MTHFR promoter hypermethylation may lead to congenital heart defects in Down syndrome

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Summary

Altered global methylation levels revealed LINE-1 methylation in young mothers of Down syndrome (DS) compared to controls suggesting the possibility of impaired DNA methylation causing abnormal segregation of chromosome 21. Methylene Tetrahydrofolate Reductase (MTHFR) is one of the major enzymes of the folate metabolism pathway. MTHFR gene polymorphism has been associated with maternal risk for DS. Studies have revealed that increased MTHFR promoter methylation results in the reduction of MTHFR protein activity further leading to increased risk of various diseases. The aim of this study is to compare the levels of MTHFR promoter methylation in all three study groups. A total of 120 subjects were recruited for the study and was divided into the following three groups: Group I (mothers of DS without Congenital Heart Defects (CHD), n = 40); Group II (mothers of DS with CHD, n = 40); and Group III (age matched control mothers, n = 40). Genomic DNA was isolated from 2 ml peripheral blood and bisulfite treatment was done to convert all unmethylated cytosines into uracil followed by PCR amplification for MTHFR promoter region and Sanger’s sequencing. Results showed that there was a two fold increase in methylated promoter region of MTHFR gene in group II compared to other groups. None of the methylation pattern was observed in the control group. MTHFR promoter methylation affects folate metabolism which is known to play a role in chromosomal breakage, abnormal chromosomal segregation and genomic instability and therefore a developmental defect in the form of congenital cardiac anomaly.

Keywords: Down syndrome, congenital heart defects, MTHFR promoter, sequencing

1. Introduction

Aberrant DNA methylation has been associated with several diseases like cancers (1,2), diabetes (3) or neurological diseases including Down syndrome (DS) (4). DNA methylation leads to the addition of a methyl group at 5’ carbon of cytosine, which can bring changes in DNA structure, altering the set patterns of gene expression.

Congenital heart defects (CHD) account for a major portion of life-threatening birth defects. Atrioventricular Septal Defect (AVSD) and Ventricular septal defects (VSD) are common cardiac malformations in DS cases (5,6). The relationship between DS and maternal genetic polymorphism in folate/homocysteine metabolism is well established. Folate is essential for various cellular processes viz., synthesis of DNA, RNA, methylation and embryonic developmental processes including the cardiovascular system (7,8). Folate deficiency may lead to stunted growth, anemia, weight loss, digestive disorders and behavioral issues. Reports of in vivo studies showed a decreased level of folate causes hypomethylation leading to DNA strand breakage and abnormal segregation of chromosomes (9,10). Changes in folate metabolism lead to an increase in DNA hypomethylation due to an altered DNA methylation pattern thereby further increasing the risk of chromosome nondisjunction (11).

Altered global methylation levels reveal LINE-1 methylation in young mothers of DS thus suggesting...
the possibility of impaired DNA methylation in mothers of DS children causing abnormal segregation of chromosome 21 (12). Also maternal folic acid supplementation has been associated with a decreased risk of congenital heart defects in DS babies (13).

The methylenetetrahydrofolate reductase (MTHFR) gene is reported to be associated with the risk of CHD in DS. It was also reported that promoter methylation regulates MTHFR gene and increased MTHFR promoter methylation was seen in DNA isolated from cancer patients, patients with cardiovascular or renal disorders, and placental DNA from women with pre-eclampsia. It was also observed that as the level of promoter methylation in MTHFR gene increases, the MTHFR protein activity is reduced, thereby increasing the risk of various diseases (14,15). In this study, we investigated MTHFR promoter methylation levels in mothers of DS, mothers of DS with CHD and matched healthy control mothers.

2. Materials and Methods

2.1. Study Subjects

A total of 120 subjects recruited in the present study included three groups: Group I comprised of mothers of DS without CHD (n = 40); Group II had mothers of DS with CHD (n = 40); and Group III included age-matched control mothers (n = 40). DS subjects were recruited from outpatient clinics of 2 tertiary care institutes in India, after obtaining informed consent. The DS were enrolled in the study after confirmation of karyotype and echocardiography to confirm the presence or absence of AVSD. Quantitative Fluorescent – Polymerase Chain Reaction (PCR) was also done on DNA samples for confirmation of DS using standard protocols. Ethical clearance was from the ethics committee of the Institute where the work was performed. Peripheral blood samples were available from all hundred and twenty women and the age of all the women ranged from 18 to 44 years.

