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ORIGINAL ARTICLE

Components of the folate metabolic pathway and ADHD core traits: an exploration in eastern Indian probands

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We investigated role of the folate–homocysteine metabolic pathway in the etiology of attention-deficit hyperactivity disorder (ADHD) due to its importance in maintaining DNA integrity as well as neurotransmission. Functional gene variants in MTR (rs1805087), CBS (rs5742905), MTHFR (rs1801133 & rs1801131), MTHFD (rs2236225), RFC1 (rs1051266), plasma vitamin B12, folate and homocysteine were analyzed. rs1805087 'A' showed strong association with ADHD. Vitamin B12 deficiency of ADHD probands ($P = 0.01$) correlated with rs1801133 'T' and rs1805087'GG'. Mild hyperhomocysteinemia ($P = 0.05$) in the probands was associated with rs1805087 'AA'. Probands having rs1805087 'GG' and rs1051266 'G' was more inattentive. Hyperactivity–impulsivity score revealed association with rs5742905 'TT' and rs2236225 'CC', while rs1801133 'CC' showed association with inattentiveness and hyperactivity–impulsivity. rs1801131 exhibited strong synergistic interaction with rs1051266 and rs2236225. This indicated that the folate–homocysteine pathway gene variants may affect ADHD etiology through mild hyperhomocysteinemia and vitamin B12 deficiency, factors known to be associated with cognitive deficit.
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INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD), a neurobehavioral disorder characterized by developmentally inappropriate inattention, impulsivity and hyperactivity,[1] is also frequently associated with cognitive deficit. Symptoms are expressed in childhood and often persist through adulthood[2] affecting behavioral, emotional and social functioning.[3]

The American Psychiatric Association reported ADHD in about 5% children,[1] while worldwide prevalence rate was reported to vary between 2.2% and 17.8%.[2,4–6] In India also, the prevalence rate was reported to vary between 11% and 15.5%.[7–9] ADHD is detected more frequently in males than in females with a 3:1 margin.[10]

Heritability rate of ADHD was reported to vary between 76% and 80%, making it one of the most highly heritable neuropsychiatric disorders.[11,12] Though the exact cause still remain unknown, several prenatal and perinatal factors, exposure to toxins and heavy metals, socio-psychological stress, diet, gene variants and structural/functional abnormalities of the brain, neurotransmitter deficiency and deregulation in the frontostriatal as well as frontocerebellar catecholaminergic circuit were reported to contribute to the etiology.[4,11,13] Imaging studies indicated that ADHD patients often have smaller prefrontal cortex volume.[14] In addition to the prefrontal cortex, the basal ganglia, cerebellum, temporal and parietal cortex also exhibited changes in ADHD subjects.[15]

Cognition includes a complex set of activities such as attention, memory, thinking, learning and perception[16] and is influenced by several factors, including diet. Deficiency in dietary nutrients such as folate and vitamin B had shown association with neurodevelopmental disorders, including ADHD and autism.[17] Folate is an essential nutrient regulating neural stem cell proliferation and differentiation, apoptosis, numerous biochemical pathways including neurotransmitter synthesis, DNA biosynthesis, myelin synthesis and repair, regulation of gene expression, amino-acid synthesis and metabolism.[18,19] Maternal folate deficiency during gestation was reported to confer childhood hyperactivity.[20] Amino-acid supplementation, leading to generation of S-adenosylmethionine (SAM) was found to alleviate major depression, bipolar disorder, schizophrenia and anxiety disorders, eating disorders, addiction, ADHD and autism.[17] Participation of folate (specifically 5-methylenetetrahydrofolate) in neurotransmitter synthesis is thought to be the most crucial factor for its effects on mood and cognition. Folate also appears to be important in regenerating tetrahydrobiopterin through folate-metabolizing enzyme, dihydrofolate reductase,[21] which as a co-factor aids in the formation of monoamine neurotransmitters, such as serotonin, dopamine, norepinephrine and epinephrine.[22]

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We have observed significant impact of few folate system gene variants, such as reduced folate carrier (*RFC1*), methylenetetrahydrofolate dehydrogenase (*MTHFD*) and methylenetetrahydrofolate reductase (*MTHFR*), on the cognitive function of ADHD probands.[23] As an extension to the previous study, presently we have investigated functional variants in methionine synthase (*MTR*) and cystathionine β-synthase (*CBS*) genes, vitamin B12 (VB12), folic acid (FA) and homocysteine (Hcy) in a group of eastern Indian subjects in order to identify whether functional gene variants and metabolites as well as co-factors of the folate–homocysteine metabolic system has any contribution in ADHD-associated traits.

MATERIALS AND METHODS

Subject recruitment

A total 866 subjects were recruited in the present study, including 286 controls and 580 individuals from unrelated nuclear families with ADHD probands. ADHD probands ($N=221$, M: F = 10.05:1 ratio) were recruited by mental health professionals based on the Diagnostic and Statistical Manual for Mental Disorders-IV-text revised (DSM-IV-TR) criteria[24] followed by psychological evaluation through: (1) The Conners' Parents Rating Scale-revised (CPRS-R)[25] for the inattention/hyperactivity level and (2) Wechsler Intelligence Scale for Children[26] to assess the Intelligence Quotient (IQ) of cases aged >5 years. Children aged <5 years were assessed for the developmental quotient using the Developmental Screening Test.[27] Age, sex and ethnically matched controls ($N=286$, M: F = 1:1.2 ratio) evaluated similarly were also recruited. Patients with any other neuropsychiatric disorders, pervasive developmental disorder and intellectual disability (ID) (IQ<70) including Fragile-X syndrome were excluded. The Human Ethical Committee of the Institute approved the study protocol.

