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Original Article

Folate System Gene Variant rs1801394 66A>G may have a Causal Role in Down Syndrome in the Eastern Indian Population

Mahasweta Chatterjee[1], Tanusree Saha[1, 2], Subhamita Maitra[1, 3], Swagata Sinha[1], Kanchan Mukhopadhyay[1*]

1. Manovikas Biomedical Research and Diagnostic Centre, MRIH, Kolkata, India.
2. Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, USA.
3. Mahidol University, Thailand.

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Down syndrome (DS) is associated with trisomy of the 21st chromosome in more than 95% cases. The extra chromosome mostly derives due to abnormal chromosomal segregation, i.e. non-disjunction, during meiosis. Earlier reports showed that abnormal folate metabolism can lead to DNA hypomethylation and abnormal chromosomal segregation. We analyzed three functional folate gene variants, namely 5-methyltetrahydrofolate-homocysteine methyltransferase rs1805087, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase rs1801394, and reduced folate carrier 1 rs1051266, for contribution in the etiology of DS. Ethnically matched subjects including DS probands (N=183), their parents (N=273), and controls (N=286) were recruited after obtaining informed written consent for participation. Karyotype analysis confirmed trisomy 21 in DS patients recruited. Genomic DNA, purified from peripheral blood leukocytes was used for genotyping of the target sites by PCR based methods, and data obtained was subjected to population- as well as family-based association analysis. Frequency of rs1801394 'G' allele and 'GG' genotype was higher in DS probands ($P < 0.0001$). Statistically significant higher occurrence of the 'G' allele in parents of DS probands ($P < 0.0001$) and maternal bias in transmission of the "G" allele was also noticed ($P < 0.0001$). Genetic model analysis demonstrated rs1801394 "G" as a risk allele under both dominant and recessive models. DS probands also showed higher occurrence of rs1051266 "G" ($P = 0.05$). Quantitative trait analysis revealed significant negative influence of rs1805087 "A" on birth weight. Screening for rs1801394 "G" could be useful in monitoring the risk of DS, at least in the studied population.

Key words: Down syndrome, DNA hypomethylation, folate, rs1805087, rs1801394, rs1051266

Subjects with Down syndrome (DS) often exhibit deficit in intelligence, delayed psychomotor development and some dysmorphic features (1). Worldwide prevalence of DS is about 1/1000 live births (1, 2). A number of clinical conditions like congenital heart disease, respiratory

*Corresponding author: Manovikas Biomedical Research and Diagnostic Centre, Kolkata, India.
Email: drkanchanm@gmail.com

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infections, gastrointestinal defects, hypothyroidism, and neurological complications such as early manifestation of Alzheimer's disease were also reported (1).

Major causal factor for DS related phenotypes are speculated to be the triplication of genes located on chromosome 21 and in around 90% of the cases, this change is imputed to imperfect non-disjunction and abnormal maternal segregation during meiosis I (3). Both *in vitro* and *in vivo* studies proved that DNA methylation is an important process for genomic stability; genome wide DNA hypomethylation was reported to induce aneuploidy and chromosomal rearrangements (4), loss of heterozygosity (5), and chromosomal segregation (6). Folate deficiency showed association with DNA hypomethylation (7), DNA instability, strand breakage, uracil misincorporation (7, 8), as well as aneuploidy of chromosome 17 and 21 (9). Investigators mostly reported association between folate metabolism and polymorphisms in 5-methyltetrahydrofolate reductase (*MTHFR*) rs 1801133 677C>T (10-12), methionine synthase (*MTR*) rs1805087 2756 A>G (12-15), methionine synthase reductase (*MTRR*) rs1801394 66 A>G (10, 12, 15, 16), and reduced folate carrier (*RFC1*) rs1051266 80 G>A in different ethnic groups (16-18). In the Indian population, analysis on *MTRR* rs1801394 showed a risk of association with DS related congenital heart disease (19). Excepting for few meta-analysis (14-16), investigators mainly analyzed one or two gene variants in limited number of DS cases and controls (10, 12, 17-19). Additionally, either population- or family-based analyses were done. We for the first time attempted to analyze three functional gene variants, namely *MTR* rs1805087, *MTRR* 1801394 and *RFC1* rs1051266, in a larger group of Eastern Indian patients with DS, and tried to identify the risk contributed by these variants by population- as well as family-based statistical methods.

