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Research Article

ANALYSIS OF *MTHFR* C677T AND A1298C POLYMORPHISM IN DOWN SYNDROME AND OTHER INTELLECTUALLY DISABLED CHILDREN

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ABSTRACT

Down syndrome or trisomy 21, the most common genetics cause for mental retardation, occurs mainly due to error during meiosis. Despite of plethora of studies the exact understanding of meiotic nondisjunction is still unclear. Some studies have suggested the role of faulty folate metabolism leads to risk of Down syndrome. Methylene tetrahydrofolate reductase (*MTHFR*), in human encoded by *MTHFR* gene, plays an important role in folate metabolism. Present study analyzed the occurrence of individual and combined *MTHFR* C677T and A1298C polymorphism in 32 Down syndrome, 100 other intellectually disabled (ID) and 100 healthy children from Gujarat. The genotypic analysis for *MTHFR* C677T and A1298C was carried out using PCR-RFLP analysis. The 677T allele frequency was observed higher in Down syndrome and intellectually disabled children as compared to control. Whereas, 1298C allele frequency was higher in intellectually disabled and lower in Down syndrome children as compared to control. The double heterozygote for C677T and A1298C observed more frequent in Down syndrome and intellectually disabled children as compared to control children.

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INTRODUCTION

Down syndrome (DS) is the most common genetic cause of intellectual disability (ID) in human and occurs due to either complete or partial copy of an additional chromosome 21 which is resulted from the error during meiosis (Antonarakis *et al.*, 1992). In spite of substantial efforts, the mechanism causing meiotic nondisjunction and role of maternal age on trisomy 21 are poorly recognized. Study by James *et al.*, (1999) mentioned that chromosomal instability and abnormal segregation may be resulted from genomic DNA hypomethylation. In contrast, the defective folate metabolism and affected gene-nutrient interaction in one-carbon metabolism may leads to abnormal DNA methylation, nucleic acid synthesis and DNA repair (Kim, 1999, 2000; Fenech, 2001).

The 5, 10 methylenetetrahydrofolate reductase (*MTHFR*), a key enzyme involved in folic acid metabolism (Wagner, 1995), plays catalytic role in conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-methyltetrahydrofolate is a main circulating form of folic acid and act as a substrate for the methionine synthase to regenerate methionine from the homocysteine (Hcy), in S-Adenosyl

methionine (SAM) cycle. Methionine in SAM cycle, subsequently produce SAM, which is a universal methyl donor in humans and requires for methylation of DNA (Schwahn and Rozen, 2001; Shane *et al.*, 1989). Thus, *MTHFR* plays a central role in the provision of methyl groups to the body. *MTHFR* also plays role in the production of nucleotides required for DNA synthesis by means of thymidylate synthase (Wagner, 1995).

The two most common and extensively studied *MTHFR* gene polymorphisms are C677T (A222V) and A1298C (E429A) and reported to decrease 50% to 60% catalytic activity of *MTHFR* (Frosset *et al.*, 1995). Substitution of cytosine to thiamin at 677 position leads to change in amino acid alanine to valine at 222nd codon which affect the catalytic domain of *MTHFR* and reduce the catalytic activity of enzyme. Homozygosity of valine at 222 amino acid position produces thermolabile form of enzyme and found to influence the occurrence of hyperhomocysteinemia, mainly during insufficient folate intake (Jacques *et al.*, 1996; Christensen *et al.*, 1997). Individuals with the variant *MTHFR* TT genotype have ~30% of the *in vitro* enzyme activity than in those with the CC wild-type. Heterozygotes (CT) show ~65% of normal enzyme activity (Frosset *et al.*, 1995). In case of A1298C, adenine is

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substituted by cytosine at 1298 nucleotide leading to the replacement of glutamate to alanine at 429th codon, which found to affect regulatory domain of *MTHFR* (Weisberg *et al.*, 1998). Several studies reported that, though homozygosity for alanine at 429 amino acid position leads to the lower enzymatic activity, it does not found to increase plasma Hcy levels, with the exception when it occurs together with occurrence homozygosity of valine at 222 (Schwahn and Rozen, 2001; van der Put *et al.*, 1998; Weisberg *et al.*, 2001). The combined heterozygous genotype for C677T and A1298C is found to be associated with increased plasma Hcy levels and reduced plasma folate levels (van der Put *et al.*, 1998; Weisberg *et al.*, 1998).