Selection of single-nucleotide polymorphisms (SNPs)

SNPs in the *MTR* (rs1805087; A2756G) and *CBS* (rs5742905; 833 T>C 844ins68) were selected based on: (1) previous report of association with neurodevelopmental disorders[28–30] and (2) deleterious effects indicated by *in silico* analysis using the F-SNP (http://compbio.cs.queensu.ca/F-SNP/).

Genomic DNA isolation and genotyping

Peripheral blood was collected from ADHD probands, their parents and control individuals after obtaining informed written consent for participation and genomic DNA was extracted from leukocytes.[31] PCR amplification was carried out using Applied Biosystems Gene Amp no. 9700 thermal cycler and analyzed by restriction digestion (described in Supplementary Table 1).

Statistical analyses of data

All the genotypes were tested for the Hardy–Weinberg equilibrium (HWE). The chi-square value of HWE was calculated using a freely available online software Online Encyclopedia for Genetic Epidemiology studies (http://www.oege.org/software/hwe-mr-calc.shtml) and *P*-value of HWE was calculated using the online Graph Pad software (http://graphpad.com/quickcalcs/PValue1.cfm). Population-based allelic frequency analysis by Cocaphase was performed using UNPHASED v 3.1.7.[32] Genotypic frequencies were compared using the rxc contingency table (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html). Family-based analysis by Transmission Disequilibrium Test[33] was performed using UNPHASED v 2.404.[34] Comparisons were tested for multiple corrections while running the UNPHASED (1000 permutations).

Stratified analysis on familial allelic transmission

Families with ADHD probands were grouped according to the age of the parents; families with maternal age <26 years and paternal age <31 years were considered as group 1 and those with higher age were considered as group 2. Comparative analysis on allelic transmission from these two groups was performed by Transmission Disequilibrium Test.

Analysis of plasma metabolites/co-factors

Plasma samples collected in EDTA from ADHD cases ($N=48$, age 6–12) and age-matched controls ($N=30$, age 7–12 yrs) were used for enzyme-linked immunosorbent assay (ELISA). Solid-phase, sandwich ELISA was used for measuring VB12 and FA using kits (MyBiosource, San Diego, CA, USA, Cat. no. MBS021583 and MBS260674, respectively). Hcy was also assayed by competitive ELISA using the kit (MyBiosource, Cat. no. MBS7252797). Optical density of the end products was measured at 45 nm using ELISA reader (Genetix, New Delhi, India). The online *t*-test calculator (http://studentsttest.com/) was used for comparing the values obtained for case and control. Genotypes of functional SNPs reported previously for *RFC1*, *MTHFD* and *MTHFR*[23] were updated and association between metabolite/co-factor levels and genotypes were calculated by online *t*-test calculator (http://studentsttest.com/).

Analysis of association between birth weight (B_w), IQ and behavioral score

Chi-square test was used to generate frequencies of ADHD probands with different levels of B_w (<2.5 kg/⩾2.5 kg) and IQ (<80/⩾80) followed by calculation of association between the groups using the online software rxc contingency table (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html). Inattention and hyperactivity–impulsivity were measured through questions selected from the DSM-IV-TR as reported previously[35] and analyzed for association with genotypes using the one-tailed *T*-test (http://studentsttest.com/). *T*-score for cognitive problem/inattention and hyperactivity was obtained from the CPRS-R and association with genotypes was calculated using the one-tailed *T*-test (http://studentsttest.com/). The online *t*-test calculator was used to analyze correlation between cognitive problem/inattention and hyperactive score obtained through DSM-IV-TR/CPRS-R and B_w (<2.5 kg/⩾2.5 kg) as well as IQ.

SNP–SNP interaction using Multifactor Dimensionality Reduction (MDR) test

The MDR program was used to evaluate gene–gene interactions using case–control data set.[36] It is a data mining approach in which balanced accuracy with random seed 1 was used to avoid spurious results due to chance divisions of the data, as the number of affected and unaffected individuals was not equal in the present data set.[37] At 0.05% significance level, best models were chosen. Finally, measures of information were used to generate a statistical interpretation of the SNP–SNP interaction model.[36] Interaction graphs using the MDR algorithm (version 2.0 beta 8.1) were generated to visualize the nature of the dependencies or interactions.[38] The MDR interaction model describes percentage of entropy contributed by each factor (information gain (IG)) independently as well as through additive or synergistic interactions.

RESULTS

Both rs1805087 and rs5742905 were nonsynonymous, and *in silico* analysis predicted effect on RNA-binding protein-mediated regulation as well as protein coding, splicing and transcriptional regulation.

Allelic/genotypic association analysis

Genotypes in all groups followed the HWE ($P>0.05$). Frequency of rs1805087 'A' allele was higher in the probands ($P=0.05$) as compared with the controls (Table 1). Stratification based on gender revealed higher frequency of the 'AA' genotype in the female probands ($P=0.03$) as compared with the female controls. Allelic and genotypic frequencies of rs5742905 failed to show any marked difference as compared with the control population even after gender-based stratification (Table 1). Family-based analysis (Table 2) revealed a bias in transmission of rs1805087 'A' allele from the parents to the ADHD probands, more so for the male probands ($P=0.001$ and $P=0.003$, respectively). This bias in transmission was both paternal ($P=0.02$) and maternal ($P=0.02$) in nature. No such significant bias in transmission of any allele was observed for rs5742905 (Table 2).

