Role of folate metabolizing genes and homocysteine in mothers of Down syndrome children

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ABSTRACT

Objectives: Folates are essential nutrients required for the synthesis of DNA/RNA in cell division and segregation. Folic acid is absorbed and transported in the liver with the help of enzymes such as methylenetetrahydrofolate reductase (MTHFR), MTR MTRR, reduced folate carrier 1, and cystathionine-β-synthase. Variants in the genes encoding these enzymes may lead to hypomethylation, resulting in nondisjunction which in turn increases the risk for Down syndrome (DS). The present study was conducted to genotype these genes and to see their association with homocysteine levels. Materials and Methods: A total of 213 mothers having DS children and 220 mothers having normal children were enrolled in the study. Genomic DNA was isolated from lymphocytes followed by polymerase chain reaction/Restriction Fragment Length Polymorphism for genotyping. Homocysteine levels were checked by chemosassay utilizing coumarin-based fluorescent probe. Results: Genotypic frequency of MTHFR 1298 A > C polymorphism was significantly different among cases and controls ($\chi^2 = 5.83$, $P = 0.01$), presence of C instead of A allele provided protection against DS in mothers (odds ratios = 0.57, 95% confidence interval = 0.35–0.91, $P = 0.01$). Higher levels of homocysteine were independently associated with the risk of having DS child ($P = 0.0001$). Conclusion: Homocysteine acted as an independent risk factor in the present study and was not associated with folate metabolizing gene variants.

KEYWORDS: Cystathionine β synthase, Down syndrome, Homocysteine, Methionine synthase reductase

INTRODUCTION

Genetic disorders are the leading cause of infant morbidity and mortality affecting nearly 8% of all conceptions, the common among them is Down syndrome (DS). DS results from the presence of an extra chromosome 21, in most of the cases, nondisjunction leads to trisomy 21 in the mother during meiosis I or II with an incidence of 1/600–1000 live births [1].

Folates are essential nutrients required for the distribution of genetic material during cell division and regulate segregation and other processes. After intestinal absorption, folate metabolism requires reduction and methylation into the liver to form 5-methylenetetrahydrofolate and then released into the blood for cellular uptake, where it can be used for the synthesis of DNA and RNA [2]. The most important enzyme, methylenetetrahydrofolate reductase (MTHFR) regulates the conversion of homocysteine (hcy) to methionine, and the enzyme methionine synthase (MTR) catalyzes the remethylation of homocysteine to methionine and tetrahydrofolate [3]. Methionine synthase reductase (MTRR) catalyzes the regeneration of methylcobalamin, a cofactor of MTR. Thus, MTR activity is maintained by MTRR. Folate transporting proteins are also important in the maintenance of DNA methylation. The reduced folate carrier 1 (RFC 1) is the protein located in the intestinal mucosa membrane and plays a role in the folic acid absorption and transportation of 5-MTHFR into the cells. If the folate pathway is impaired, the transsulfuration pathway condenses homocysteine with serine to form cystathionine in the reaction catalyzed by enzyme cystathionine-β-synthase (CBS). Variations in the genes encoding enzymes alter their activity, resulting in hypomethylation which is a possible risk factor for nondisjunction [2]. The present study was conducted to find out the association of SNPs in these genes and homocysteine levels were measured in mothers of DS children (MDS). To

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the best of our knowledge, this is the first study evaluating the role of these polymorphisms from the Punjab region.

**MATERIALS AND METHODS**

The study enrolled 213 MDS after confirming trisomy 21, and 220 mothers having normal children (without any miscarriage) as controls. Informed consent and approval of the institutional ethical committee were obtained before all the investigations (EC-94/HG dated 9.1.2014.). Detailed family history and pedigree analysis were done. Genomic DNA was isolated from peripheral blood lymphocytes by the phenol extraction method [4] with modifications. Polymerase chain reaction was carried out using specific primers [Table 1] followed by restriction digestion.

**Homocysteine measurement**

To measure the levels of homocysteine, fasting serum samples were collected from MDS and controls. Samples were then stored at −80°C till further analysis.

