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# Analysis of polymorphisms in genes involved in folate metabolism and its impact on Down syndrome and other intellectual disability

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ABSTRACT

Folate is one of the B vitamins and essential for cellular functioning because it donates one-carbon for *de novo* purine and pyrimidine synthesis, required for DNA synthesis and repair. Deficiency or mutation in one of the cofactor or enzyme involved in folate/homocysteine metabolism may leads to DNA hypomethylation, abnormal DNA synthesis, DNA strand breakage, impaired DNA repair and neurological damages as well as abnormal chromosomal segregation and chromosomal breaks. *MTR*, *MTHFD1* and *CBS* are the key enzymes involved in the folate/homocysteine metabolism. The present study analyzed the risk association of *MTR* A2756G, *MTHFD1* G1958A and *CBS* 844ins68 in the Down syndrome and other intellectually disabled children from Gujarat, using PCR/PCR-RFLP technique. The results of the present study revealed the significant association between *MTR* A2756G polymorphism and risk for intellectual disability. The significant negative associations were observed among *MTR* A2756G or *MTHFD1* G1958A polymorphisms and Down syndrome. The *CBS* ins + /ins + genotype showed 4.09 fold increased risk of intellectual disability.

## 1. Background

The consequences of both deficiencies of micronutrients and the genetic polymorphisms in genes involved in folate/homocysteine metabolism have been established since last few years (Clarke et al., 1998; Selhub et al., 2000; Morris et al., 2003; Obeid and Herrmann, 2006; Stover, 2011; Coppedè, 2015). Several studies suggested that genetic polymorphisms in the enzymes involved in folate/homocysteine metabolism may leads to an increased risk of adult and developmental neurodevelopmental disorder, Down syndrome, intellectual disability (mental retardation), neural tube defect, coronary artery disease and several types of cancer (Scala et al., 2006; Obeid and Herrmann, 2006; Fonseca et al., 1999; Breteler, 2000; Miller, 2000; Wenstrom et al., 2000). Folate is one of the B vitamins and essential for cellular functioning as it donates one-carbon for *de novo* purine and pyrimidine synthesis, required for DNA synthesis and repair (de Jonge et al., 2009). 5-methyl tetrahydrofolate, a major circulating form of folate, donates the methyl group required for the regeneration of methionine form the homocysteine. Methionine is one of the essential amino acids, playing a vital role in one carbon metabolism. One carbon metabolism is the string of biosynthetic pathways, essential for DNA synthesis and repair as well as various methylation reactions including DNA and neurotransmitter methylation (Mattson and Shea, 2003). The supplement of methionine from the normal diet cannot fulfill the demands for the various biochemical reactions involving methyl groups, hence, the additional methionine are provided by the one-carbon folate pool that regenerates methionine. In the *S*-adenosyl methionine (SAM) cycle, the methionine further generates SAM which is a major methyl group donor for most of the methylation reactions. Homocysteine, a sulfur-containing non-essential amino acid, is derived from the de-methylation of SAM. Folate deficiency leads to deleterious effect on cells by accumulation of homocysteine, which is a potential toxic substance. The nervous system is sensitive to the extracellular homocysteine as it leads to neuronal DNA damage and triggers apoptosis (Ho et al., 2002; Kruman et al., 2000; Obeid and Herrmann, 2006). Thus, deficiency or mutation in one of the cofactor or enzyme involved in folate/homocysteine metabolism may leads to DNA hypomethylation, abnormal DNA synthesis, DNA strand breakage, impaired DNA repair and neurological damages. Moreover, DNA hypomethylation is also associated with chromosomal instability as well as abnormal chromosomal segregation (Scala et al.,

*Abbreviations:* MTR, 5-methyl tetrahydrofolate-homocysteine methyl transferase; MTHFD, methylene tetrahydrofolate dehydrogenase; MTHFC, methylene tetrahydrofolate cyclohydrolase; FTHFS, formyl tetrahydrofolate synthase; THF, tetrahydrofolate; CBS, cystathionine-beta-synthase; SAM, *S*-adenosyl methionine; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; DS, Down syndrome; ID, intellectual disability

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Methionine synthase (MTR), a key enzyme involved in one carbon metabolism, catalyze the conversion of homocysteine to methionine. Regeneration of methionine is carried out in two steps in a ping pong reaction in which methyl group of 5-methly tetrahydrofolate initially transferred to cobalamin by converting 5-methly tetrahydrofolate to tetrahydrofolate. Cobalamin now converted in methyl cobalamin transfer its methyl group to homocysteine to regenerate methionine (Matthews et al., 2003). Methionine synthase in human is encoded by the 5-methyl tetrahydrofolate-homocysteine methyl transferase (*MTR*) gene (Li et al., 1996). The most common polymorphism in *MTR* gene is A2756G leads to a change from aspartic acid at codon 919 to glycine (D919G) and reported to be associated with DNA hypomethylation (Leclerc et al., 1996; Paz et al., 2002). Methylenetetrahydrofolate dehydrogenase (MTHFD1) also known as C1-tetrahydrofolate synthase, a key enzyme involved in folate metabolism is encoded by *MTHFD1* gene in human. It is a trifunctional enzyme having 5,10-methenyl tetrahydrofolate cyclohydrolase (MTHFC) and dehydrogenase (MTHFD) activity at N-terminal domain and formyl tetrahydrofolate synthase (FTHFS) activity at C– terminal domain. FTHFS catalyze the conversion of format and tetrahydrofolate (THF) in to 10-formyl tetrahydrofolate (10-FTHF), which is a cofactor for purine biosynthesis. MTHFC catalyze the interconversion of 10-FTHF in to 5,10-methenyl tetrahydrofolate. While MTHFD catalyze the NADPH dependent conversion of 5,10-methenyl tetrahydrofolate in to 5,10-methylene tetrahydrofolate, which is a substrate for thymidylate synthase (Coppedè, 2015). G1958A is the most common polymorphism reported in the human *MTHFD1* gene leads to substitution of arginine to glutamine at 653 amino acid position which is located in FTHFS domain of MTHFD1. The polymorphism forms the thermolabile form of enzyme reducing 25% of the enzymatic activity (Stover, 2011). Cystathionine-beta-synthase, encoded by *CBS* gene, is a main enzyme involved in the transsulfuration of homocysteine to form cystathionine. The 844ins68 is the most common polymorphism reported in *CBS* gene. Cystathionine further generate cysteine which is the precursor for the glutathione, an antioxidant compound (Blom and Smulders, 2011).