Table 1 Population-based comparative analysis on allelic and genotypic frequencies

ID	*Allele/ genotype*	*All control*	*All probands*	χ^2(P)	*Male control*	*Male probands*	χ^2(P)	*Female control*	*Female probands*	χ^2(P)
MTR rs1805087	A	0.68	0.74	3.62 (**0.05**)	0.71	0.74	0.50 (0.47)	0.66	0.78	2.20 (0.13)
	G	0.32	0.26		0.29	0.26		0.34	0.22	
	AA	0.47	0.55	1.47 (0.48)	0.53	0.54	0.59 (0.74)	0.42	0.60	6.88 (**0.03**)
	AG	0.43	0.38		0.37	0.39		0.48	0.35	
	GG	0.10	0.07		0.10	0.07		0.10	0.05	
CBS rs5742905	T	0.95	0.97	3.12 (0.07)	0.95	0.97	2.35 (0.12)	0.95	0.98	0.51 (0.47)
	C	0.05	0.03		0.05	0.03		0.05	0.02	
	TT	0.90	0.94	1.09 (0.29)	0.90	0.94	1.09 (0.29)	0.90	0.95	1.80 (0.17)
	CT	0.10	0.06		0.10	0.06		0.10	0.05	
	CC	0.00	0.00		0.0	0.0		0.0	0.0	

Note: Statistically significant differences are presented in bold.

Table 2 Transmission disequilibrium test performed for informative nuclear families with ADHD probands

SNPs	*Groups*	*Probands*	*Allele*	T	*NT*	*χ2* (P)
rs1805087	Both	All probands	A	0.66	0.34	10.2 (**0.001**)
			G	0.34	0.66	
		Male probands	A	0.65	0.35	8.46 (**0.003**)
			G	0.35	0.65	
		Female probands	A	0.80	0.20	1.92 (0.16)
			G	0.20	0.80	
	Father	All probands	A	0.65	0.35	5.34 (**0.02**)
			G	0.35	0.65	
		Male probands	A	0.66	0.34	5.55 (**0.01**)
			G	0.34	0.66	
		Female probands	A	0.50	0.50	0.0 (1.0)
			G	0.50	0.50	
	Mother	Allprobands	A	0.66	0.34	4.87 (**0.02**)
			G	0.34	0.66	
		Maleprobands	A	0.63	0.37	2.98 (0.08)
			G	0.37	0.63	
		Female probands	A	0.83	0.17	2.91 (0.08)
			G	0.17	0.83	
rs5742905	Both	All probands	T	0.56	0.44	0.22 (0.63)
			C	0.44	0.56	
		Male probands	T	0.56	0.44	0.25 (0.61)
			C	0.44	0.56	
		Female probands	T	0.0	1.0	1.38 (0.23)
			C	1.0	0.0	
	Father	All probands	T	0.44	0.56	0.11 (0.73)
			C	0.56	0.44	
		Male probands	T	0.50	0.50	0.0 (1.0)
			C	0.50	0.50	
		Female probands	T	1.0	0.0	1.38 (0.23)
			C	0.0	1.0	
	Mother	All probands	T	0.67	0.33	1.01 (0.31)
			C	0.33	0.67	
		Male probands	T	0.63	0.37	0.50 (0.47)
			C	0.37	0.63	
		Female probands	T	1.0	0.0	1.38 (0.23)
			C	0.0	1.0	

Abbreviations: ADHD, attention-deficit hyperactivity disorder; SNP, single-nucleotide polymorphism.
Note: Statistically significant differences are presented in bold.

Comparative analysis on metabolites and co-factors

Plasma VB12 concentration was significantly low in the ADHD probands ($P=0.01$) as compared with the controls (Table 3) while Hcy concentration was marginally elevated in the probands ($P=0.05$) in comparison to the controls. Stratified analysis based on genotypes (Table 4) revealed that individuals with *MTHFR* rs18011133 'CT' and 'TT' genotypes had significantly lower VB12 levels ($P=0.01$ and $P=5.0E-5$, respectively), whereas probands with the 'CC' genotype had lower FA level ($P=0.008$). *MTR* rs1805087 'AA' genotype ($P=0.03$) showed association with mild hyperhomocysteinemia, while individuals with the 'GG' genotype revealed a marked deficiency in VB12 concentration ($P=0.0001$). rs1051266, rs2236225, rs1801131 and rs5742905 failed to show any association.

Comparative analysis on behavioral score

Comparative analysis between different genotypes and trait scores (Table 5) obtained through DSM-IV-TR revealed that probands having rs1051266 'GG' genotype were more inattentive ($P=0.02$) while probands having rs2236225 'CC' ($P=0.003$) and rs1801131 'CC' ($P=0.05$) genotypes were more hyperactive–impulsive than those having other genotypes. Probands with rs1801133 'CC' genotypes also showed higher level of inattention, though the differences were statistically insignificant. Comparative analysis using CPRS-R scores revealed that probands with rs1801133 'CC' ($P=0.01$) and rs1805087 'GG' ($P=0.008$) genotypes were more inattentive than other genotypes, while probands with rs1801133 'CC' ($P=0.02$) and rs5742905 'TT' ($P=0.014$) were more hyperactive–impulsive than the rest (Table 5).

Analysis of association between B_w, IQ and behavioral trait scores

Low B_w; that is, <2.5 kg, showed positive correlation with low IQ ($P<0.0001$) in higher frequency of subjects (Table 6) as compared with those with higher B_w (that is, >2.5 kg). On the other hand, DSM-IV-TR inattentiveness score was higher in probands with higher B_w ($P=0.01$).