Many studies have been conducted on maternal risk of *MTHFR* gene polymorphisms in occurrence of DS but very few studies conducted to check the prevalence of variation in DS and ID children. Hence, the present study was focused on the analysis of prevalence of individual and combined *MTHFR* C677T and A1298C genotype in DS and ID children of Gujarati population.

METHODOLOGY

Samples

Genotyping was done for C677T (A222V) and A1298C (E429A) SNPs of *MTHFR* gene using PCR-RFLP. Present study was ethically approved by S. G. Patel Ayurveda Hospital and Maternity Homes, New Vallabh Vidyanagar, Anand, Gujarat, India. Total 232 blood samples of the children from different area of Gujarat were collected using the trained technicians. The consent forms were signed by the parents of the volunteers before the sample collection. From 232 (142 males and 90 females) children, 32 (20 males and 12 females) were of Down syndrome (with characteristic facial features), 100 (67 males and 33 females) were of other intellectual disability (IQ less than 70) and 100 (55 males and 45 females) were control (normal healthy). The samples of DS and ID children were collected from the special schools for the mentally retarded children.

Molecular analysis

DNA from the blood samples was isolated using standard phenol-chloroform method. PCR was carried out for A222V using the primer sequence: Forward- TGA AGG AGA AGG TGT CTG CGG GA; Reverse- AGG ACG GTG CGG TGA GAG TG. Similarly, primer sequence Forward - CTT TGG GGA GCT GAA GGA CTA CTA C; Reverse - CAC TTT GTG ACC ATT CCG GTT TG were used for E429A (Matsuo *et al.*, 2001). PCR reaction mixture for A222V and E429A were prepared using 1X master mix (Takaea Bio Inc.), 0.4 pM of each primer (Sigma-Aldrich) and 100 ng of DNA. Total volume 25µL was adjusted using nuclease free sterile distilled water. The PCR was carried out using the following thermal cycler parameters: Initial denaturation for 4 minutes at 94°C and final extension at 72°C for 7 minutes. Denaturation was carried out at 94°C for 30 seconds, annealing at 67°C (A222V) and 62°C (E429A) for 40 seconds followed by extension for 55 seconds at 72°C for 35 cycles. The PCR products of both A222V and E429A were digested by *Hinf* I and *Mbo* II restriction enzymes (New England Biolabs, UK) respectively

(Table 1). The digestion was carried out using master mix containing 1X buffer, 10 µL of respective PCR products and 1 µL of the respective restriction enzyme. The mixture was kept the water bath at 37°C for 2 hours for the incubation. The digested PCR products were analyzed on 3.5% agarose gel prepared in 1X TBE buffer.

Table 1 Details of PCR primers and RFLP pattern

Variant	Primer Sequence	PCR product size	Restriction enzyme	Pattern of genotype with size in bp
C677T (A222V)	F: 5' tga agg aga agg tgt ctg cgg ga 3'	198 bp	<i>Hinf</i> I	CC : 198
	R: 3' agg acg gtg cgg tga gag tg 5'			CT : 198+175+23 TT : 175+23
A1298C (E429A)	F: 5' ctt tgg gga gct gaa gga cta cta c 3'	163 bp	<i>Mbo</i> II	AA : 56+30+30+28+19
	R: 3' cac ttt gtg acc att cgg gtt tg 5'			AG : 84+56+30+30+28+19 GG : 84+30+30+19

Statistical Analysis

The gene and genotype frequency for single and two alleles for males and females of all three groups were calculated manually. The odd ratio was calculated to show any association of *MTHFR* polymorphism with DS and ID.

