

Cytogenetic and Molecular Study of Children with Down's Syndrome in India: Influence of Maternal Hypothyroidism

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ABSTRACT

Down's syndrome (DS) has lately been gaining attention among researchers due to its genetic significance leading to dementia and Alzheimer's disease. The aim of the present study was to investigate the possible interaction between thyroid hormone and total homocysteine levels by analyzing the patterns of chromosomal abnormalities and genetic polymorphisms of the methionine synthase reductase (*MTRR*) gene among DS children, mothers of DS children (MDS) and their respective controls. Free trisomy was present in 57%, translocation in 10% and mosaicism in 33% of DS children from mothers with abnormal thyroid hormonal and homocysteine levels. A combination of the heterozygous and homozygous *MTRR* variant (AG+GG) showed a highly significant increase in the prevalence of the A66G mutation among MDS. The prevalence of the GG homozygous variant was significantly higher in MDS compared to controls. Although the confinement of this study to a specific geographic region of India and the relatively small sample size, our observations support the integration of genetic analysis and biochemical tests for a potential prognosis of DS that could enable the implementation of prophylactic treatments.

Keywords: Down's Syndrome, Cytogenetics, Homocysteine, Hypothyroidism, Micronuclei, *MTRR* genotype

INTRODUCTION

Down's syndrome (DS; trisomy 21) is the second commonest serious birth defect, after neural tube defects, and is the most common survivable chromosomal abnormality, with an estimated >217,000 live births per year. In the USA, DS occurs in one of every 800 infants with as many as 6,000 children born with DS each year. Approximately 85–90% of individuals born with DS can be expected to survive to one year of age and over 50% will be expected to survive beyond 50 years [1]. According to the National Down's Syndrome Society, there are more than 350,000 people living with DS in the USA.

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DS is registered in nearly all surveillance programs for birth defects as a paradigm for aneuploid mutations. The conspicuous DS phenotype and the high proportion of new mutants make surveillance of trisomy 21 particularly suitable for assessing mutagenic hazards and identifying genetic factors influencing non-disjunction. Approximately 95% of the cases are due to non-disjunction, resulting in an extra copy of a chromosome 21 (trisomy 21) as described by Lejeune et al. [2,3]. The remainders are due to translocations involving chromosome 21 and somatic mosaicism [4]. Most trisomy 21 cases are due to an error in maternal meiosis, whereby about 70% originate during maternal meiosis I, about 20% during maternal meiosis II and 8-10% due to defective paternal meiosis [4,5]. Even though significant progress has been made in recent years, the causes of the increased non-disjunction rate resulting in trisomy 21 are far from understood. Maternal age, germline mosaicism, and altered recombination remain the only well-established risk factors for non-disjunction of chromosome 21 [4].

An important factor relating DS with one-carbon metabolism is the cystathionine beta synthase (*CBS*) gene located on chromosome 21. This location would explain the functional homocysteine (Hcy) deficiency observed in infants with trisomy 21, due to the over-expression of the *CBS* gene [6]. Therefore, the functional Hcy deficiency in embryos with three chromosomes 21 could diminish the levels of S-adenosylmethionine (SAM), provoking unrestricted 5-methyltetrahydrofolate formation and conserving Hcy for methionine production, thereby affecting DNA synthesis. This destabilization of Hcy metabolism might alter cell division and growth and, therefore, embryo survival, which may be related to the well-known high lethality of trisomy 21 at conception [7].

Among the genetic factors that could be related to centromeric hypomethylation are the polymorphic variants of enzymes in the methionine homocysteine metabolic pathway, such as methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*) [8]. These enzymes are involved in the regulation of S-adenosylmethionine (SAM), the main intracellular donor of methyl groups to DNA and other substrates. These enzymatic variants could alter normal metabolic pathways, resulting in increased levels of homocysteine, decreased SAM synthesis, and abnormal DNA methylation.