Analyses of correlation between gene variants and parental age

Mothers belonging to group 1 (maternal age <26 years) showed significant preferential transmission of rs1801133 'T' allele to the probands ($P=0.03$; Supplementary Table 2). Statistically significant bias in transmission of rs1805087 'A' allele was also noticed when paternal age was <31 years and maternal age was <26 years

Table 3 Comparative analysis on plasma vitamin B12, folic acid and homocysteine level by *t*-test

	Vitamin B12 (pg ml^{-1})			*Folic acid (ng ml^{-1})*			*Homocysteine (μ mol l^{-1})*		
Subjects (N)	*Mean*	*s.e.*	P-*value*	*Mean*	*s.e.*	P-*value*	*Mean*	*s.e.*	P-*value*
Controls (30)	371.08	44.81	**0.01**	5.11	0.20	0.06	28.01	1.72	**0.05**
Probands (48)	232.61	44.23		5.52	0.15		36.80	5.19	

Note: Statistically significant differences are presented in bold.

Table 4 Comparative analysis on the concentration of metabolites in subjects harboring different genotypes

			Vitamin B12 (pg ml^{-1})		*Folic acid (ng ml^{-1})*		*Homocysteine(μmol l^{-1})*	
Gene	*SNP ID*	*Genotype*	*Mean* ± *s.e.*	P-*value*	*Mean* ± *s.e.*	P-*value*	*Mean* ± *s.e.*	P-*value*
RFC1	rs1051266	GG[a]	188.88 ± 92.31	0.37	5.81 ± 0.12	0.09	41.51 ± 11.23	0.32
		AG[b]	225.50 ± 61.0	0.21	5.44 ± 0.25	0.36	35.60 ± 6.66	0.43
		AA[c]	314.07 ± 91.39	0.17	5.28 ± 0.37	0.10	33.22 ± 13.06	0.31
MTHFD	rs2236225	CC[a]	133.33 ± 87.02	0.16	5.74 ± 0.21	0.26	28.36 ± 5.81	0.18
		CT[b]	239.19 ± 56.72	0.35	5.56 ± 0.19	0.30	37.22 ± 7.94	0.39
		TT[c]	282.56 ± 98.44	0.13	5.33 ± 0.39	0.18	40.61 ± 9.83	0.14
MTHFR	rs1801131	AA[a]	212.78 ± 84.90	0.36	5.31 ± 0.46	0.27	38.15 ± 10.51	0.46
		AC[b]	253.01 ± 78.87	0.43	5.62 ± 0.22	0.35	39.42 ± 8.59	0.32
		CC[c]	235.77 ± 71.15	0.41	5.51 ± 0.20	0.35	33.59 ± 9.12	0.37
	rs1801133	CC[a]	272.13 ± 58.22	0.07	5.32 ± 0.22	**0.008**	35.97 ± 6.08	0.42
		CT[b]	152.29 ± 55.84	**0.01**	5.95 ± 0.11	0.41	33.75 ± 10.05	0.27
		TT[c]	10.8 ± 0.0	**5.0E-5**	5.88 ± 0.23	0.08	70.41 ± 42.31	0.28
MTR	rs1805087	AA[a]	228.60 ± 56.96	0.41	5.59 ± 0.19	0.30	45.89 ± 8.47	0.06
		AG[b]	247.18 ± 71.01	**0.001**	5.41 ± 0.27	0.12	29.12 ± 6.38	0.22
		GG[c]	10.8 ± 0.0	**0.000**	5.85 ± 0.20	0.20	20.66 ± 7.43	**0.03**
CBS	rs5742905	TT[a]	233.28 ± 45.71	0.31	5.55 ± 0.16	0.39	35.07 ± 5.38	0.39
		CT	367.73 ± 251.03		5.38 ± 0.56		39.25 ± 13.04	

Abbreviation: SNP, single-nucleotide polymorphism.
[a]As compared with the heterozygote genotype.
[b]As compared with the derived homozygote genotype.
[c]As compared with the wild-type homozygote genotype.
Statistically significant differences are presented in bold.

($P<0.01$) at the time of birth of the probands (Supplementary Table 2).

Analysis of interaction between the sites

Independent main effects of rs1801133 (IG = 3.42%) followed by rs2236225 (IG = 1.79%), rs1805087 (IG = 0.60%), rs5742905 (IG = 0.62%), rs1051266 (IG = 0.51%) and rs1801131 (IG = 0.22%) were observed in all the ADHD cases (Figure 1a). Moderate positive synergistic interactive effect was also observed between rs1051266 and rs1801131 (IG = 0.51%) in all the ADHD cases (Figure 1a). Entropy graph for the male probands indicated redundant interaction of rs5742905 with rs1051266, rs2236225, rs1801131, rs1801133 and rs1805087, with a strong single effect of rs1801133 (Figure 1b). In the female probands, strong positive synergistic interactions were observed between rs1801131-rs1051266 (IG = 2.64%) and rs1801131-rs2236225 (IG = 1.98%), while rs2236225-rs1805087 (IG = 1.27%) and rs1805087-rs1801131 (IG = 0.78%) exhibited moderate synergistic interactive effects (Figure 1c).

