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The Bipolar Association Case–Control Study (BACCS) and Meta-Analysis: No Association With the 5,10-Methylenetetrahydrofolate Reductase Gene and Bipolar Disorder

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Bipolar disorder (BD) is a complex genetic disease for which the underlying pathophysiology has yet to be fully explained. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is a crucial enzyme in folate-mediated one-carbon metabolism and folate deficiency can be associated with psychiatric symptoms. A single base variant in *MTHFR* gene (C677T) results in the production of a mildly dysfunctional thermolabile enzyme and has recently been implicated in BD. We conducted an association study of this polymorphism in 897 patients with bipolar I or bipolar II disorder, and 1,687 healthy control subjects. We found no evidence for genotypic or allelic association in this sample. We also performed a meta-analysis of our own, and all published data, and report no evidence for association. Our findings suggest that the *MTHFR* C677T polymorphism is not involved in the genetic etiology of clinically significant BD.

Key words: mood disorder; bipolar disorder; manic depression; genetics; *MTHFR*

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INTRODUCTION

Narrowly defined bipolar disorder (BD) has a life-time prevalence estimated between 0.3% and 1.6%, with mean age-of-onset around 21 and equal prevalence amongst men and women [Smith and Weissman, 1992; Weissman et al., 1996]. BD is characterized by severe alterations in mood, with periods of depression and mania or, less commonly, mania only. It has been well established that multiple genetic and environmental factors play a role in the etiology of BD [Licinio, 2002], and as a complex genetic disease the underlying pathophysiology has yet to be fully explained.

5,10-Methylenetetrahydrofolate reductase (MTHFR) is a necessary enzyme in folate-mediated one-carbon metabolism, catalyzing the irreversible MTHFR-dependent reaction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [Godfrey et al., 1990]. 5-Methyltetrahydrofolate acts as a cofactor alongside methionine synthase and methylcobalamin (active form of vitamin B12) for

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the methylation of homocysteine to methionine, and is linked to biopterin-dependent neurotransmitter synthesis [van Praag, 1982]. The conversion of homocysteine to methionine via the transfer of a methyl group from methylcobalamin is crucial for the biosynthesis of *S*-adenosylmethionine (SAMe) [Andreoli and Maffei, 1975], a major methyl group donor for biogenic amines and phospholipids in the central nervous system [Bottiglieri et al., 1994; Anguelova et al., 2003]. In addition to this, homocysteine and its metabolites may have a direct excitotoxic effect on the *N*-methyl-D-aspartate (NMDA) glutamate receptors in the brain, and may inhibit methylation processes in the central nervous system [Bottiglieri et al., 1994], therefore its conversion is critical.

Patients suffering folate metabolic disorders (i.e., hyperhomocysteinemia) exhibit psychiatric symptoms [Rosenblatt, 1995], and patients with folate deficiency have been reported to present psychiatric manifestations [Freeman et al., 1975; Pasquier et al., 1994; Regland et al., 1997]. Mania has been associated with depleted folate levels and deficient functioning [Coppen and Abou-Saleh, 1982; Coppen et al., 1986; Hasanah et al., 1997], and independent reports detail symptoms of folate deficiency that resemble schizophrenia [Freeman et al., 1975; Pasquier et al., 1994; Regland et al., 1997]. Furthermore elevated homocysteine levels unaccounted for by nutrient deficiency (in folate or cobalamin) or medication have been reported in individuals diagnosed with schizophrenia [Regland et al., 1994, 1995], and folate has been reported to enhance clinical improvement in schizophrenia as with unipolar depression [Godfrey et al., 1990]. Additionally, although the folate metabolite SAMe has been reported to be an effective and well-tolerated antidepressant, in vulnerable patients it can precipitate mania [Goren et al., 2004]. Overall, therefore, genetic variants in MTHFR represent excellent potential candidates for implication in BP etiology.