2.2. Bisulfite treatment and sequencing

Genomic DNA was isolated from 2 mL peripheral blood using standard Phenol Chloroform method and quantified using Nanodrop spectrophotometer (NanoDrop Thermo Scientific). The EpiTect Bisulfite Kit (Qiagen, Milan, Italy) was used to convert all unmethylated cytosines into uracil. Bisulfite treatment deaminated cytosine to uracil and 5' Methyl cytosine residues to cytosine, further distinguishing methylated CpGs from unmethylated CpGs by the presence of a cytosine "C" versus thymine "T" residue after sequencing.

PCR amplification was done for the MTHFR promoter region. The CpG islands present in the 5'-untranslated promoter region of MTHFR; from + 30 to 184 from the TSS (transcription start site) was studied. This region contains seven CpG islands and the methylation levels of these CpG islands were found to be associated with gene expression levels in human lung cancer cells (16).

Table 1 shows the details of PCR reaction and primers including: the sequence of primers used, annealing temperature (Ta), amplicon length, the location of promoter region studied and the number of CpG sites present.

3. Results and Discussion

The age range of the mothers with DS babies was 18-45 years, with median age of 28.5 years. In the DS babies with cardiac defects, the most common cardiac defect was AVSD, comprising 62.5% cases. Other cardiac defects included VSD, atrial septal defects (ASD) and Tetralogy of Fallot (TOF). Mothers of DS with cardiac defects were in Group II for the study.

The sequencing results of bisulfite converted genomic DNA for all three groups showed that there was a two fold increase in methylated promoter region of MTHFR gene in group mothers of DS having CHD compared to mothers of DS without CHD. However, no methylation was observed in age matched control mothers group (Figure 1). Figure 2 shows the pictorial representation of CpG island frequencies in all three study groups. CpG island methylation frequencies

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primer Sequences</th>
<th>Ta</th>
<th>Amplicon length</th>
<th>CpG sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: 5'TTTAAATTCGGTTGAGGTTAG-3' R: 5'AAAAAAACCACCTTACCAAAATTIC-3'</td>
<td>55°C</td>
<td>155bp</td>
<td>7</td>
</tr>
</tbody>
</table>
Several congenital complications are reported in individuals with DS. Most of them are affected by maternal carbon metabolism and consequently lead to epigenetic changes and hence impaired chromosome segregation (24). The promoter methylation of a gene is an epigenetic event which leads to reduced expression of genes.

A similar study was conducted by Coppede et al. in 2015 (7) in Italian mothers of DS along with age-matched controls who conceived before 35 years of age. Authors have investigated MTHFR promoter methylation levels and also searched for correlation between MTHFR promoter methylation and micronucleus frequency and found an increase in the methylation level in mothers of DS when compared to controls (33.3 ± 8.1% vs. 28.3 ± 5.8%; p = 0.001). The frequency of micronucleated lymphocytes was also found to be higher in mothers of DS group than control mothers (16.1 ± 6.9% vs. 10.5 ± 4.3%; p = 0.0004) and it correlated with MTHFR promoter methylation levels (r = 0.33; p = 0.006). The study concluded that MTHFR methylation is likely to contribute to the increased genomic instability observed in DNA isolated from mothers of DS, and could play a role in the risk of birth of a child with DS as well as in the onset of age related diseases in those women. We investigated a CpG island in the MTHFR promoter region in three groups (mothers of DS with CHD, mothers of DS without CHD and controls) and the methylation status was found to be elevated in mothers of DS with CHD highlighting the essential role of MTHFR promoter methylation in occurrence of both CHD and DS in women of reproductive age. These studies indicate that MTHFR promoter methylation can be used as a biomarker for screening mothers of DS for the presence of CHD in these populations. However, this is the only study reported in the literature which shows the association of MTHFR promoter methylation with the occurrence of CHD in mothers of DS. Furthermore, these studies can also be conducted in other ethnic groups with a larger sample size to confirm these findings.

In conclusion, MTHFR promoter methylation affects folate metabolism, which is known to play a role in chromosomal breakage, abnormal chromosomal segregation and genomic instability and therefore a developmental defect in the form of congenital cardiac anomaly. The present study thus clearly highlights the association of MTHFR promoter hypermethylation in mothers of DS having DS babies with cardiac defects. We reported increased promoter methylation in mothers of DS with CHD when compared to mothers of DS without CHD and healthy control mothers.

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