MDR considering IQ and B_W as phenotypic covariates

Analysis of gene–gene interaction considering IQ as a phenotypic co-variate revealed independent effect of all the gene variants as well as IQ (Figure 2a). B_W as a phenotypic covariate revealed highest independent effect of rs2236225 (IG = 1.30%) in all the groups (Figure 3a). rs1051266-rs1801131 exhibited moderate synergistic interaction (IG = 0.69%) in all the probands with IQ (Figure 2a) and B_w (Figure 3a) as phenotypic co-variates. rs1051266, rs2236225, rs1801131, rs1801133 and rs1805087 showed redundant interaction with rs5742905 in the male probands considering IQ (Figure 2b). On the other hand, difference in B_w showed independent main effects of rs1805087 (IG = 2.13%) in the male group (Figure 3b) and rs1801133 (IG = 1.82%) in the female group (Figure 3c) with redundant interactions with B_W. Moderate positive synergistic interactions were observed between rs1805087-rs1801131 (IG = 1.24%), rs1805087-rs2236225 (IG = 0.93%) and rs2236225-rs5742905 (IG = 0.79%) in the female group when B_W was considered (Figure 3c).

DISCUSSION

ADHD is an intricate disorder speculated to be controlled by both gene and environment.[10,11] We tried to find out contribution of the folate–homocysteine metabolic system gene variants and co-factors/metabolites in the core traits of ADHD and the data obtained indicate significant involvement of gene variants as well as metabolites/co-factors with IQ, inattention and hyperactivity/impulsivity levels of eastern Indian ADHD probands. Age of the parents exhibited correlation with allelic transmission while probands with low B_w showed association with low IQ and thus could be considered as environmental factors affecting the disease etiology.

Different enzymes and co-factors of the folate metabolic pathway are involved in the maintenance of DNA methylation, myelination,

Table 5 Comparative analysis between different genotypes and trait scores obtained through DSM-IV-TR and CPRS-R

		DSM-IV-TR				*CPRS-R*			
		Inattention		*Hyperactivity–impulsivity*		*Inattention*		*Hyperactivity–impulsivity*	
SNP ID	*Genotype*	*Mean ± s.e.*	P	*Mean ± s.e.*	P	*Mean ± s.e.*	P	*Mean ± s.e.*	P
rs1051266	AA[a]	10.34 ± 0.75	0.18	10.48 ± 0.68	0.17	71.25 ± 1.52	0.48	71.65 ± 2.16	0.07
	AG[b]	11.08 ± 0.36	**0.03**	11.24 ± 0.40	0.15	71.32 ± 1.07	0.06	75.41 ± 1.38	0.20
	GG[c]	12.31 ± 0.57	**0.02**	10.55 ± 0.54	0.46	74.0 ± 1.42	0.09	73.59 ± 1.70	0.24
rs2236225	CC[a]	11.20 ± 0.84	0.33	12.41 ± 0.58	**0.003**	70.63 ± 1.98	0.15	75.53 ± 2.07	0.26
	CT[b]	11.6 ± 0.34	0.10	10.41 ± 0.39	0.31	72.86 ± 0.97	0.23	73.95 ± 1.29	0.37
	TT[c]	10.66 ± 0.64	0.30	10.75 ± 0.60	**0.02**	71.56 ± 1.48	0.35	73.21 ± 1.99	0.21
rs1801131	AA[a]	11.42 ± 0.41	0.23	10.38 ± 0.50	0.23	73.11 ± 1.25	0.17	74.47 ± 1.71	0.29
	AC[b]	10.97 ± 0.46	0.22	10.85 ± 0.44	0.14	71.42 ± 1.25	0.37	73.28 ± 1.43	0.20
	CC[c]	11.63 ± 0.71	0.40	11.68 ± 0.64	**0.05**	72.02 ± 1.29	0.27	75.38 ± 2.07	0.36
rs1801133	CC[a]	11.52 ± 0.34	0.15	11.12 ± 0.33	0.06	73.33 ± 0.90	**0.01**	75.60 ± 1.10	**0.02**
	CT[b]	10.8 ± 0.63	0.19	10.02 ± 0.65	0.16	69.87 ± 1.32	0.14	71.04 ± 1.91	**0.05**
	TT[c]	8.8 ± 1.98	0.12	12.0 ± 1.70	0.31	61.33 ± 6.0	0.09	61.0 ± 4.04	**0.02**
rs1805087	AA[a]	11.33 ± 0.40	0.24	11.24 ± 0.37	0.11	71.53 ± 1.05	0.42	73.89 ± 1.27	0.38
	AG[b]	12.28 ± 1.04	0.20	9.85 ± 1.04	0.11	71.82 ± 1.22	**0.013**	73.31 ± 1.59	0.19
	GG[c]	10.88 ± 0.49	0.11	10.46 ± 0.52	0.30	77.3 ± 1.89	**0.008**	77.2 ± 3.97	0.22
rs5742905	TT[a]	11.19 ± 0.31	0.11	10.75 ± 0.31	0.15	72.26 ± 0.79	0.39	74.12 ± 1.01	**0.014**
	CT	12.41 ± 0.92		11.91 ± 1.03		71.33 ± 3.34		65.66 ± 3.14	

Abbreviations: CPRS-R, Conners' Parents Rating Scale-revised; DSM-IV-TR, Diagnostic and Statistical Manual for Mental Disorders-IV-text revised; SNP, single-nucleotide polymorphism.
Note: Statistically significant differences are presented in bold.
[a]As compared with the heterozygote genotype.
[b]As compared with the derived homozygote genotype.
[c]As compared with the wild-type homozygote genotype.