RESULTS

The size of the PCR product for both the allele of C677T was 198 bp and of A1298C was 163 bp. The wild type allele with C at 677th position doesn't have site for the restriction enzyme *Hinf* I. While the substitution of cytosine to thiamin at 677th position, leads to T (mutant) allele, created the site for *Hinf* I restriction enzyme. The PCR product with CC genotype produced only single band of 198 bp after the digestion with *Hinf* I. However, the mutant genotype (TT) showed two bands of 175 and 23 bp (Fig 1).

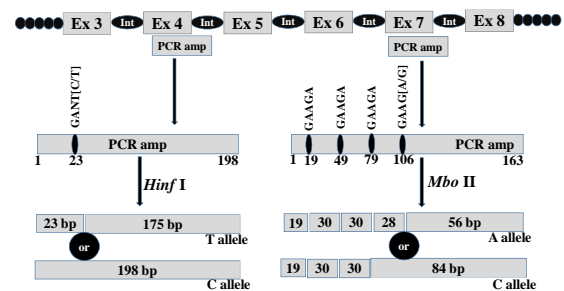


Fig 1 Diagrammatic representation of PCR-RFLP pattern of *MTHFR* C677T and A1298C.

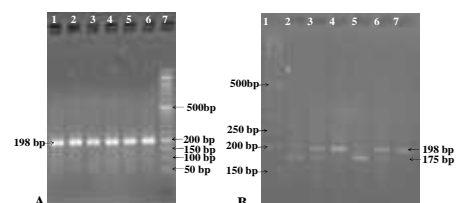


Fig 2 *MTHFR* C677T polymorphism: A- PCR bands on 1.5% agarose gel and B- RFLP bands on 3.5% agarose gel. In A lane 1-6 are PCR products of 198 bp and lane 7 is 50 to 1500bp DNA ladder. In B lane 1 is 50 to 1500bp DNA ladder, lane 2 & 5 are homozygous mutant with TT genotype, lane 3 & 6 are heterozygous with CT genotype and lane 4 & 7

The heterozygous individuals (CT) produced 3 bands of 198, 175 and 23 bp after the digestion of PCR products with *Hinf* I. The banding pattern of the *Hinf* I digested PCR products is shown in Fig 2. The summary of genotyping of *MTHFR* C677T is shown in Table 2. Out of 100 control sample, 44 males and 31 females found with CC genotype, 9 males and 13 females with CT genotype and 2 males and 1 female with TT genotype. From 100 ID children, 48 males and 22 females found with CC genotype, 16 males and 11 females with CT genotype and 3 males and none of the females with TT genotype. Out of 32 DS samples, 13 males and 8 females found with CC genotype, 7 males and 4 females with CT genotype. While, none of the DS sample were found with TT genotype.

Table 2 Allelic and genotypic distribution of *MTHFR* polymorphisms in patients and control

Variant	Allele/Genotype	Frequency in DS (n=32)			Frequency in ID (n=100)			Frequency in Control (n=100)		
		Male	Female	Total	Male	Female	Total	Male	Female	Total
C677T	C	0.825	0.833	0.828	0.835	0.833	0.835	0.881	0.833	0.860
	T	0.175	0.166	0.171	0.164	0.166	0.165	0.118	0.166	0.410
	CC	0.650	0.666	0.656	0.716	0.666	0.700	0.800	0.688	0.750
	CT	0.350	0.333	0.343	0.238	0.333	0.270	0.163	0.288	0.220
	TT	0.000	0.000	0.000	0.044	0.000	0.030	0.036	0.022	0.030
A1298C	A	0.650	0.666	0.656	0.604	0.560	0.590	0.600	0.611	0.615
	C	0.350	0.333	0.343	0.395	0.439	0.410	0.400	0.388	0.395
	AA	0.400	0.333	0.375	0.313	0.212	0.280	0.309	0.288	0.300
	AC	0.500	0.666	0.562	0.582	0.696	0.620	0.581	0.644	0.610
	CC	0.100	0.000	0.062	0.104	0.090	0.100	0.109	0.066	0.090

Adenine at 1298th position in A (wild type) allele has a site for the restriction enzyme *Mbo* II. While the substitution of adenine to cytosine at 1298th position, leads to C (mutant) allele, abolish the site of *Mbo* II restriction enzyme. The PCR product with genotype (AA) produced five bands of 56, 30, 30, 28 and 19 bp after the digestion with *Mbo* II. However, the mutant genotype (CC) showed four bands of 84, 30, 30 and 19 bp. The heterozygous individuals (AC) produced 6 bands of 84, 56, 30, 30, 28 and 19 bp after the digestion of PCR products with *Mbo* II (Fig 1). The banding pattern of the *Mbo* II digested PCR products are shown in Fig 3.