The focal aim of the present study was directed at the population of Coimbatore, South India, to detect the different patterns of chromosomal abnormalities of suspected DS patients by cytogenetic analysis using the trypsin G-banding method; to analyze the frequency of micronucleus (MN) and binucleated cells in mothers of DS children (MDS), DS cases and controls, and to investigate the possible influence of thyroid hormone [Free Thyroxine (FT4), thyroid stimulating hormone (TSH)] and plasma total

homocysteine (tHcy) levels in MDS, DS and controls. The study also investigated single nucleotide polymorphisms (SNP) in the methionine synthase reductase (*MTRR*) gene.

MATERIALS AND METHODS

Subject recruitment and sample collection

Experimental samples were selected from the hospitals in and around Coimbatore City, Tamilnadu, India, from September 2015 to August 2016. Mentally normal and physically healthy individuals residing in the same area were selected as controls, with their ages ranging within ± 2 years of their DS counterparts. 100 samples taken for this study included 30 children with DS who had been reported to the clinic during the study period, 30 mothers of DS children (MDS), 20 normal children and 20 normal mothers. The minimum onset age of MDS experimental samples was considered at 30 years. A detailed questionnaire was directed to both subject groups to obtain relevant details such as smoking and alcohol consumption. Other health effects such as thyroid dysfunction, respiratory issues, skin disorders, reproductive effects, child age, birth order, parental age, marital status, family history and clinical features were also recorded.

For the present study, 5 mL of blood was collected intravenously from the individuals and brought to the culture laboratory in airtight ice-packed containers to perform the chromosomal (peripheral blood leukocyte culturing and micronucleus analysis), biochemical (Enzyme Immunoassay for Quantitative Determination of Serum Free T4 (FT4) and Immunoassay for the Quantitative Determination of Thyroid Stimulating Hormone (TSH)), and genotypic (PCR-RFLP analysis of *MTRR* gene polymorphism) analyses. Serum was separated from the blood samples and assayed for various hematological parameters (Enzyme Immunoassay for the Quantitative Determination of (tHcy) in Human Serum).

Human Peripheral Blood Leukocyte culture for chromosomal analysis

5 mL of peripheral blood was collected from exposed and control subjects using a heparinized syringe, for peripheral blood leukocyte culture following the method of Moorhead et al. [9]. The chromosomal preparations obtained were processed and stained with Giemsa to obtain G-bands.

A modified method of Seabright [10] was employed to obtain chromosomal bands. The slides bearing chromosome spreads were treated with 0.25% trypsin for 3 to 10 s to enable the digestion of the cell membrane, the cytoplasm and to enhance good exposure of the metaphase chromosomes. After trypsin treatment, the slides were stained in 4% buffered Giemsa solution for 3 min, washed with distilled water and then air-dried.

Micronucleus study

Scoring of micronuclei was limited to binucleated lymphocytes with preserved cytoplasm [11], and stained for 5 to 8 min using 2% Giemsa solution made in 0.025 M phosphate buffer pH 6.8 according to the criteria proposed by Countryman and Heddle [12]. The results are expressed as the average percentage of micronucleated cells per binucleated cells. To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of micronuclei in binucleated lymphocytes (MNL), a total of 1,000 binucleated cells (500 per replicate) with well-preserved cytoplasm were scored per subject on coded slides.

Biochemical assays

Enzyme immunoassay for quantitative determination of serum free T4 (FT4) and immunoassay for the quantitative determination of thyroid stimulating hormone (TSH) were performed according to the methods described by Sati et al. [13] and Soos [14], respectively. The ELISA-based test for analyzing the FT4 was carried out using patient serum samples, standards, and thyroxine-enzyme conjugate reagent was added to wells coated with monoclonal T4 antibody. The colored enzyme-substrate complex was read spectrophotometrically at 492 nm. The Genix TSH EIA test was read at 450 nm using a goat anti-TSH antibody in the antibody-enzyme (horse radish peroxidase) conjugate solution. Total homocysteine biochemical assay was carried out using a Cell Biolabs, Inc kit, using the patient's serum in reaction with recombinant methionine α , γ -lyase (HCY enzyme) to produce H₂S which was, in turn, measured optically at 660 nm.