Table 6 Comparative analysis on birth weight, IQ and trait scores of ADHD probands

	Birth weight		
Traits	<2.5 Kg	⩾2.5 Kg	χ^2 (P-*value*)
IQ (frequency of subjects)			
<80	75.51	24.49	20.1 (**0.0001**)
⩾80	45.11	54.88	
DSM-IV (trait score)			
Inattentiveness	10.26 ± 0.63	11.87 ± 0.32	**0.01**
Hyperactivity–impulsivity	10.38 ± 0.57	10.88 ± 0.39	0.23
CPRS (T-score for traits)			
Inattentiveness	70.47 ± 1.56	71.92 ± 1.11	0.22
Hyperactivity–impulsivity	72.5 ± 2.11	73.32 ± 1.31	0.37

Abbreviations: ADHD, attention-deficit hyperactivity disorder; IQ, intelligent quotient.
Note: Statistically significant differences are presented in bold. DSM-IV score varies between 0 and 36 and a score of 10 was used as a cutoff mark CPRS-T score ranges between 0 and 81 and score >60 falls under the risk zone.

regulation of gene expression, amino-acid synthesis and metabolism. Deficiency in any of these components can lead to changes in the DNA methylation or misincorporation of bases in the DNA leading to chromosomal breakage.[39,40] Though cellular uptake of folate is essential for cell growth and tissue regeneration,[19,41,42] it cannot be synthesized *de novo* in humans. Everyday foods, such as leafy greens and legumes, contain the natural form of vitamin B9, folate. 5-Methyl tetrahydrofolate, the biologically active form of vitamin B9, is the main circulatory form of folate, which can be recycled by MTR/MTR reductase (MTRR) to generate tetrahydrofolate and methionine respectively. MTR reductase maintains the active form of MTR, which requires vitamin B12 or cobalamin as a co-factor, and results in the formation of SAM, the primary methyl donor for DNA methylation[43] while also being required for neurotransmitter synthesis. SAM is demethylated to form S-adenosylhomocysteine and eventually hydrolyzed to form adenine and Hcy. Under normal physical conditions, the enzyme CBS mediates conversion of Hcy into cystathionine, which is finally metabolized to cysteine via the trans-sulfuration pathway.[44] Increase in the trans-sulfuration pathway of Hcy, resulting from overexpression of CBS, may generate a folate trap by decreasing cellular Hcy concentration and its subsequent remethylation pathway while an increase in Hcy concentration may lead to impairment in folate metabolism.[45] The remethylation reaction is catalyzed by MTR where methyl cobalamine (III) acts as a methyl donor,[46] a vital reaction important for maintaining low Hcy level. In the brain, methylation reactions are highly dependent on methionine synthase activity, as this is the sole enzyme controlling the level of brain Hcy level reviewed by Waly *et al.*[47]

The *MTR* gene is located at 1q43.[48] A 2756A>G polymorphism (rs1805087) in *MTR*, replacing aspartic acid with glycine (D919G), was first identified among patients with a deficiency of MTR and among normal controls, though the biological impact remained unknown.[49] Later investigation revealed that this non-synonymous substitution reduces affinity of the enzyme for its co-factor methylcobalamin.[50] As *MTR* activity is required for dopamine-stimulated phospholipid methylation, a step critical for synchronization of brain activity during attention,[51,52] functional deficit in MTR was hypothesized to affect the attention process. Impaired synchronization of the dopamine receptor 4 was linked to autism and ADHD,[53] thus indicating an important role of MTR in these developmental disorders. In patients with bipolar disorder type I and schizophrenia, significantly higher frequency of 2756GG or 2756AG genotypes ($P=0.008$ and $P=0.016$, respectively) were noticed.[54] In Brazilian

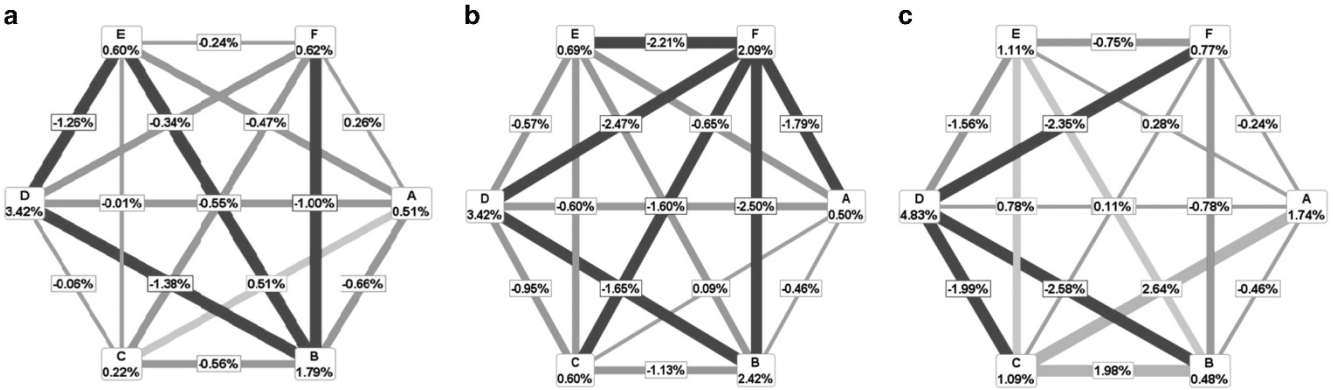

Figure 1 Gene–gene interaction analyzed using case–control data set: (**a**) all, (**b**) only male, (**c**) only female. (A) rs1051266, (B) rs2236225, (C) rs1801131, (D) rs1801133, (E) rs1805087, (F) rs5742905. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