Materials and methods

Subject's recruitment

Unrelated nuclear families (N=183) with DS probands (106 trios, 61 duos – 54 without father and 7 without mother, 16 probands without parents) were recruited from the outpatient department based on the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (1). After obtaining informed written consent for participation from parents, peripheral blood was collected from the ante-cubital vein and trisomy for the 21st chromosome was confirmed by karyotyping. Ethnically matched healthy control individuals (N=286) were recruited following similar protocol. The study was approved by the institutional Human Ethical Committee (PR-04-09).

In silico analysis on studied SNPs

All three studied sites harbor missense substitution of base leading to change in amino acid, and were predicted to alter splicing. Details on the functional role of studied variants were analyzed by F-SNP (https:// compbio. cs.queensu. ca/F-SNP/) and SNPinfo (https://snpinfo. niehs. nih. gov/).

Genomic DNA isolation and genotyping

Genomic DNA was isolated from peripheral leukocytes by standard phenol/chloroform method, and was stored at -20 °C until analysis. Genotyping was performed by PCR amplification using oligonucleotides followed by digestion with restriction enzymes and gel electrophoresis (20, 21).

Statistical analysis

Population-based comparative analyses on allelic and genotypic frequencies were performed using Unphased version 3.1.7. (22). Allelic transmission pattern from parents to DS probands was analyzed by Haplotype based Haplotype Relative Risk method under the Unphased program (version 3.1.7) (22). Odd's ratio (OR) was calculated online using the OR calculator (http:// www. H utchon. net/ ConfidORnulhypo. htm) and

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relative risk was calculated using Medcalc's Relative risk calculator (https://www. medcalc. org/ calc/ relative_risk.php). Quantitative Trait analysis (QTA) between the SNPs and birth weight was done using the UNPHASED program (version.3.1.7). The multifactor dimensionality reduction (MDR) (version.3.0.2) program (23) was employed for detecting effects of studied SNPs on DS. While studying genes of complex disorders, multiple genetic models help to explore the biological rationale of the study. Hence, we have explored the dominant and recessive models to identify the risk conferred by studied variants.

Results

In silico analysis of studied SNPs

MTR rs1805087, located in 1q43, harbors missense base substitution of A2756>G leading to change in amino acid asparagine>glycine. This change was predicted to alter splicing regulation and post translational modification. rs1805087 A2756>G was found to be associated with disorders of vitamin D metabolism and gastrointestinal stromal tumor. Polyphen analysis indicated that the substitution could be damaging. Regulatory potential score: 0.188, conservation score: 1 (where 0.0 is the lowest score).

MTRR rs1801394, located in 5p15.31, harbors missense base substitution of 66A>G leading to change in amino acid isoleucine>methionine. The derived variant was predicted to alter splicing regulation and transcriptional regulation. This mutation was detected in patients with deficiency in vitamin D metabolism, DS, gastrointestinal stroma tumor, and neural tube defect. Polyphen analysis indicated that the substitution is benign, regulatory potential score: 0.24, conservation score: 0.161 (where 0.0 is the lowest score).

RFC1 rs1051266, located in 21q22.3, harbors missense base substitution of G80>A leading to change in amino acid histidine>arginine. *In silico* analysis predicted that this substitution can affect splicing regulation, transcriptional regulation, and post-translational modification. Mutation showed association with gastrointestinal stromal tumor. Polyphen analysis indicated possible damaging effect of the substitution. Regulatory potential score: 0, conservation score: 0 (where 0.0 is the lowest score).