The *MTHFR* gene is located on the short arm of chromosome one at 1p36.3. A common polymorphism previously investigated in psychiatric disease is a C → T transition at nucleotide 677 (*C677T*) in exon 4 identified by Frosst et al. [1995]. The *C677T* SNP results in an alanine to valine substitution (*A222V*) which has been associated with a reduction in *MTHFR* activity due to the production of a mildly dysfunctional thermolabile enzyme that exhibits reduced activity at temperatures above 37°C [Ueland et al., 2001]. Given this report, one would expect stability at body temperature, however, *677TT* homozygotes demonstrate just 30% of enzyme activity relative to homozygote *677CC* individuals, whilst heterozygotes retain 65% of the wild-type enzyme activity [Rozen, 1996]. Although significantly different enzymatic activity and thermolability were reported, overlapping profiles for the T/T and C/T genotypes have been described with C/C remaining distinct [Frosst et al., 1995]. This indicates that the T allele follows a co-dominant model, or a dominant model with incomplete penetrance [Tan et al., 2004]. The *MTHFR 677TT* genotype has also been associated with a significant elevation in the circulating concentrations of homocysteine, and linked with lower levels of red blood cell folate, plasma folate, and vitamin B12 [Van Tonder et al., 1975; Ozbek et al., 2008]. This may parallel a similar reduction in 5-methyltetrahydrofolate in the CNS and lead to a reduction in methyl group donors and monoamine neurotransmitter function, which in turn may elevate the risk of BD.

Most studies investigating BD in association with *C677T* present no evidence for association [Arinami et al., 1997; Tan et al., 2004; Reif et al., 2005; Jonsson et al., 2008; Ozbek et al., 2008; Chen et al., 2009], with case samples ranging from just 117 bipolar subjects up to 501. One study reports an ''unconvincing trend'' for association with the *677TT* genotype (n = 258) bipolar [Kunugi et al., 1998]. A recent meta-analysis including four studies [Arinami et al., 1997; Kunugi et al., 1998; Tan et al., 2004; Reif et al., 2005] reports no overall association between *C677T* and the risk of BD, although the East Asian population presents a marginal association with the T allele [Zintzaras, 2006]. This meta-analysis did not include the investigation of the *C677T* SNP and the risk of developing BD that reported a significant association with the dominant model for the T allele ($P = 0.0006$; OR = 1.988 [CC vs. CT and TT] [Kempisty et al., 2006]), with a more recent meta-analysis including this study presenting evidence for association [Gilbody et al., 2007] although this has not been supported in the most recent study and meta-analysis to date [Chen et al., 2009]. We present here the largest clinical case–control comparison investigating the possible role of the *MTHFR* C677T polymorphism in the risk of developing BD, and an up-to-date meta-analysis.

METHODS

Study Subjects

Eight hundred ninety-seven individuals (319 men and 578 women; mean age ± SD: 47.15 ± 11.94) with BD were recruited from two sites: London, UK and Toronto, Canada. The mean age for Canadian subjects was 45.99 years (SD ± 12.54 years; n = 383 total, 143 men and 240 women), and for UK subjects 48.02 years (SD ± 11.40; n = 514 total, 176 men and 338 women). Age upon entry to study differed significantly between centers of ascertainment ($t = 2.526$, $P = 0.012$), gender did not ($\chi^2 = 0.916$, $P = 0.360$). Subjects were recruited following identical protocols and were identified from psychiatric clinics, hospitals, primary care physicians, patient support groups, and from volunteers responding to media advertisements. All subjects were interviewed in person. Subjects were included if they were over the age of 18 and had been diagnosed with Bipolar I or Bipolar II disorder as defined by the Diagnostic and Statistical Manual 4th edition operational criteria (DSMIV) [American Psychiatric Association, 1994] or the International Classification of Diseases 10th edition operational criteria (ICD10), for bipolar depression [World Health Organisation, 1993]. The age of onset of disease was 21.28 ± 10.48. All subjects were interviewed using the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) [Wing et al., 1990; World Health Organisation, 1998]. All were white and of European parentage, in an attempt to minimize population stratification effects. Subjects were only included if informed written consent to participate in the study was obtained. Exclusion criteria were: (1) first-degree relative having fulfilled criteria for schizophrenia; (2) psychotic symptoms that were mood incongruent or present when there was no evidence for mood disturbance; (3) intravenous drug use with a lifetime diagnosis of drug dependency; (4) mania or depression occurred solely in relation to, or a consequence of, alcohol or substance abuse/dependence and/or medical illness; (5) being related to an individual already included in the study. High inter-rater reliability for diagnosis was achieved with a mean kappa of 0.83. All participants gave written informed consent and the study was approved by the Local Ethical Committees of all centers.