**Reagents**

D,L-Homocysteine was purchased from Sigma Chemicals Co. (St. Louis MO, USA). Sodium borohydride, 10% meta-phosphoric acid, NaOH, EDTA, n-amyl alcohol were obtained from SRL Pvt. Ltd. India.

Deproteinization of serum: To 400 μL serum, 100 μL of freshly prepared 1.43 M sodium borohydride solution (1.5 μM EDTA, 66 mM NaOH and 10 μL n-amyl alcohol) was added. These were mixed and incubated in the water bath at 40°C for 30 min and 250 μL of ice-cold 10% of meta-phosphoric acid was added to precipitate the proteins. After keeping it on ice for 30 min, centrifuged at 21000 g for 15 min to separate the upper phase and filtered it through 0.2 μ filter [5].

**Fluorescence measurement of serum samples**

The coumarin-based fluorescent probe 1 was synthesized using the described procedure in the literature [6]. Serum (150 μL) from MDS was mixed with an equal amount of cold acetonitrile. The standard calibration curve was prepared by measuring the fluorescence of standard solutions containing 100, 150, 200, and 250 μM of homocysteine using fluorescence spectroscopy. The fluorescent intensities of the serum samples were plotted in the standard calibration graph and calculated the homocysteine level of the samples.

**Statistical analysis**

Genotypic and allelic frequencies were calculated under the assumption of Hardy’s Weinberg Equilibrium. Chi-square test was employed to evaluate the relationship between cases and controls, and to estimate the relative risk for DS, odds ratios (OR) at 95% confidence interval (CI) was calculated. To check the difference in the levels of homocysteine between cases and controls, Student’s t-test and ANOVA were used to analyze gene-environment interaction. All the analyses were performed using SPSS software (Statistical Package for the Social Sciences Inc. 20, Chicago, IL, USA).

**RESULTS**

**Genotype frequencies and Down syndrome risk**

In the present study, 213 cases and 220 controls were enrolled and analyzed. Individual analysis of genotype frequencies among MTHFR 677 C > T, MTR 2756 A > G, MTRR 66 A > G, RFC I 80 G > A, and CBS844ins68 showed no difference among cases and controls. Genotype frequency of MTHFR 1298 A > C showed a significant difference between cases and controls (χ²=5.83, P = 0.01). The allele frequency, OR = 0.53, 95% CI = 0.32–0.87, P = 0.01 indicated that substitution of alanine instead of glutamine provided protection against the occurrence of the disorder [Table 2].

**Diploptote analysis**

In diploptote analysis, gene-gene interaction showed that presence of MTHFR 677 CT/CBS-/+ and MTR2756 AA/RFC I 80 GA combinations in an individual significantly increases the risk of DS child (OR = 3.89, 95% CI = 1.06–14.24, P = 0.04 and OR = 1.89, 95% CI = 1.02–3.48, P = 0.04). On the other hand, the presence of MTHFR 1298 AC/MTR 2756 AA and MTHFR 1298 AC/CBS-/+ combination in an individual confers protection against the birth of DS child [Table 3].

**Haplotype analysis**

Haplotype analysis was performed between MTHFR 677 C > T/1298 A > C and the results indicated that both SNPs are not in linkage disequilibrium [Tables 4 and 5].

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**Table 1: Primer sequences for markers and their respective enzymes**

| Genes       | Primer sequences                  | Annealing temp (°C) | Enzyme | Fragment size (bp) |
|-------------|-----------------------------------|---------------------|--------|--------------------|
| MTHFR C677T | F5’TGAAGGAGAAAGTGTCGCGGGA-3’      | 62                  | Hinf I | C - 198            |
|             | R5’- AGGACGGTGCGTGAGATG-3’        |                     |        |                    |
| MTHFR A1298C| F5’CTTTGGGAGACTGGAAGGACTAAT3’    | 60                  | Mbo II | A - 56,31,30,28,18 |
|             | R5’CAGCTTTGAGACATCCCCTTTGG-3’    |                     |        |                    |
| MTR A2756G  | F5’CAGTGGTTCGCAGCTGTTAAT3’       | 60                  | Hae III| A - 252            |
|             | R5’GGGAACTTAAAGACACTGAGGCTTCTG-3’|                     |        |                    |
| MTRR A66G   | F5’CAGCGAGGCAAGGCAAAGGCTACGCAGAAGACAT3’ | 58      | Nde I     | A - 196          |
|             | R5’CTGGTGATCTCTTACATCAGACACACAC3’|                     |        |                    |
| RFC I G80A  | F5’AGTGTCACCTCTTCGCC-3’          | 58                  | Hha I   | G - 125,68,37     |
|             | R5’CTGGCGCGGATGACATCTG-3’        |                     |        |                    |
| CBS844ins68 | F5’CTGGATCGCTGCAGTGACACAC3’      | 60                  | -       | DD - 174           |
|             | R5’CTGGACTGCACCTACCGTCTC-3’      |                     |        |                    |