MTRR genotyping

Whole blood was collected in EDTA-coated collection tubes to avoid clotting, and DNA was extracted using a Genei kit.

Standard DNA was prepared using salmon sperm DNA at various concentrations (10, 25, 50, 75 μ g/mL of distilled water) and was used to quantify the DNA extracted from patient's blood. The ratio of reading at OD 260 and 280 nm provided an estimate of DNA purity.

The primers (Genei, India) used for amplifying genomic DNA were chosen according to Ulvik et al. [15] for the MTRR gene polymorphism assay. 4 μ L of template DNA, 1 μ L each of forward (Primer F–5' CAAAGGCCATCGCAGAAG 3') and reverse primer (Primer R– 5'AAGATCTGCAGAAAATCCATGT 3'), 12.5 μ L of 2X PCR master mix and 8.5 μ L of MilliQ water were subjected to an initial denaturing step at 95°C (10 min) followed by 30 cycles of Denaturation step at 95°C (30s), Annealing step at 55°C (30 s), Extension step at 72°C (1min) and Final elongation step at 72°C for 10 min. The PCR products were electrophoresed on 1% agarose gels containing EtBr and viewed under ultraviolet light.

For digestion of PCR product with *NdeI* restriction enzyme, 10 μ L of PCR reaction product, 18 μ L of nuclease-free water, 2 μ L of 10X restriction enzyme buffer and 2 μ L of *NdeI* enzyme were mixed gently and centrifuged for a few seconds. The reaction mix was then incubated at 37°C for 8 to 12 h. The digestion products were viewed on 4% Metaphor agarose gel containing EtBr.

RESULTS

A ratio of 56.33% of DS children and 55% of the respective controls were females. Interestingly, statistical differences ($p < 0.01$) were observed between male and female DS children in terms of clinical features such as flat facial profile, slightly open mouth, small or absence of earlobes and protruding tongue. 95% significance was observed between slanted palpebral fissures and short hands and also between wide feet and wide neck or broad hands (**Figure 1**).

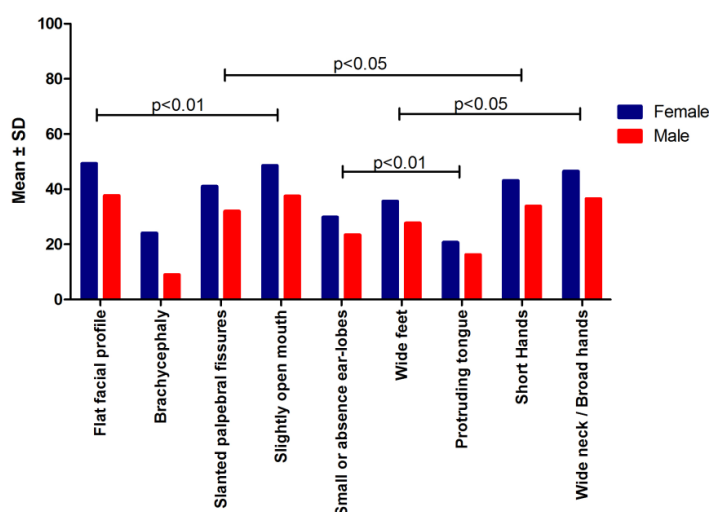


Figure 1. Phenotypic features of children with Down's syndrome

Table 1 represents the chromosomal alterations found among DS children. Trisomy 21, translocation and mosaicism were the major alterations observed in the experimental subjects. Among them, 57% ($n=17$) of DS patients were found to have trisomy 21 (47, XX, +21 or 47, XY, +21), 10% ($n=3$) translocations (46, XY, der (14; 21) or

46, XX, der (14;21), and 33% ($n=10$) Mosaicism (46, XX/47, XX, +21 or 46, XY/47, XY, +21) (Table 1). The birth frequency of DS babies was higher in the older mothers (≥ 35 years, $n=18$) 60%, than in younger mothers (<35 , $n=12$) 40%.

