248 (32.3%)			384	177 (46.1%)	166 (43.2%)	41 (10.7%)			0.823
SPX probands	410	234 (57.1%)	176 (42.9%)	12.666	0.0004	205	68 (33.2%)	98 (47.8%)	39 (19.0%)	12.847	0.0016	0.727
SPX parents	800	513 (64.1%)	287 (35.9%)	2.082	0.149	400	167 (41.8%)	179 (44.8%)	54 (13.5%)	2.234	0.326	0.584
MPX AFFs*	614	410 (66.8%)	204 (33.2%)	0.096	0.757	307	137 (44.6%)	136 (44.3%)	34 (11.1%)	0.151	0.927	1
MPX Parents	1,168	784 (67.1%)	384 (32.9%)	0.072	0.788	584	270 (46.2%)	244 (41.8%)	70 (12.0%)	0.462	0.794	0.198

* One affected individual was randomly chosen from each MPX family

^a Chi-square value with continuity correction

^b Chi-square test

^c Two-sided test

Table 2 Allele and genotype distributions of *MTHFR* A1298C in children with autism, their parents and the comparison group

	Allele				Genotype					HWE		
	N	A	C	χ^2 ^a	p ($df = 1$)	N	A/A	A/C	C/C		χ^2	p^b ($df = 2$)
Comparison group	764	515 (67.4%)	249 (32.6%)			382	170 (44.5%)	175 (45.8%)	37 (9.7%)			0.405
SPX probands	410	299 (72.9%)	111 (27.1%)	3.567	0.059	205	109 (53.2%)	81 (39.5%)	15 (7.3%)	4.168	0.124	0.993
SPX parents	802	554 (69.1%)	248 (30.9%)	0.429	0.513	401	200 (49.9%)	154 (38.4%)	47 (11.7%)	4.505	0.105	0.043
MPX AFFs*	614	405 (66.0%)	209 (34.0%)	0.259	0.610	307	134 (43.6%)	133 (43.3%)	40 (13.1%)	1.612	0.447	0.530
MPX Parents	1,168	782 (67.0%)	386 (33.0%)	0.044	0.834	584	272 (46.6%)	238 (40.8%)	74 (12.7%)	3.39	0.184	0.057

* One affected individual was randomly chosen from each MPX family

^a Chi-square value with continuity correction

^b Chi-square test

^c Two-sided test

Table 3 *MTHFR* C677T and A1298C genotypes in children with autism from SPX families and the comparison group

	N	Compound genotypes		χ^2	p ($df = 1$)
		TT-AA	Other combinations		
Control	382	41 (10.73%)	341 (89.17%)		
Probands	204	39 (19.12%)	165 (80.88%)	7.236	0.007

	N	Compound genotypes		χ^2	p ($df = 1$)
		CT-AC	Other Combinations		
Control	382	76 (19.90%)	306 (80.10%)		
Probands	204	43 (21.08%)	161 (78.92%)	0.054	0.817

We also investigated the haplotype distributions of the two SNPs in the SPX and control groups (Table 5). The markers are in moderately strong linkage disequilibrium. Significant differences were found in the frequencies of the haplotypes between the individuals with an ASD in SPX families and the control population. The 677T-1298A haplotype, which contains both risk alleles from the two SNPs, was more frequent in individuals with autism (42.5%) than in the control population (31.6%) ($p = 0.0004$). Haplotype FBAT (Table 6) demonstrated an excess transmission of the risk-allele-containing T-A haplotype from parents to affected offspring ($p = 9.1 \times 10^{-5}$) in the SPX families.

MPX Families

Allele and genotype frequencies in individuals with an ASD and their parents from MPX families are shown in Table 1 for C677T and in Table 2 for A1298C. The samplings were under Hardy–Weinberg equilibrium. The allele and genotype distributions of these polymorphisms in affected individuals were very similar to those in the control group. Therefore we did not compare the compound genotype frequencies and the haplotype frequencies between cases and controls. Family-based association tests did not reveal any significant preferential allele or

Table 4 Results of single marker FBAT for the two *MTHFR* SNPs in children with autism from SPX and MPX families

	SNP	Allele	S	E(S)	Var(S)	Z	p
SPX Families	C677T	C	134	160.5	44.05	-3.994	0.000065
	C677T	T	136	109.5	44.05	3.994	0.000065
	A1298C	C	77	92	38	-2.433	0.015
MPX Families	A1298C	A	175	160	38	2.433	0.015
	C677T	C	391	384.8	106.5	0.598	0.55
	C677T	T	259	265.17	106.5	-0.598	0.55
	A1298C	C	287	280	112.5	0.66	0.51
	A1298C	A	413	420	112.5	-0.66	0.51

S: test statistics for the observed number of transmitted alleles

E(S): expected value of S under the null hypothesis

The test was performed under an additive model

haplotype transmission from parents to affected children in single marker FBAT tests (Table 4) or haplotype FBAT tests (Table 6).