MTHFR: Methylene tetrahydrofolate reductase, MTR: Methionine and enzyme methionine synthase, MTRR: Methionine synthase reductase, RFC 1: Reduced folate carrier 1, CBS: Cystathionine-β-synthase
Homocysteine analysis

Statistically significant difference was observed in levels of homocysteine in cases and controls which suggested that homocysteine acted as an independent risk factor for the birth of DS child [Table 6]. To assess the influence of genetic variations in folate metabolizing genes on homocysteine levels, ANOVA was carried out. There was no significant association between homocysteine levels and SNPs [Table 7].

Discussion

The variations in genes encoding enzymes involved in folate metabolism play an important role in the etiology of birth defects. Gene expressions are regulated by DNA or histone methylation, thus hypomethylation of DNA/histone affects DNA repair, replication, expression, segregation, chromatin conformation, leading to disease conditions. MTHFR is a key enzyme that plays a critical role in DNA/RNA synthesis and methylation pathways. In its homozygous condition, it is responsible for vascular events, NTDs, bad obstetric history, and possibly Down syndrome. Among North Americans, younger mothers with 677 TT were at higher risk of having DS baby [7].

Many studies have suggested that TT genotype could be considered as a risk factor for DS [8,9]. However, Tayeb [10], Kaur and Kaur [11], Coppedè et al. [12], and Cretu et al. [13] did not observe any association between cases and controls, but the presence of the MTHFR 677T allele slightly increased the risk for DS child in mothers. In the present study, MTHFR 1298 A > C was significantly associated with DS, conferring protection against the disorder, similar to the reports in the literature [14,15]. On the contrary, according to Scala et al. [16] presence of 1298A  > C allele escalates 2.29 fold risk for the birth of DS. However, Izci et al. [8], da Silva et al. [17], Santos-Rebouças et al. [18], Sukla et al. [19] reported that MTHFR 1298 A  > C was not associated with the risk of DS child.

The presence of linkage disequilibrium provided evidence that both SNPs (MTHFR 677 C > T and 1298 A > C) have strong interaction with protein stability and activity [16,20]. Scala et al. [16] reported that in their population T-C haplotype significantly increased the risk of DS child among mothers. Biselli et al. [21] reported the absence of T-C haplotype in

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**Table 2: Distribution of genotype and allele frequency of methylenetetrahydrofolate reductase, methionine and enzyme methionine synthase, reduced folate carrier 1 and cystathionine-β-synthase 844ins68 genes**