One thousand six hundred eighty-seven control individuals (721 men and 966 women; mean age ± SD: 42.07 ± 13.17) were recruited from two sites: London, UK and Toronto, Canada. The mean age of the Canadian control group was 43.66 years (SD ± 13.12 years; n = 311 total, 142 men and 169 women), and for the UK control group 41.70 years (SD ± 13.16, n = 1,376 total, 579 men and 797 women). Age upon entry to study differed significantly between centers of ascertainment ($t = -2.358$, $P = 0.019$), gender did not ($\chi^2 = 1.324$, $P = 0.254$). Subjects were from both sites were screened for lifetime absence of psychiatric disorder using a modified version of the Past History Schedule [PHS; McGuffin et al., 1986]. Those recruited in London were contacted via the MRC general practice research framework and screened using the Sham et al. [2000] composite index (G) of depressive and anxiety symptoms then telephone interviewed using the PHS (n = 865), or were interviewed in person and were healthy volunteers who were staff or students of King's College London, screened using the PHS. All controls subjects were White and of European parentage. Exclusion criteria were: (1) if they, or a first-degree relative, ever fulfilled criteria for BD, depression or any other psychiatric disorder; (2) if they scored 10 or above on the Beck Depression Inventory [Beck and Steer, 1984]; (3) did not return consent; (4) failed to return cheek swabs or successfully give blood. All participants gave written informed consent and the study was approved by the Local Ethical Committees of all centers.

Phenotypic Data Analysis

All phenotypic information from SCAN interviews and questionnaires was coded by assigning a number to each subject, and removing any personal identifying information. The same codes were used on the blood sample or cheek swab tubes using a bar code system. The phenotypic information was entered into a data file created using Statistical Procedures for the Social Sciences (SPSS) version 12 for Windows for the statistical analyses.

Statistical Analysis

To test for deviation from Hardy–Weinberg equilibrium (HWE) the Pearson (χ^2) test statistic and an exact statistic were calculated with the computer program FINETTI (available on http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Using Stata (Version 9.0), genotype and allele frequencies were assessed for association using standard contingency table analysis incorporating Chi-squared test of independence, producing a χ^2 statistic with 1 or 2 degrees of freedom depending on the number of parameters. To calculate the power of the case–control sample size we used the PS V2.1.31 [Dupont and Plummer, 1990].

Meta-Analysis

A meta-analysis was conducted combining all case–control studies published to date investigating the C677T polymorphism in association with BD. The PubMed database was searched including published studies up to and including March 4, 2010, using the search terms "MTHFR" and "bipolar." All results were carefully reviewed, and any studies investigating the *C667T* polymorphism in association with BD were extracted, and the reference list scrutinized. Seven out of the eight studies applied DSM-III-R or DSM-IV criteria for diagnosis, with just one applying ICD-10 [Reif et al., 2005]. The meta-analysis compares *677TT* and *677CC* genotype frequencies, *677TT* combined with *677CT* genotype frequencies, and *677T* and *677C* allele frequencies for the patients versus controls. Odds ratios were combined using random-effects model due to the significant between-study heterogeneity estimated using the I^2 statistic [Higgins et al., 2003] that was observed between the Caucasian studies and when all the studies were combined. In contrast odds ratios were combined using fixed-effects model when the Asian studies were combined, due to a lack of such heterogeneity. Genetic association studies may be susceptible to publication bias [Salanti et al., 2005], and so a funnel plot and rank correlation test [Begg and Mazumdar, 1994] were applied to assess this. All analyses were conducted in Stata (Version 9.0) using the *metan* and *metabias* commands.

Genotyping

Buccal mucosa swabs were collected for DNA via the mail for 858 control individuals from the UK. All the remaining control individuals and the bipolar cases were interviewed in person. At the time of interview, interviewers obtained 25 ml of whole blood collected in 37.5 ml (EDTA containing) monovettes. In addition, drops of blood were placed on a Guthrie blood spot card. The blood samples were labeled with a bar code, gently mixed and stored frozen upright in a −20°C freezer pending DNA extraction. The swab containers were labeled with a bar code pending DNA extraction. Genomic DNA was extracted from bloods and cheek swabs collected as described previously [Freeman et al., 1997, 2003].

Outsourced genotyping of the *MTHFR* C677T SNP was performed by KBioscience (Hoddesdon, Herts, UK; www.kbioscience.

TABLE I. Genotype and Allele Frequencies of the C677T SNP in the Control and Bipolar Samples, and Single Marker Case–Control Association Analyses

Group	N	CC (%)	CT (%)	TT (%)	C (%)	T (%)
Controls	1,576	642 (40.7)	719 (45.6)	215 (13.6)	2,003 (63.5)	11,49 (36.5)
Bipolar disorder	846	362 (42.8)	386 (45.6)	98 (11.6)	1,110 (65.6)	582 (34.4)

Genotype distribution CC versus CT versus TT: $\chi^2 = 2.37$, df = 2, $P = 0.31$.
Genotype distribution CC versus CT/TT: $\chi^2 = 0.96$, df = 1, $P = 0.96$; odds ratio = 0.92 (0.78–1.09).
Genotype distribution CC versus TT: $\chi^2 = 2.36$, df = 1, $P = 0.12$; odds ratio = 0.81 (0.62–1.06).
Allele distribution C versus T: $\chi^2 = 2.01$, df = 1, Cochran-Armitage $P = 0.16$; common odds ratio = 0.9.