Table 1. Observations obtained after karyotyping of DS for trisomy 21, translocation and mosaicism

S.No	Age (months)	Gender	Trisomy 21	Translocation	Mosaicism Abnormal cells (%)	Karyotype
1DS [#]	8	F	Φ	-	-	47,XX,+21
2DS	7	F	-	-	85%	46,XX / 47,XX,+21
3DS [¶]	5	M	Φ	-	-	47,XY,+21
4DS [#]	12	F	Φ	-	-	47,XX,+21
5DS ^{*#}	9 days	M	Φ	-	-	47,XY,+21
6DS	6	F	-	Φ	-	46,XX,der (14;21)
7DS [#]	14	M	Φ	-	-	47,XY,+21
8DS ^{*¶}	8	M	Φ	-	-	47,XY,+21
9DS [¶]	18	F	-	-	85%	47,XX,+21/46,XX
10DS	13	F	-	Φ	-	46,XX,der (13;21)
11DS [*]	11	M	-	-	65%	47,XY,+21/46,XY
12DS [¶]	16	F	Φ	-	-	47,XX,+21
13DS	9	F	-	-	50%	46, XX/47,XX, +21
14DS	19	M	-	-	75%	46,XY /47,XY,+21
15DS [*]	12 days	F	-	-	70%	46,XX/ 47,XX,+21
16DS [#]	5	F	Φ	-	-	47,XX,+21
17DS [¶]	8	M	Φ	-	-	47,XY,+21
18DS [¶]	7	F	-	-	80%	46,XX /47,XX,+21
19DS [#]	12	M	Φ	-	-	47,XY,+21
20DS [*]	20 days	M	-	-	70%	46,XY/ 47,XY,+21
21DS [#]	13	F	Φ	-	-	47,XX,+21
22DS [¶]	17	M	Φ	-	-	47,XY,+21
23DS	20	F	-	Φ	60%	46,XX,der (14;21)
24DS [#]	15	M	Φ	-	-	47,XY,+21
25DS [*]	28 days	M	-	-	75%	47,XY,+21
26DS [¶]	3	M	Φ	-	-	47,XY,+21
27DS [#]	6	F	Φ	-	-	47,XX,+21
28DS	4	F	-	-	50%	46, XX/47,XX,+21
29DS [¶]	7	F	Φ	-	-	47,XX,+21
30DS ^{*#}	27 days	F	Φ	-	-	47,XX,+21

*- Age in days, #- Patients with Hypothyroidism, F – Female, M – Male, DS – Down's syndrome.

The Micronucleus Assay (MN) was carried out with peripheral lymphocytes of patients and their controls (Figure 2D). The mean MN/1000 binucleated cells were found to be significantly increased ($p<0.05$) in peripheral lymphocytes of DS patients when compared to controls and it was significantly higher in MDS (2.25 ± 1.63), when compared to DS children (0.2 ± 0.40) and their respective controls (Control children: 0.05 ± 0.22 and control mothers: 0.25 ± 0.44).