Population based comparative analysis

Population based analysis on rs1805087 failed to show any significant difference in allelic or genotypic frequencies (Table 1). Comparative analysis on rs1801394 revealed higher occurrence of the "G" allele (χ^2 = 39.42, P = 3.4e-010, OR = 2.56; 95% CI = 1.89-3.46) and "GG" genotype (χ^2 = 57.8, P = 2.8e-013, OR= 2.74; 95% CI 1.80-4.20) in DS probands (Table 1). Parents of DS probands also showed higher occurrence of the "G" allele with respect to control (Table 1; χ^2 =15.75, P=7. 22e-005, OR= 1.98; 95% CI = 1.40-2.80 in father; χ^2 = 52.35, P = 4.62e-013, OR = 3.13; 95% CI = 2.27- 4.31 in mother), resulting in 99% occurrence of the risk "G" allele in families with DS probands. For rs1051266, heterozygous "GA" genotype was higher in the probands (χ^2= 5.8, P = 0.05, OR = 1.58; 95% CI = 1.07-2.35). On the other hand, in mother of DS probands, frequency of the "A" allele (χ^2 = 9.54, P = 0.002, OR = 1.57, 95%CI = 1.17-2.10) and "AA" genotype (χ^2 = 11.36, P=0.003, OR = 2.49, 95%CI = 1.56-3.98) was higher as compared to the controls.

Family based transmission analysis

Family-based analysis (Table 2) showed significant higher maternal transmission of rs1801394 'G' allele (χ^2 = 5.82, P = 0.01, RR= 1.68; 95% CI = 1.244- 2.27). Stratified analysis based on the age of the mother at the time of birth of the probands indicated that this over-transmission was due to biased transmission from mothers below 28 years (χ^2 = 3.45, P = 0.06, RR = 1.50; 95% CI = 1.17-1.92).

Association with birth weight

QTA revealed significant negative effect of

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Table 1. Population based comparative analysis on the studied variants.

Gene (ID)	Allele/ genotype	Control (N=286)	DS probands (N=183)	X^2 P	Parents			
					Father (N=113)	X^2 (P)	Mother (N=160)	X^2 (P)
MTR (rs1805087)	A	0.69	0.70	0.13	0.69	0.001	0.70	0.03
	G	0.31	0.30	(0.71)	0.31	(0.97)	0.30	(0.85)
	AA	0.49	0.46	5.37	0.44		0.45	5.18
	AG	0.41	0.49	(0.06)	0.50	3.69	0.50	(0.07)
	GG	0.10	0.05		0.06	(0.15)	0.05	
MTRR (rs1801394)	A	0.50	0.29	**39.42**	0.34	**15.75**	0.25	**52.35**
	G	0.50	0.71	**(3.40906e-010)**	0.66	**(7.2186e-005)**	0.75	**(4.62511e-013)**
	AA	0.23	0.01	**57.80**	0.01		0	**74.82**
	AG	0.55	0.55	**(2.80275e-013)**	0.66	**35.34 (2.1117e-008)**	0.49	**(5.64921e-017)**
	GG	0.22	0.44		0.33		0.51	
RFC 1 (rs1051266)	G	0.56	0.56	0.02 (0.88)	0.51	1.08 (0.29)	0.44	**9.54 (0.002)**
	A	0.44	0.44		0.49		0.56	
	GG	0.29	0.24	**5.80 (0.05)**	0.22		0.16	
	GA	0.53	0.65		0.59	2.06 (0.36)	0.57	**11.36 (0.003)**
	AA	0.18	0.11		0.19		0.27	

X^2 = Chi square; P = p value; N= Number of individuals. Statistically significant differences are presented in bold.

Table 2. Family based analysis on studied variants to identify transmission pattern.