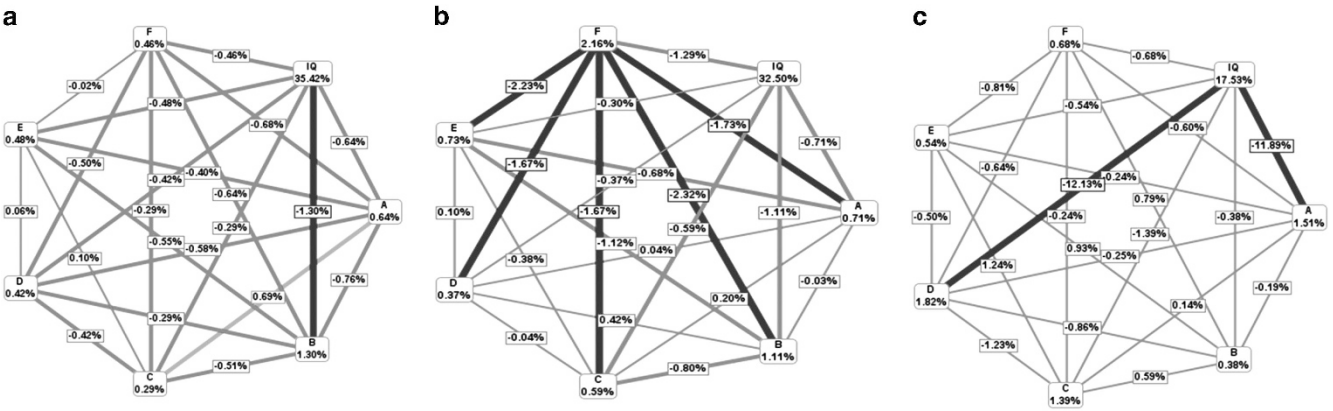

Figure 2 Gene–gene interaction analyzed using case–control data set and IQ as a phenotypic co-variate: (**a**) all, (**b**) only male, (**c**) only female. (A) rs1051266, (B) rs2236225, (C) rs1801131, (D) rs1801133, (E) rs1805087, (F) rs5742905. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

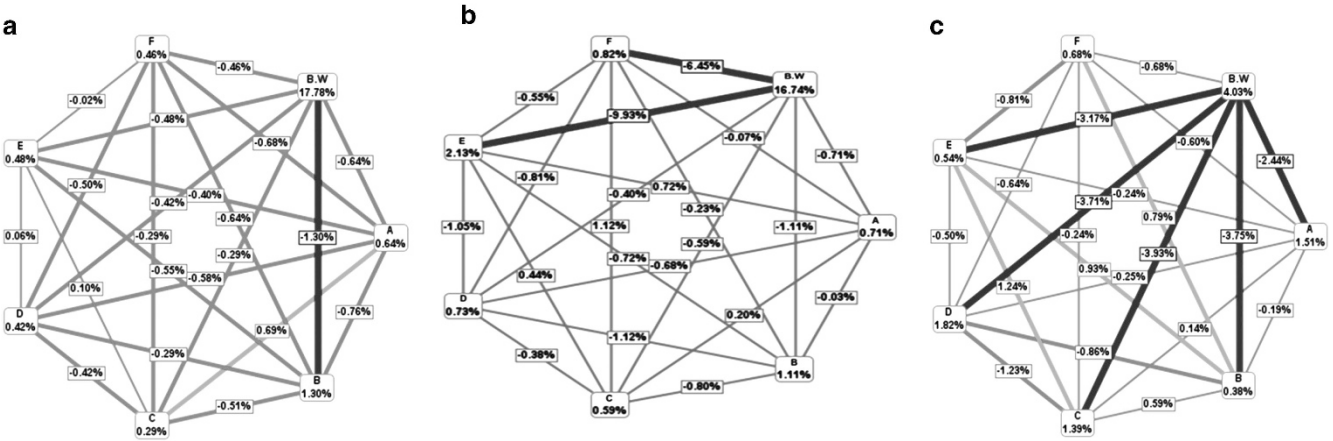

Figure 3 Gene–gene interaction analyzed using case–control data set and birth weight (B_W) as a phenotypic co-variate: (**a**) all, (**b**) only male, (**c**) only female. (A) rs1051266, (B) rs2236225, (C) rs1801131, (D) rs1801133, (E) rs1805087, (F) rs5742905. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Down syndrome patients, increased plasma Hcy concentration was found to be associated with heterozygous *MTR* 2756AG genotype.[55] However, a study on colorectal cancer patients failed to identify any association between *MTR* polymorphism and plasma levels of folate, VB12 or tHcy, suggesting that this aspartic acid-to-glycine change may not significantly affect MTR activity.[56] In the Pakistani population also, no significant association was found between *MTR* A2756G genotypes and hyperhomocysteinemia.[57] A meta-analysis on Alzheimer's disease also failed to observe any association between *MTR* A2756G polymorphism and the disorder.[58] However, in the present study on Indo-Caucasoid ADHD probands, the 'A' allele and 'AA' genotype of *MTR* rs1805087 showed association by both

population- and family-based analysis. The mild hyperhomocysteinemia observed in ADHD probands correlated with the presence of 'A' allele, indicating a role of this allele with cognitive dysfunction due to mild hyperhomocysteinemia. On the other hand, ADHD probands with the 'G' allele showed markedly reduced level of VB12, which may eventually inhibit remethylation of Hcy to 5-methyl tetrahydrofolate thus affecting neurotransmitter synthesis and cognition. This hypothesis is supported by the remarkable inattention observed in the ADHD probands. On the basis of the present observations, it could be inferred that the MTR rs1805087 could be a modulator of ADHD-associated phenotypic traits, thus warranting more detailed examination.