| Genotype | Cases | Controls | χ² | Alleles | Cases | Controls | OR  | P (95% CI) |
|----------|-------|----------|----|---------|-------|----------|-----|-----------|
| MTHFR C677T | | | | | | | | |
| CC | 163 (76.53) | 178 (80.91) | 3.68 | C | 372 (87.32) | 390 (88.64) | 1.32 | 0.09 (0.95-1.85) |
| CT | 46 (21.59) | 34 (15.46) | 0.159 | | | | | |
| TT | 4 (1.88) | 8 (3.64) | | | | | | |
| MTHFR A1298C | | | | | | | | |
| AA | 183 (85.91) | 168 (76.36) | 5.83 | A | 396 (92.96) | 388 (88.18) | 0.57 | 0.01 (0.35-0.91) |
| AC | 30 (14.09) | 52 (23.64) | 0.01 | C | 30 (7.04) | 52 (11.82) | | |
| CC | 0 | 0 | | | | | | |
| MTR A2756G | | | | | | | | |
| AA | 101 (47.42) | 113 (51.37) | 0.69 | A | 304 (71.36) | 324 (73.64) | 1.12 | 0.45 (0.82-1.51) |
| AG | 102 (47.89) | 98 (44.55) | 0.71 | G | 122 (28.64) | 116 (26.36) | | |
| GG | 10 (4.69) | 9 (4.09) | | | | | | |
| MTRR A66G | | | | | | | | |
| AA | 8 (3.76) | 8 (3.64) | 0.07 | A | 146 (34.27) | 153 (34.77) | 1.02 | 0.88 (0.77-1.35) |
| AG | 130 (61.03) | 137 (62.27) | 0.97 | G | 280 (65.73) | 287 (65.23) | | |
| GG | 75 (35.21) | 75 (34.09) | | | | | | |
| RFC I G80A | | | | | | | | |
| GG | 55 (25.82) | 66 (30) | 1.09 | G | 238 (55.87) | 254 (57.73) | 0.93 | 0.58 (0.71-1.21) |
| GA | 128 (60.09) | 122 (55.46) | 0.58 | A | 188 (44.13) | 186 (42.27) | | |
| AA | 10 (4.69) | 14 (6.15) | | | | | | |
| CBS 844ins68 | | | | | | | | |
| DD | 171 (80.28) | 177 (80.46) | 0.006 | D | 384 (90.14) | 394 (90.23) | 1.00 | 0.99 (0.64-1.57) |
| DI | 42 (19.72) | 43 (19.55) | 0.94 | | | | | |
| II | 0 | 0 | | | | | | |

MTHFR: Methylenetetrahydrofolate reductase, MTR: Methionine and enzyme methionine synthase, MTRR: Methionine synthase reductase, RFC 1: Reduced folate carrier 1, CBS: Cystathionine-β-synthase, OR: Odds ratio, CI: Confidence interval

**Table 3: Gene-gene interactions between different genotypes studied**

| Combinations | Cases | Controls | OR  | 95% CI | P |
|--------------|-------|----------|-----|--------|---|
| MTHFR C677T/CBS844ins68 | CTID | 11 | 3 | 3.89 | 1.06-14.24 | 0.04 |
| MTR A2756G/RFC I G80A | AAGA | 69 | 57 | 1.89 | 1.02-3.48 | 0.04 |
| MTHFR A1298C/MTR A2756G | ACAA | 12 | 25 | 0.48 | 0.23-1.00 | 0.05 |
| MTHFR A1298C/CBS844ins68 | ACID | 2 | 13 | 0.15 | 0.03-0.67 | 0.01 |

MTHFR: Methylenetetrahydrofolate reductase, MTR: Methionine and enzyme methionine synthase, OR: Odds ratio, CI: Confidence interval
the Brazilian population suggesting negative selection of this haplotype. In this study, the C-C haplotype conferred risk for the birth of DS child [Figure 1].

Another variant in the folate metabolic pathway is MTR 2756 A > G and it has been suggested that the presence of

MTR 2756 AG + GG genotype increased the risk of DS child by 3.5 folds [22]. In the present study, no association was observed between DS and this genotype and similar results have been reported [7,12,17,19]. However, Coppède et al. [12] noticed that MTR 2756 G allele frequency among European and Brazilian ranged from 18% to 21% whereas Liao et al. [23] reported allele frequency of <10% among Asians. Moustafa et al. [24] observed that mothers with MTR 2756 G genotype could be considered at risk for DS in Egyptians.

There are limited studies on the variant MTRR 66 A > G but has been found to be associated with DS [1,25]. We did not observe any association between the SNP and the risk of DS and these results are in conformity with other studies [7,21].

Scala et al. [16] did not find any association between disease and RFC I 80 G > A genotype but suggested that the presence of RFC I G > A allele increased 2.05 folds risk of DS child. Wang et al. [1] and Coppède et al. [26] observed that the presence of RFC I G > A genotype in women increased the risk of delivering DS child. We did not observe any association between the risk of DS child and the presence of RFC I 80 G > A.