SNP ID	Parent	ALLELE	T	NT	X^2 (P)
rs1805087	Both	A	0.70	0.65	1.11 (0.29)
		G	0.30	0.35	
	Father	A	0.70	0.66	0.62 (0.42)
		G	0.30	0.34	
	Mother	A	0.71	0.67	0.35 (0.55)
		G	0.29	0.33	
	Mother< 28years age (N=72)	A	0.71	0.68	0.11 (0.73)
		G	0.29	0.32	
	Mother ≥ 28years age (N=85)	A	0.70	0.68	0.04 (0.82)
		G	0.30	0.32	
rs1801394	Both	A	0.33	0.33	1.13687e-013 (1)
		G	0.67	0.67	
	Father	A	0.29	0.31	0.20 (0.65)
		G	0.71	0.69	
	Mother	A	0.30	0.17	**5.82 (0.01)**
		G	0.70	0.83	
	Mother <28years age (N=72)	A	0.25	0.11	**3.44 (0.06)**
		G	0.75	0.89	
	Mother ≥ 28years age (N=85)	A	0.32	0.19	2.36 (0.12)
		G	0.68	0.81	
rs1051266	Both	G	0.50	0.48	0.17 (0.68)
		A	0.50	0.50	
	Father	G	0.54	0.54	0.002 (0.95)
		A	0.46	0.46	
	Mother	G	0.53	0.43	2.55 (0.10)
		A	0.47	0.57	
	Mother < 28years age (N=72)	G	0.52	0.51	0.01 (0.91)
		A	0.48	0.49	
	Mother ≥ 28years age (N=85)	G	0.52	0.39	2.00 (0.15)
		A	0.48	0.61	

T= Transmitted; NT = Not Transmitted; X^2 = Chi square; P = p value. Statistically significant differences are presented in bold.

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rs1805087 "A" on birth weight (Table 3; AV = 0.24, CI = 0.008 to 0.47, χ^2 = 4.40, P = 0.035). rs1051266 "A" also showed negative impact on birth weight (AV= -0.17), though the value was statistically non-significant.

Multidimensionality reduction (MDR) analysis

MDR using case–control data revealed strong independent effects of all three variants evidenced from high nodal values of 16.24, 7.17, and 5.25 for rs1801394, rs1051266, and rs1805087 respectively (Figure 1). However, studied variants did not show any significant additive or interactive effects.

Genetic model analysis

Risk allele of *MTR* rs1805087 (A/G), *MTRR* rs1801394 (A/G) and *RFC1* rs1051266 (G/A), as identified by *in silico* analysis, were considered for calculating the dominant (rs1805087 AA+AG versus GG; rs1801394 GG+GA versus AA; rs1051266 AA+AG versus GG) as well as the recessive model (rs1805087 AA versus AG+GG; rs1801394 GG versus GA+AA; rs1051266 AA versus AG+GG) (Table 4). Significant contribution of rs1801394 was observed under both dominant (χ^2 = 38.73; P < 0.00001; OR = 5.38; 95% CI: 3.16-9.16) and recessive (χ^2 = 21.72; P < 0.00001; OR = 2.73; 95% CI: 1.78-4.17) models. rs 1805087 and

Table 3. Quantitative Trait analysis to identify association between SNPs and birth weight.

Trait	SNP	Allele	AV	X^2 (P)
Birth Weight	rs1805087	A	-0.24	**4.40 (0.03)**
		G	0.24	
	rs1801394	A	0	0.009 (0.92)
		G	0.01	
	rs1051266	G	0	2.44 (0.11)
		A	-0.17	

AV= AddValue; X^2 = Chi square; P = p value. Statistically significant differences are presented in bold.

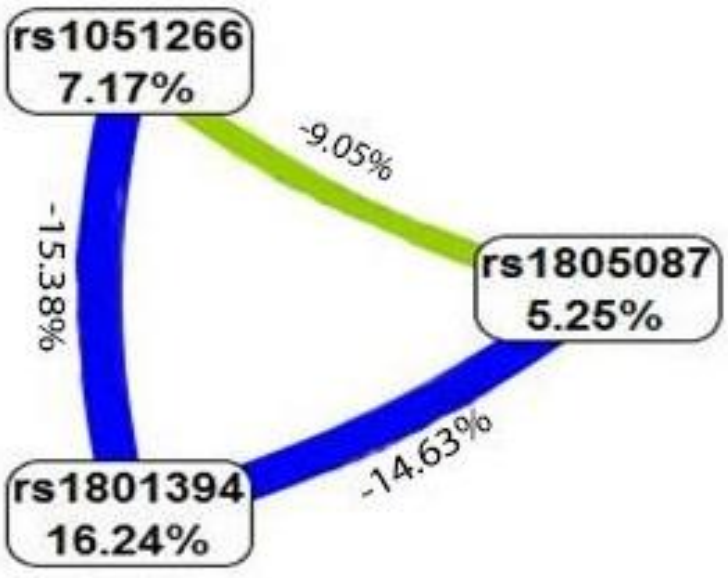

Fig. 1. Multidimensionality reduction analysis using case-control data to identify relation between the studied sites.