The *CBS* gene is localized at chromosome 21q22.3[59] and the protein encoded is a homotetramer of 63-kD subunit, which requires pyridoxal phosphate and heme for activity. It can also be stimulated by the addition of SAM.[60,61] In humans, the *CBS* gene is known to have a large number of mutations, majority of which are missense in nature.[62] A transition mutation, T833C, is known to segregate in *cis* with 844ins68 mutation in exon 8,[63] resulting in Ile278Thr change in the protein harboring a BsrI restriction site and was reported to be associated with premature occlusive arterial disease.[64] *CBS* rs5742905 T>C causes isoleucine to threonine transition in exon 8. Ethnic heterogeneity of 844ins68 polymorphism is highly prevalent in African, European and North American populations.[65,66] In healthy Pakistani individuals, heterozygous genotype of *CBS* 844ins68 was reported to confer protection against hyperhomocysteinemia as compared with the ancestral homozygous genotype.[57] On the other hand, in the eastern Indian children with idiopathic intellectual disability, a higher relative risk and biased transmission of the mutated allele was noticed from heterozygous mothers to the male probands indicating a risk conferred by this variant.[28] In the present study on ADHD probands, both population- and family-based analysis failed to show any association of this variant. However, probands with the ancestral 'TT' genotype were more hyperactive–impulsive, a trait most common for ADHD probands, as compared with the rest.

A previous study reported association of lower paternal age and advanced maternal age with severity of hyperactivity/impulsivity in children and adolescents with ADHD while none of the parent's age showed association with severity of inattentiveness.[67] Maternal age at first birth also showed association with development of ADHD; teenage childbirth (<20 years) conferred 78% increased risk of ADHD.[68] In a recent case–control study from Finland, the researchers also found that fathers aged <20 years had a 1.5-fold increased risk of having offspring with ADHD as compared with fathers aged 25–29 years.[69] Mothers of the same age group also had a 1.4-fold increased risk of having a child with ADHD.[69] In the present study on Indo-Caucasoid probands, younger mothers (<26 years) showed statistically significant biased transmission of rs1801133 'T' and rs1805087 'A' allele to the probands. As ADHD has already been proved to have a strong hereditary basis,[4,11] it could be inferred that the observed risk of association between ADHD and younger parents could be due to presence of risk gene variants in parents, with higher level of impulsivity and or novelty seeking behavior, which is preferentially transmitted to the probands.

In a study on Swedish twins, low B_w was found to be a risk factor for symptoms of ADHD.[70] In 252 ADHD children from United States also, low B_w was found to be an independent risk factor for the disorder.[71] Similar association between very low B_w and increased risk of psychiatric symptoms, especially ADHD, were reported in British children.[72] A follow-up longitudinal study revealed an increased risk of attention problems with low B_w only in the urban community residing at Detroit, USA, while in the suburban community no increased risk for attention problems was found.[73] A meta-analysis on the field, involving 4125 children, revealed that very preterm or very low B_w children have moderate-to-severe attention problems along with executive function deficit as compared with those born at term and with normal B_w.[74] In the present investigation on eastern Indian population, low B_w; that is, <2.5 kg, showed positive correlation with low IQ in significantly higher number of probands. On the other hand, comparative analysis revealed higher DSM-IV-TR inattentiveness score in probands with >2.5 kg B_w, while this difference was insignificant for CPRS-R inattention score. Whether this difference in association between inattention and B_w is due to ethnic differences or due to the difference in sample number merits further study using large cohort of samples.

Major limitations of the present study are (1) limited number of samples; (2) high plasma Hcy level in apparently healthy children; and (3) inadequate information regarding other causal factors, including gene variants. However, this pilot project was carried out on the Indo-Caucasoid ADHD probands who were never explored for these factors. The study revealed statistically significant differences between age and ethnically matched controls and ADHD probands for plasma vitamin B12 and Hcy levels for the first time indicating the necessity for investigating the folate–homocysteine pathway metabolites/co-factors in the ADHD probands. Degradation of Hcy is dependent on availability of folate, vitamin B12 and vitamin B6, thus making plasma Hcy concentration an effective indicator of the nutritional status of B vitamins.[75] An elevation in Hcy and methylmalonic acid (a functional metabolic marker for vitamin B 12 deficiency) were reported long back in neuropsychiatric population in the absence of hematological manifestations.[76] In the Indian population, total Hcy levels were reported to be higher,[77] which the authors speculated to be due to dietary deficiency of vitamin B6, B12 and folate.[78,79] The present study provides further evidence for the fact by identifying gene variants that may lower the vitamin levels thereby increasing Hcy concentration. In view of these findings, we propose further in-depth investigation on the folate–homocysteine pathway to understand its contribution in the etiology of ADHD.

From this pilot investigation, it can be inferred that *RFC1* rs1051266, *MTHFD1* rs2236225, *MTHFR* rs1801133 and *MTR* rs1805087 variants, together with co-factors/metabolites of the folate–homocysteine pathway, may contribute to the etiology of ADHD in this population by affecting folate transport as well as neurotransmitter synthesis. The case–control comparative analysis revealed statistically significant deficiency in vitamin B12 level along with mild hyperhocysteinemia in the ADHD probands that may eventually influence ADHD-associated traits, especially cognition. The data obtained in the present study thus warrants further in-depth analysis of the folate–homocysteine pathway for its contribution in the development of ADHD and further elucidation of the pathway may help in devising better strategy for management of subjects with ADHD, a complex disorder difficult to deal with.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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