Discussion

Two common polymorphisms (C677T and A1298C) were selected for testing because they are associated with reduced MTHFR enzyme activity, affect folate metabolism/DNA methylation, and have previously been reported to be associated with autism susceptibility in case–controls studies. This population- and family-based study first examined these polymorphisms in a cohort of 205 North American SPX families defined as having only a single case of ASD in at least three generations. Overall, the findings demonstrated: (a) a significantly higher frequency of the *MTHFR*, low-activity 677T allele ($p = 0.0004$); (b) a higher frequency of the 677TT genotype ($p = 0.0016$); (c) higher frequencies of the 677T-1298A haplotype (42.55% vs. 31.60%; $p = 0.0004$) and compound 677TT/1298AA genotypes (19.12% vs. 10.73%; $p = 0.007$) in ASD subjects compared to controls. The haplotype

Table 5 Haplotype distributions of two *MTHFR* SNPs in individuals with autism in SPX families and controls (risk haplotype vs. others)

	N	Haplotypes		χ^2	p ($df = 1$)
		T-A	Other haplotypes		
Control	764	241 (31.60%)	523 (68.4%)		
Probands	408	174 (42.55%)	234 (57.45%)	12.702	0.0004

Table 6 Haplotype FBAT for two *MTHFR* SNPs

	Haplotype	S	$E(S)$	$\text{Var}(S)$	Z	p
SPX Families	T-A	142.8	115.8	47.5	3.914	0.000091
	C-A	100.2	113.2	42.1	-2.00	0.045
	C-C	84.8	97.8	36.5	-2.15	0.031
	T-C	*	*	*	*	
MPX Families	C-A	265.8	280.9	107.2	-1.46	0.14
	T-A	272.2	272.5	87.0	-0.03	0.97
	C-C	304.2	285.3	94.9	1.95	0.072
	T-C	*				

S : test statistics for the observed number of transmitted haplotypes

$E(S)$: expected value of S under the null hypothesis (i.e., no linkage or association)

*: The frequency is lower than the minimum default value of 5%

The test was under additive model

frequency distribution revealed a higher prevalence of the 677T-1298A haplotype in affected individuals compared to controls (42.55% vs. 31.60%; $p = 0.0004$). Single marker FBAT demonstrated excess transmission of the 677T and 1298A *MTHFR* alleles ($p = 6.5 \times 10^{-5}$, $p = 0.015$ respectively) to affected offspring. Haplotype FBAT also showed excess transmission of the 677T-1298A haplotype to affected offspring ($p = 9.1 \times 10^{-5}$).

The allele and genotype frequencies for both markers in our controls are similar to those published by two other independent studies in the US and Canada (Boris et al. 2004; Kotsopoulos et al. 2008), indicating our control sample is representative of the North American population. The preferential transmission of the T-A haplotype to affected offspring ($p = 9.1 \times 10^{-5}$) is in keeping with previously reported findings from a case-control study (Boris et al. 2004) in North American families. Our allele, genotype, and haplotype distributions in both population- and family-based association tests suggest that the low-activity T allele at C677T and A allele at A1298C are “risk alleles” in the *MTHFR* gene, significantly increasing the risk of autism in our North American SPX family cohort.

Curiously, none of the above significant findings were replicated in our study of 307 MPX families suggesting that the T-A haplotype is a risk factor for autism only in SPX families. Several studies have suggested different etiologies for MPX and SPX families, with MPX families

having a higher genetic component. Interestingly, studies on de novo copy number variants (CNVs) in individuals with ASDs has pointed to a higher prevalence in SPX families, providing a clue to some of the genetic differences (Sebat et al. 2007; Marshall et al. 2008; Christian et al. 2008). Since DNA hypomethylation is associated with chromosome instability in many cancers (Wilson et al. 2007), and low-expressing *MTHFR* alleles have also been implicated in chromosome instability (Kimura et al. 2004), we propose that the difference between MPX and SPX families is, in part, due to the higher prevalence of low-expressing *MTHFR* alleles in the latter groups of families.

One limitation of this study is the small sample size of sporadic ASD families. Nevertheless, the results are significant for the *MTHFR* 677TT/1298AA genotype and the 677T-1298A haplotype and in agreement with the Boris et al. (2004) findings in North American families. All reported case-control studies examining these *MTHFR* polymorphisms have to date encompassed smaller ASD cohorts, and consistently associate the 677T allele and 677TT genotype with autism (Boris et al. 2004; James et al. 2006; Mohammad et al. 2009; the present study), making it unlikely that such corroborative findings are false-positives. Differences due to genetic heterogeneity, recruiting strategies, family type (SPX vs. MPX), sample size, ethnic variation, and other factors could account for some differences in the respective study results. Larger replication studies in phenotypically well-characterized (i.e. examining the whole body “phenome”) (Qiao et al. 2009) single incidence families, allowing comparison between defined subgroups of diverse ethno-cultural background, are needed to determine whether the presence or absence of the two *MTHFR* SNPs (C677T and A1298C) moderates the risk of autism as suggested by our population- and family-based studies.

Acknowledgments We extend our sincere appreciation to our research subjects and their extended family members for their enthusiastic support of this study and acknowledge the resources provided by the AGRE (Autism Genetics Resource Exchange) consortium and the participating AGRE families. AGRE is a program of Cure Autism Now and supported, in part, by grant MH64547 from the NIMH to Daniel H. Geschwind (PI). This work was supported by an OMHF grant (JJAH principal investigator) and a CIHR Interdisciplinary Health Research Team grant (RT-43820) to the Autism Spectrum Disorders Canadian-American Research Consortium (ASD-CARC: www.autismresearch.ca) (JJAH, principal investigator); MESL sincerely appreciates the support provided by a Michael Smith Foundation for Health Research Career Investigator (Scholar) Award (2005–2010); and we appreciate the support of the New York State Office for People with Developmental Disabilities (OPWDD).

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