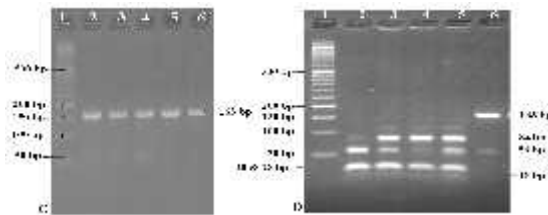


Fig 3 *MTHFR* A1298C polymorphism: C- PCR bands on 1.5% agarose gel and D- RFLP bands on 3.5% agarose gel. In C lane 1 is 50 to 1500bp DNA ladder, lane 2-6 are PCR products of 163 bp. In D lane 1 is 50 to 1500bp DNA ladder, lane 2 is homozygous normal with AA genotype, lane 3 & 5 are heterozygous with AC genotype, lane 4 is homozygous mutant with CC genotype and lane 6 is PCR product of *MTHFR* A1298C.

The summary of genotyping of *MTHFR* A1298C is shown in Table 2. Out of 100 control sample, 17 males and 13 females found with AA genotype, 32 males and 29 females with AC genotype and 6 males and 3 females with CC genotype. From 100 ID children, 21 males and 7 females found with AA genotype, 39 males and 23 females with AC genotype and 7 males and 3 females with CC genotype. Out of 32 DS samples, 8 males and 4 females found with AA genotype, 10 males and 8 females with AC genotype. Only 2 males and none of the females with DS were found with CC genotype. Combined genotype frequency of *MTHFR* polymorphisms in males and females of all three groups are shown in Table 3.

The association of *MTHFR* polymorphism with DS or ID is shown in **Table 4**.

DISCUSSION

Down syndrome (DS), the most common genetic cause of intellectual disability, mainly occurs due to non-disjunction of chromosome 21. Although high prevalence and social impact of DS, understanding of etiology and pathogenesis is still incomplete. A known risk factor for DS syndrome is maternal age of more than 35 years. However, DS children are also born to young age mothers (EL-Gharib *et al.*, 2012). Hence, it is a major challenge to identify possible risk factors for DS (Rai *et al.*, 2006).

Some clinical studies have suggested that individuals with DS have unusual one-carbon metabolism (Pogribna *et al.*, 2001; James *et al.*, 1999) and folate is directly related metabolic factor for this metabolism. A clinical study by Hobbs *et al.*, (2000) has more directly concerned folate deficiency as a risk factor for human aneuploidy. Moreover, results of EL-Gharib *et al.*, (2012) depicted that Hcy concentration is significantly higher in mothers of infants with DS having the homozygous normal CC genotype than their

Table 3 Combined genotypic analysis of *MTHFR* polymorphisms in patients and control

MTHFR Polymorphism		Frequency in DS (n=32)			Frequency in ID (n=100)			Frequency in Control (n=100)		
C677T	A1298C	Male	Female	Total	Male	Female	Total	Male	Female	Total
CC	AA	0.250	0.166	0.218	0.179	0.090	0.150	0.181	0.133	0.160
CC	AC	0.300	0.500	0.375	0.432	0.484	0.450	0.509	0.488	0.500
CC	CC	0.100	0.000	0.062	0.104	0.090	0.100	0.109	0.066	0.090
CT	AA	0.150	0.166	0.156	0.104	0.121	0.110	0.109	0.155	0.130
CT	AC	0.200	0.166	0.187	0.134	0.212	0.160	0.054	0.133	0.090
CT	CC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TT	AA	0.000	0.000	0.000	0.029	0.000	0.020	0.018	0.000	0.010
TT	AC	0.000	0.000	0.000	0.014	0.000	0.010	0.018	0.022	0.020
TT	CC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 4 Association of *MTHFR* C677T and A1298C polymorphism in DS & ID