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Table 4. Genetic model analysis of the studied variants in DS probands.

Genetic Model	SNP ID	Alleles	X^2 (P)	OR (95% CI)
Dominant	rs1805087	AA+AG versus GG	3.32 (0.06)	1.88 (0.95-3.72)
	rs1801394	GG+GA versus AA	**38.73 (<0.00001)**	**5.38** (3.16-9.16)
	rs1051266	AA+AG versus GG	0.97 (0.32)	1.24 (0.80-1.93)
Recessive	rs1805087	AA versus AG+GG	0.10 (0.74)	0.93 (0.63-1.37)
	rs1801394	GG versus GA+AA	**21.72 (<0.00001)**	**2.73** (1.78-4.17)
	rs1051266	AA versus AG+GG	3.21 (0.07)	0.61 (0.36-1.04)

X^2 = Chi square; P = p value (P<0.05); OR= Odds ratio; 95% CI= 95% Confidence Interval. Statistically significant differences are presented in bold.

rs1051266 failed to show any significant role (Table 4).

Discussion

Data obtained in the present study indicated that *MTRR* rs1801394 "G" allele could be a major risk factor for DS in the studied population. *In silico* analysis revealed that the 66 A>G transition can alter splicing as well as transcriptional regulation. Frequency of this allele was higher in families with DS probands; the risk "G" allele was detected in 99% of DS probands either in homozygous or heterozygous conditions. Maternal transmission of the derived 'G' allele was significantly higher and genetic model analysis showed significant risk of DS in presence of the "G" allele. Till date it remains uncertain whether increasing maternal age is a contributory factor to DS or not. Our study revealed a bias in transmission of rs1801394 "A" from younger mothers (age < 28 years at the time of birth of the proband) while other analyses revealed a risk of DS conferred by rs1801394 "G". Our analysis by MDR showed significant independent effect of all the studied variants.

Folate metabolism is a complex pathway involving multiple enzymes and water-soluble B vitamins such as vitamin B9, vitamin B6, and vitamin B12 playing key roles as cofactors or substrates in the pathway. It includes two main cycles: purine and pyrimidine synthesis, necessary for synthesis and repair of DNA, and DNA methylation, an epigenetic process that modulates gene expression and genomic stability essential for normal cellular methylation reactions. Congenital heart defect (CHD), found in almost half of DS probands, is predicted to occur due to peri-conceptional folic acid deficiency leading to epigenetic modification of several genes (24). Increased risk of CHD in DS subjects in association with *MTRR* C524T and A66G polymorphisms were also reported (19). Polymorphism of folate genes were predicted to increase the risk of neurodevelopmental disorders and chromosomal abnormalities (9-15, 17,18). Adequate folate intake around the time of conception and early pregnancy was found to reduce the risk of certain problems detected in patients with neurodevelopmental disorders like attention deficit hyperactive disorder and autism spectrum disorder (25- 27).

In the *MTR* gene, rs1805087 (2756 A>G) was reported to be associated with increased maternal risk for DS in presence of AG or GG genotypes, as well as when combined with *MTRR* rs1801394 polymorphism (*MTR* 2756AG/*MTRR* 66AG) (28). Additionally, the "G" allele was proved to be more frequent, both in homozygous as well as heterozygous conditions, in mothers with DS probands as compared to mothers of individuals without the syndrome (29). Reports on allelic association with homocysteine concentration also varied widely; while some investigators found

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association of the "A" allele with higher homocysteine concentration (30), similar associations with the "G" allele was noticed by others (31). In the present study on Eastern Indian families with DS probands, no significant difference in allelic/genotypic frequencies was noticed by case-control analysis of rs1805087. Family based analysis also failed to show any biased allelic transmission. However, stratified analysis revealed negative impact of the "A" allele on birth weight of probands and genetic model analysis showed marginally higher risk of DS in presence of the "A" allele with higher OR under the dominant model. Further in depth analysis on the site would be necessary to decipher the role of this variant in DS etiology.