CBS enzyme in the transulfuration pathway converts homocysteine to cystathionine, thus regulating homocysteine levels [27] which otherwise leads to homocysteinemia. More than 17 mutations have been identified in the CBS gene and 68 bp insertion in the coding region of exon 8 is most common. Some studies have reported that CBS844ins68 polymorphism was not associated with the risk of birth of DS child [16,20,28]. Similarly, the nonsignificant association of CBS844ins68 polymorphism with the risk of DS child was observed in the present study when analyzed independently.

When studied in combination, MTHFR 677 C and MTHFR 1298 C, MTRR 66 AA, RFC I 80 AG, and CBS844ins68, these may manifest their effect and result in increased risk of DS offspring. MTR 2756 A > G in amalgamation with other variants (MTHFR 677, 1298, MTRR 66, RFC I 80, and CBS844ins68), they may manifest their effect and result in increased risk of DS offspring. MTR 2756 A > G in amalgamation with other variants (MTHFR 677, 1298, MTRR 66 AA > G and CBS844ins68) increased the maternal risk up to 1.2 and 1.7 folds, respectively [17,21,29]. Similarly, Brandalize et al. [30] also suggested that the presence of these genotypes confers 4.8–6.9 folds risk for DS. The combination of MTR2756 AA + MTHFR 677 TT genotype elevated the risk by 3.0 folds for DS child [15]. A diplotype analysis showed that MTHFR 677 CC when combined with MTR 2756 (AG or GG), elevated the risk by 6.7 folds and when MTR 2756 AA unites with MTHFR 677 (CT or TT), it confers 4.2 folds risk [23]. In 2006, Coppède et al. [25] observed a slight increase in maternal risk for DS child when mothers carried RFC I 80GG/MTHFR 677 TT and reduced risk when carried RFC I 80 (AA or AG)/MTHFR 1298AA. In our study, MTHFR CT genotype when combined with CBS844ins68+/+ genotype, the risk for the syndrome increased by 3.89 folds and when MTR 2756 AA amalgamated with RFC I 80 AG, the risk escalated to 1.89 folds. On the other side, MTHFR 1298 AC in alliance with MTR 2756 AA and CBS844ins68 provided protection against DS.

Homocysteine, Vitamin B12, and folate are metabolic and nutritional factors directly related to the folate

### Table 4: Haplotype analysis between methylenetetrahydrofolate reductase C677T/A1298C

| Haplotype | Frequency | Case ratio | Control ratio | χ² | P  |
|-----------|-----------|------------|---------------|----|----|
| CA        | 0.794     | 345/81     | 342/96        | 1.041 | 0.3077 |
| TA        | 0.112     | 52/375     | 45/393        | 0.702 | 0.4021 |
| CC        | 0.085     | 28/399     | 46/392        | 4.583 | 0.0323 |

Table 5: Linkage disequilibrium between two markers of single gene

| Gene          | SNPs          | Cases  | Controls | D'   | LOD | R² | D'  | LOD | R² |
|---------------|---------------|--------|----------|------|-----|----|-----|-----|----|
| MTHFR C677T  | 0.555         | 0.12   | 0.002    | 0.115 | 0.01 | 0.0 |      |     |    |
| MTHFR A1298C |               |        |          |      |     |    |      |     |    |
| MTR A2756G   |               |        |          |      |     |    |      |     |    |
| MTRR A66G    |               |        |          |      |     |    |      |     |    |
| RFC I 80     |               |        |          |      |     |    |      |     |    |
| CBS844ins68  |               |        |          |      |     |    |      |     |    |

### Table 6: Comparison of means (student t-test) of homocysteine levels between cases and controls

| Cases | Controls | Difference | 95% CI | T    | Df  | P    |
|-------|----------|------------|--------|------|-----|------|
| 104   | 109      | 9.05       | 8.93-9.17 | 145.17 | 211 | 0.0001 |

### Table 7: Comparison of serum homocysteine levels with different genotypes

| Genes          | 1/1¹ | 1/2² | 2/2² | F   | P     |
|----------------|------|------|------|-----|-------|
| MTHFR C677T    | 15.7±6.3 | 15.2±4.5 | 13.25±3.7 | 0.38 | 0.69  |
| MTHFR A1298C   | 15.14±5.7 | 17.44±5.8 | -    | 2.13 | 0.15  |
| MTR A2756G     | 14.39±5.9 | 16.17±5.3 | 18.03±8.07 | 1.7 | 0.19  |
| MTRR A66G      | 16.33±6.5 | 15.20±5.6 | 15.68±6.15 | 0.15 | 0.86  |
| RFC I G80A     | 17.02±5.4 | 14.45±6.2 | 16.19±4.9 | 2.1 | 0.13  |
| CBS844ins68    | 15.56±6.1 | 15.36±5.2 | -    | 0.01 | 0.92  |