Polymorphism Allele	Control	DS	OR (95% CI)	P Value	ID	OR (95% CI)	P Value
<i>MTHFR</i> C677T	T	28	11	0.53	33	1.21 (0.70-2.09)	0.48
	C	172	53		167		
	Total	200	64		200		
<i>MTHFR</i> A1298C	C	79	22	0.46	82	1.06 (0.71-1.58)	0.75
	A	121	42		118		
	Total	200	64		200		

control counterparts with identical heterozygous CT genotype. Contradictory to those, Scala *et al.*, (2006) didn't analyze any significant difference between blood Hcy levels of DS and control mother, indicating direct impact of certain genotypes as a risk factor for DS.

Several studies reported the association of *MTHFR* C677T and A1298C polymorphism and risk of having Down syndrome children (James *et al.*, 1999; Al-Gazali *et al.*, 2001; Scala *et al.*, 2006). The results of Patterson (2008) showed 2.5 fold increases in maternal risk of Down syndrome due to *MTHFR* C677T polymorphism. Similarly, results of Saxena (2011) revealed heterozygous (CT) condition at 677th position in *MTHFR* is an independent risk factor for intellectual disability. Contradictory to that, several studies did not found any association between *MTHFR* C677T polymorphism and risk of DS (Elsayed *et al.*, 2014; Pandey *et al.*, 2013)

Studies by EL-Gharib *et al.*, (2012) suggested that abnormal folic acid metabolism coupled with *MTHFR* gene polymorphisms may leads to significant decrease in methionine and increase in Hcy levels which in turn seems to be involved in increasing the risk of DS. However, Scala *et al.*, (2006) suggested that the impact of genotype might be influenced by blood folate and Hcy levels. In addition, Shashi *et al.*, (2001) suggested low folate intake with *MTHFR* C677T polymorphism as a maternal risk factor for human aneuploidy. Plethora of studies has been carried out to understand the impact of deranged folate metabolism in the risk of having DS children. However, because of conflicting emergence of outcomes in different populations, the problem still remains unsolved (Rai *et al.*, 2006; Kohli *et al.*, 2008; Cyril *et al.*, 2009; Coppede *et al.*, 2009; Mohanty *et al.*, 2012; Pandey *et al.*, 2013; Elsayed *et al.*, 2014). Variation in the data from different geographical regions may be due to different nutritional habits as well as genetic background of different population.

There are many studies on the maternal *MTHFR* C677T and A1298C polymorphism and risk of having DS children, but there are only few studies on distribution of genotype in DS individuals (Bozovic *et al.*, 2011; Rai *et al.*, 2014). Accordingly, the present study explores the genetic risk profiles for DS and ID and reports the analysis of two polymorphic variants of *MTHFR* gene involved in Hcy/folate metabolism, the allele-allele interactions among polymorphisms and their association with DS & ID. In our study, the frequency of heterozygous individuals for *MTHFR* C677T is grater in DS and ID as compared to control. Similarly, 677T allele frequency is higher in DS and ID children as compared to control children. 1298C allele frequency is higher in ID children and lower in DS children as compared to control children.

The two distinct polymorphisms in *CIS* position in the same gene may functionally interact to reduce the enzyme activity which can results in the stronger impact on the observed phenotype (Scala *et al.*, 2006). The data reported in the present study are consistent with the above hypothesis and revealed an association between DS/ID and double heterozygote.

CONCLUSION

MTHFR C677T polymorphism can be a risk for Down syndrome and intellectual disability, while *MTHFR* A1298C might be a risk factor for intellectual disability but not for the Down syndrome in Gujarati population. Double heterozygotes for *MTHFR* C677T and A1298C is more frequent in Down syndrome and intellectually disabled children as compared to control

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Ethics

The present study is ethically approved by S. G. Patel Ayurvedic Hospital and Maternity Home, New Vallabh Vidyanagar, Anand, Gujarat, India-388121

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