TABLE II. Distribution of C677T Genotypes in Association Studies of the Methylenetetrahydrofolate Reductase (*MTHFR*) Gene in Bipolar Disorder

	CC				CT				TT				T allele					
	Cases		Controls		Cases		Controls		Cases		Controls		Cases		Controls			
Refs.	(N)	%	(N)	%	(N)	%	(N)	%	(N)	%	(N)	%	(N)	%	(N)	%	HW	Odds ratio (95% CI)
Arinami et al. [1997]	15	37.5	154	36.8	20	50.0	214	51.1	5	12.5	51	12.2	30	37.5	316	37.7	0.10	0.99 (0.62–1.59)
Kunugi et al. [1998]	41	28.7	95	36.8	74	51.8	129	50.0	28	19.6	34	13.2	130	45.5	197	38.2	0.43	1.35 (1.01–1.81)
Tan et al. [2004]	99	59.3	80	66.7	60	35.9	33	27.5	8	4.8	7	5.8	76	22.8	47	19.6	0.25	1.18 (0.79–1.78)
Reif et al. [2005]	48	52.2	75	42.6	34	37.0	80	45.5	10	10.8	21	11.9	54	58.7	122	69.3	0.96	0.78 (0.53–1.15)
Kempisty et al. [2006]	108	54.0	210	70.0	73	36.5	79	26.3	19	9.5	11	3.7	111	22.8	101	16.8	0.30	1.90 (1.40–2.58)
Jonsson et al. [2008][a]	58	49.6	726	49.2	49	41.9	601	40.7	10	8.6	149	10.1	69	29.5	899	30.5	0.14	0.83 (0.58–1.18)
Ozbek et al. [2008]	104	52.8	116	48.7	76	38.6	97	40.8	17	8.6	25	10.5	110	27.9	147	30.9	0.54	0.87 (0.65–1.16)
Chen et al. [2009]	178	35.5	153	33.2	231	46.1	235	51.0	92	18.4	73	15.8	415	41.4	381	41.3	0.27	1.00 (0.84–1.20)
Cohen-Woods[b]	195	40.5	532	41.3	236	49.1	582	45.2	50	10.4	175	13.5	336	34.9	932	36.2	0.43	0.99 (0.62–1.59)
UK sample																		
Cohen-Woods[b]	167	45.8	110	38.3	150	41.1	137	47.8	48	13.1	40	13.9	246	33.7	217	37.8	0.80	1.35 (1.01–1.81)
Canadian sample																		

[a]Only Norwegian controls considered in meta-analysis as all bipolar cases in this study were from the Norwegian population.
[b]BACC Study published in this article, two populations separated for purposes of meta-analyses.

co.uk) for 858 control individuals. The genotyping system used is based on competitive allele-specific PCR in a homogenous FRET format, named KASPar. Further information on the chemistry is available on the website (http://www.kbioscience.co.uk/chemistry/kaspar_chemistry-intro.htm). SNPviewer software utilizes a clustering algorithm to enable genotype calling. This may be downloaded from the KBioscience website so that genotype calls may be viewed and checked. The remaining 1,637 individuals, all case and remaining control individuals, were genotyped internally at the London laboratory using the Taqman® SNP genotyping platform (PE Applied Biosystems, Foster City, CA). The genotyping reactions followed the instruction from manufacturer (Assay-on-Demand SNP products: C_1202883_20).

Genotyping was blind to all phenotypic information, including affected status. In total 1,576 controls and 846 cases were successfully genotyped. Five hundred thirty randomly selected samples were re-genotyped using the same system (KBioscience), there was 1 genotyping error (0.16%) and this sample was excluded from further analyses. We also used the TAQMAN® SNP genotyping system to perform in-house re-genotyping of 85 randomly selected samples with no error observed.

RESULTS

Bipolar Association Case–Control Study (BACCS)

The genotype distribution of the C677T polymorphism is in accordance with HWE in the control sample and the bipolar sample ($P > 0.05$). The genotype and allele frequencies of the C677T polymorphism are shown in Table I. No significant differences in allele or genotype frequencies are observed between the control sample and the bipolar sample. This remains the case no matter what model is considered (dominant, co-dominant, or heterozygote-only) as shown in Table I.