*Notes: ¹ Haplotype analysis between methylenetetrahydrofolate reductase, MTR: Methionine and enzyme methionine synthase, MTRR: Methionine synthase reductase, RFC I: Reduced folate carrier 1, CBS: Cystathionine-β-synthase, a: Homozygous wild; b: Heterozygous mutant; c: Homozygous mutant

MTHFR: Methylenetetrahydrofolate reductase

Figure 1: LD plot for methylenetetrahydrofolate reductase gene polymorphisms (a) LD plot for case–control combined (b) LD plot for cases (c) LD plot for controls

*Image 46x82 to 288x249*
pathway and alterations in their concentrations may lead to disturbance in folate metabolism. Various studies have shown that genetic polymorphisms influence the plasma homocysteine concentration either directly or by affecting the folate concentration. Maternal diet before or during conception provides necessary folates to complete the process of meiosis thus, inadequate intake of folic acid or impaired metabolism resulted in abnormal chromosome 21 recombination and malsegregation. A recent study compared the use of folic acid supplements among 702 mothers having DS child due to nondisjunction and 983 mothers having normal children, revealed that lack of folic acid or impaired folate metabolism is associated with risk of birth of DS child [31].

Maintenance of methylation pattern through folate cycle required the action of gene products and micronutrients such as vitamin B12, B6, and methionine obtained from the diet. These elements participate in the conversion of homocysteine to methionine, later the precursor of S-adenosylmethionine (SAM), main methyl donor for DNA, RNA, and protein. SAM, changes into S-adenosylhomocysteine by donating methyl group, whose end product is homocysteine. Thus, the presence of genetic variants alters the enzymatic activity required to convert homocysteine to methionine, predispose to genetic instability, abnormal recombination, and malsegregation. The effect of these variants depends upon the folate status of an individual and high folic acid and other dietary nutrients neutralize the effect of homocysteine and SNPs. Furthermore, variants that reduce the availability of folate/hcy in mothers before or during pregnancy have been observed to be associated with abnormalities such as NTDs, cleft palate, Down syndrome. In our previous study, various risk factors associated with Down syndrome have been studied [32] and maternal age in DS cases was found to be 27.34 ± 5.2 years, while in controls, it was 27.75 ± 4.9 years indicating that the majority of the DS children were born to younger mothers.

Significantly higher levels of homocysteine were observed in the present study which is consistent with the recent report by Kedar and Chadel [33]. However, the association of genotypes and homocysteine levels among cases was not significant in the present study; in contrast to the report suggesting significant levels in the presence of MTHFR 677TT [29]. The elevated levels of hcy are due to insufficient folic acid or reduced absorption of folates in MDS that ultimately affects the Vitamin B12 status. In other words, complex gene-environment interaction that involves maternal diet, lifestyle and genotype could result in risk for DS child [17,19,21,22]. To overcome this food fortification should be made mandatory, which is being recommended by the Indian government to improve nutrition in children, pregnant women, and lactating mothers through various programs.

**Limitations of our study**

(a) It is restricted only to Punjab; (b) analysis of gene-environment interaction was not done which would otherwise increase the power of the study.

**CONCLUSION**

This is the first report from Punjab analyzing six SNPs and their association with homocysteine levels. The alliance of SNPs was significantly associated either with the risk or protection of having DS child and homocysteine came out to be independent risk factor among women. However, individual SNPs and their association with hcy did not provide risk. A larger sample size is required to see gene-gene and gene-nutrient interaction and will help in establishing the link between disease and genotype and awareness about intake of folic acid either orally or in fortified food products, thus providing opportunities to create new strategies to improve public health and prevent the birth of DS.

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**Conflicts of interest**

There are no conflicts of interest.

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