The MTRR enzyme is responsible for maintenance of the active form of the enzyme MTR and rs1801394 *(*66 A>G) was reported to confer independent maternal risk for DS (15), especially in presence of the homozygous GG genotype (10, 29). Association between rs1801394 and increased homocysteine concentration with risk of DS in combination with other polymorphisms, such as *MTHFR* 677 C>T, were also reported (10). In Caucasian and mixed Brazilian populations, frequency of rs1801394 "G" allele ranged from 35.8 to 54.3% and 40.0 to 48.0% respectively, while in the Asians (Chinese/Japanese) the value ranged between 41.5 to 62.5% (14, 15). In the Indian control population, we have detected 50% frequency of the "G" allele. On the contrary in families with DS probands, frequency of genotypes with the "G" allele was ~99% and in the mother of the DS probands, it was 100% with total absence of the "AA" genotype. Family based stratified analysis revealed statistically significant maternal bias in transmission of the "G" allele to the probands, more specifically from younger mothers. Both OR and RR indicated significant risk of association of the "G" allele with DS. Genetic model analysis showed statistically significant risk in presence of the "G" allele under both dominant and recessive models. This observed data in Indo-Caucasoid families with DS probands indicate that this site may have a role in the pathophysiology of DS, which could be modulated by malfunctioning of the MTR enzyme culminating in methionine deficit and a disturbance in the folate-homocysteine cycle ultimately leading to DNA hypomethylation and inappropriate chromosomal segregation.

Previous investigators have also evaluated *RFC1* 80 G>A polymorphism to identify its influence in DS etiology (13, 17, 18). Though some studies found no association between this polymorphism and DS (32), a role of this polymorphism was suggested in combination with other gene variants involved in the folate metabolic cycle (33). In a later review of literature on the folate metabolic gene variants, individuals with the rs1051266 "G" allele were also reported to contribute to a deficit in folate (24). In Indian subjects, population–based comparative analysis revealed higher frequency of genotypes with the "G" allele in the DS probands with concurrent decrease in the "AA" genotype. While mother of DS probands showed higher occurrence of the "A" allele and "AA" genotype, family-based analysis showed a trend for higher transmission of the "G" allele to DS probands from both parents. The "A" allele showed mild negative impact on birth weight.

DNA hypomethylation was reported to induce aneuploidy and chromosomal rearrangements (4), loss of heterozygosity (5), and abnormal chromosomal segregation (6). This DNA hypomethylation was speculated to result from missense base substitutions like rs1801394 "G" encoding for an enzyme with reduced affinity for its substrate (34). In our study, comparative analysis on rs1801394 revealed higher occurrence of "G" allele and "GG" genotype in DS probands as compared to the control subjects. Population based analysis also showed that rs1801394 "G" allele was present in 100% of mothers with DS probands.

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Significant contribution of rs1801394 was observed under both dominant and recessive models. Moreover, family based analysis revealed significant maternal bias in transmission of the rs1801394 “G” to DS probands. Our study also revealed a bias in transmission of rs1801394 “A” from younger mothers (age < 28 years at the time of birth of the proband) while other analyses revealed that a risk of DS conferred by rs1801394 “G” could be associated with chromosomal non-disjunction in older mothers. In this backdrop of information, we hypothesize that in Eastern Indian DS probands, the risk “G” allele of rs1801394 may contribute to the etiology of DS by disrupting the folate metabolic cycle of the mother resulting in chromosomal non-disjunction as was already proposed by earlier investigators in the Caucasoid population (10). Based on our observation, further in depth investigation is warranted in this population to identify the role of folate-homocysteine metabolic pathway in the pathophysiology of DS.

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Conflict of interest

Authors declare that there is no conflict of interest to disclose.

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