Based on this evidence, the present study was undertaken to evaluate the etiology of *MTR* A2756G, *MTHFD1* G1958A and *CBS* 844ins64 into Down syndrome, other intellectually disabled and normal healthy children from Gujarat and to analyze the risk association of *MTR* A2756G, *MTHFD1* G1958A and *CBS* 844ins64 with Down syndrome, other intellectual disability.

## 2. Methodology

### 2.1. Samples

The study was conducted at Post Graduate Department of Genetics, Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Science. Inclusion criteria were male or female individuals between the age group 3 to 18 years with Down syndrome and other intellectually disabled and healthy children. Exclusion criteria were male or female with age below 3 years or above 18 years. Blood samples from total 103 cases (30 Down syndrome and 73 intellectually disabled) and 100 from the normal healthy children (Control) were collected in K3-EDTA vacutainer. The samples were collected from the normal school and special schools for intellectually disabled children from different areas of Gujarat state. The consent forms were signed by the parents of the children. The present study was ethically approved by Surajben Govindbhai Patel Ayurvedic Hospital and Maternity Home, New Vallabh Vidyanagar, Anand, Gujarat, India.

### 2.2. Genotypic analysis

DNA was isolated from 203 blood samples using standard phenol chloroform method (Shams et al., 2011). The PCR reaction mixtures for all the primers were prepared using 1 × PCR master mix form Takara (EmeraldAmp GT PCR Master Mix), 0.4 pM of each primer (Sigma Aldrich), 100 ng of DNA and nuclease free sterile distilled water up to 25 μL total volume.

#### 2.2.1. MTR A2756G

The polymorphism creates a site for restriction enzyme *Hae*III. Primers used for PCR amplification were Forward- 5′ TGT TCC AGA CAG TTA GAT GAA AAT C 3′ and reverse- 5′GAT CCA AAG CCT TTT ACA CTC CTC 3′ (Matsuo et al., 2001). PCR amplification conditions were: initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s, elongation at 72 °C for 55 s and final elongation at 72 °C for 7 min for a total of 35 cycles. The PCR products of 211 bp were digested using *Hae*III restriction endonuclease (NEB: New England Biolabs). The reaction mixture contained 1 × cutsmart buffer, 10 μL of PCR products and 2 μL of restriction enzyme in a total volume of 25 μL. The reaction mixture was incubated for 1 h at 37 °C in water bath. The Digested products were analyzed on 2.5% agarose gel prepared in 1 × TBE.

#### 2.2.2. MTHFD1 G1958A

The G to A transition abolishes a restriction site for *Msp*I. Primers used for PCR amplification were: forward 5′ CAC TCC AGT GTT TGT CCA TG 3′ and reverse 5′ GCA TCT TGA GAG CCC TGA C 3′ (Li et al., 2006). PCR amplification conditions were: initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, elongation at 72 °C for 55 s and final elongation at 72 °C for 7 min for a total of 35 cycles. The PCR products of 330 bp were digested using *Msp*I restriction endonuclease (NEB) The reaction mixture consisted of 1 × cutsmart buffer, 10 μL of PCR products and 2 μL of restriction enzyme in a total volume of 25 μL. The reaction mixture was incubated for 1 h at 37 °C in water bath. The Digested products were analyzed on 2.5% agarose gel prepared in 1 × TBE.

#### 2.2.3. CBS 844ins68

Primers used for PCR amplification were: forward 5′ CTG GCC TTG AGC CCT GAA 3′ and reverse 5′ GGC CGG GCT CTG GAC TC 3′ (Tsai et al., 1996). PCR amplification conditions were: initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 30 s, annealing at 63 °C for 45 s, elongation at 72 °C for 55 s and final elongation at 72 °C for 7 min for a total of 35 cycles. The normal individual will show 184 bp PCR product, while individuals with *CBS* 844ins68 will show 252 bp PCR product. The PCR products were analyzed on 1.5% agarose gel prepared in 1 × TBE.

### 2.3. Statistical analysis

Allele and genotype frequency were calculated for each gene polymorphism. To check the deviation from Hardy-Weinberg equilibrium, Chi square test for the goodness of the fit for case and control were calculated for each polymorphism by means of the Social Science Statistic web site (http://www.socscistatistics.com/tests/goodnessoffit/Default2.aspx). The Odds ratios for polymorphic allele as compared to wild allele, as well as heterozygous and homozygous mutant genotype as compared to homozygous normal genotype were calculated for each gene polymorphism to check the association between polymorphic allele/genotype and Down syndrome/intellectual disability by means of MedCalc statistical software (https://www.medcalc.org/calc/odds_ratio.php).

## 3. Results

### 3.1. Genotypic analysis

The PCR product of *MTR* gene showed fragment of 211 bp. The homozygous normal individual with *MTR* 2756AA genotype doesn't