Meta-Analysis

Eight previously published articles were identified that investigated the C677T polymorphism in BD [Arinami et al., 1997; Kunugi et al., 1998; Tan et al., 2004; Reif et al., 2005; Kempisty et al., 2006; Jonsson et al., 2008; Ozbek et al., 2008; Chen et al., 2009]. Together with individuals from the present study, a total of 2,303 BD cases (37% from the present study) and 3,725 controls (42% from the present study) were included in the meta-analysis. Four out of these previous eight studies investigated Asian populations [Arinami et al., 1997; Kunugi et al., 1998; Tan et al., 2004; Chen et al., 2009]. No increased risk of BD was found following meta-analyses of all samples, or with European and Asian samples considered separately, in carriers of the *677T* allele, the *677TT* genotype, or when the *677TT* and *677CT* genotypes were combined (see Table III). Between-study heterogeneity is observed when all articles are analyzed together and when European samples only are considered, although none is observed between the Asian studies (see Table III). The heterogeneity observed between the Caucasian studies, and so the total studies, appears to be attributable to an increased frequency of the *677C* allele in the control sample presented by Kempisty et al. [2006] (Table II). When this study is excluded from

TABLE III. Summary Statistics of Meta-Analyses of C677T in the Methylenetetrahydrofolate Reductase (*MTHFR*) Gene and Risk of Developing Bipolar Disorder

Comparison	No. of samples	Cases (N)	Controls (N)	OR	95% CI	*P*-value	I^2 (%)	Heterogeneity, χ^2 statistic	Heterogeneity, *P*-value
All[a]	10	2,303	3,725						
T versus C				1.03	0.88–1.20	0.73	67.7	27.88	0.001
TT versus CC				1.02	0.77–1.36	0.89	52.2	18.83	0.027
TT and CT versus CC				1.02	0.84–1.25	0.82	64.2	25.16	0.003
Asian only[b]	4	851	1,258						
T versus C				1.09	0.95–1.25	0.21	4.60	3.15	0.370
TT versus CC				1.21	0.90–1.63	0.21	0.00	2.77	0.428
TT and CT versus CC				1.07	0.88–1.30	0.51	33.2	4.49	0.213
European only[a]	6	1,452	2,467						
T versus C				0.97	0.77–1.23	0.83	78.0	22.69	<0.001
TT versus CC				0.92	0.62–1.38	0.69	61.6	13.01	0.023
TT and CT versus CC				0.96	0.72–1.29	0.79	75.3	20.21	0.001

[a]Calculated under random-effects model.
[b]Calculated under fixed-effects mode.

analyses, the between-study heterogeneity drops drastically, failing to remain significant; nevertheless, still no evidence for association is observed (data not shown). The funnel plot analyses provide no evidence for publication bias regardless of studies considered, or the genotype or allele model tested ($P > 0.05$).

DISCUSSION

This article reports the largest to date clinical case–control study investigating the association between the *MTHFR* C677T polymorphism and BD. Previous case–control reports have yielded inconsistent results regarding the C677T SNP and unipolar depression [Arinami et al., 1997; Kunugi et al., 1998; Hickie et al., 2001; Kelly et al., 2004; Tan et al., 2004; Chen et al., 2005; Reif et al., 2005], with our own data effectively excluding any but the smallest of effect [Gaysina et al., 2008]. The BD data have also been inconsistent [Arinami et al., 1997; Kunugi et al., 1998; Tan et al., 2004; Reif et al., 2005; Kempisty et al., 2006; Chen et al., 2009], with our study presenting no evidence for allelic or genotypic association with the C677T SNP and the risk of developing BD. A meta-analysis comprising of all eight studies published to date plus that published here, analyzing the Caucasian and Asian samples independently as well as combined, further fails to detect evidence for association despite the combined case population reaching in excess of 2,300, and Caucasian and Asian population 1,400, and 850 individuals, respectively. The only article to date indicative of association is by Kempisty et al. [2006], where the control population appears to have different genotype and allele distribution relative to other control populations.

The ethnic and geographic variation is considerable for the 677T allele and C677T genotypes [Botto and Yang, 2000], and although it may be possible ethnic stratification may be masking an effect, it is unlikely. To minimize the effects of population stratification, all subjects included in this study were unrelated and of white European parentage. Furthermore, no differences in genotype and allele distributions were observed between the UK and Canadian populations (analysis not shown). Previous association could simply be a consequence of chance, or a consequence of the polymorphism being associated with another disease that is highly associated with BD, as with depression [discussed in Gaysina et al., 2008]. In conclusion, the observed data, and our reanalysis of those of others, make it unlikely that the *MTHFR* C677T polymorphism influences the risk of BD.

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