Figure 2 (A, B, C) embodies the pooled data with values of TSH, FT4 and tHcy in DS patients, their mothers and in controls. Thyroid hormonal and homocysteine levels were found to be abnormal in mothers of DS subjects. TSH level was significantly higher in MDS (4.01 ± 0.77) compared to DS children (3.52 ± 0.88) and controls (0.69 ± 0.14 , 0.66 ± 0.11) $\mu\text{IU/ml}$. Levels of FT4 in the MDS group (0.73 ± 0.57) were lower than in DS (1.77 ± 0.69) and controls (0.79 ± 0.08 , 0.76 ± 0.05) ng/dl . However, tHcy levels in MDS (14.24 ± 2.89) showed higher levels than in DS (12.11 ± 3.33) and controls (6.09 ± 1.34 , 6.28 ± 1.13) μM with no significant variation. The **Figure 2D** explains the mean MN/1000 binucleated cells were found to be statistically increased ($p<0.05$) in peripheral lymphocytes of DS patients when compared to controls and it was significantly higher in MDS (2.25 ± 1.63), when compared to DS children (0.2 ± 0.40) and their respective controls (Control children: 0.05 ± 0.22 and control mothers: 0.25 ± 0.44). A ratio of 67% of MDS showed hypothyroidism and 43% displayed homocystinuria. However, DS patients only showed 33% and 30% of hypothyroidism and homocystinuria, respectively (**Table 2**).

The frequencies of the MTRR genotypes (AA, AG, and GG) in controls were as follows: control children: 15%, 55%, and 30% control mothers: 15%, 50%, 35% (**Table 3**). The corresponding frequencies among patients were 13%, 47%, and 40% in DS children and 3%, 48% and 50% in MDS (**Table 3**). Combining the heterozygous and homozygous MTRR variants (AG+GG), we observed a very significant increase in the prevalence of the A66G mutation in MDS

(98%). The prevalence of the GG homozygous variant was significantly higher in MDS compared to controls (50%). As there was only one MTRR-AA homozygote in the reference group, odds ratio values must be regarded as imprecise (as shown by the wide confidence intervals).

DISCUSSION

DS is characterized by a constellation of physical features and systemic malformations. Kava et al. [16] reported in DS from India Mongoloid slant, ear abnormalities, epicanthic folds, flat faces, and hypotonia in >50% of cases. Similar to children with DS, psychiatric disorders are common in adults with DS compared to adults with intellectual disabilities of other etiology. Symptoms of delusions and hallucinations may exist together with social isolation, bland affect, apathy, and sleep disturbance in adults with DS and psychiatric disorders, and are often wrongly diagnosed as major depression [17]. Although psychiatric disorders seem uncommon in adults with DS, there continue to be gaps in knowledge regarding the development and progression of these problems across the lifespan [18].

A review of over 5,000 cases of DS from laboratories in England and Wales between 1989 and 1993 revealed that 95% had an extra chromosome 21 resulting from a non-disjunction error during gametogenesis. Less than 1% showed somatic mosaicism while the rest carried translocations involving chromosome 21 [19]. Similar frequencies were also documented in other reports [20,21].

In the present study, our results show free trisomy in 57%, translocations in 10% and mosaicisms in 33% of the subjects. This was in accordance with earlier reports from India [16,22,23]. Individuals with non-classical karyotypes and free trisomy 21 associated with structural and/or numerical anomalies of other chromosomes have been reported by Mokhtar et al. [20] and Sheth et al. [24]. Mokhtar et al. [20] also reported a skewed male:female ratio of 0.67 among mosaics.

Table 2. Percentage of hypothyroidism and homocystinuria observed in the experimental population of this study

Condition	DS Children (n=30)	Mothers of DS children (n=30)
Hypothyroidism	33.33%	67%
Homocystinuria	35%	65%

A higher incidence of DS in aged mothers reflects meiotic error as a common cause. The birth frequency of DS babies in younger (<35 years of age, n =12) mothers is 40% and in mothers of older than 35 years of age, n=18) is 60%.

The mean maternal age was higher in free trisomy 21, but not in translocations [19]. Chromosomal studies of parents

having children with DS in 13 couples revealed chromosomal variants in 23% (6 of 26 individuals) which did not involve chromosome 21. Chromosomal variants may predispose to non-disjunction in DS because of an interchromosomal effect [25].

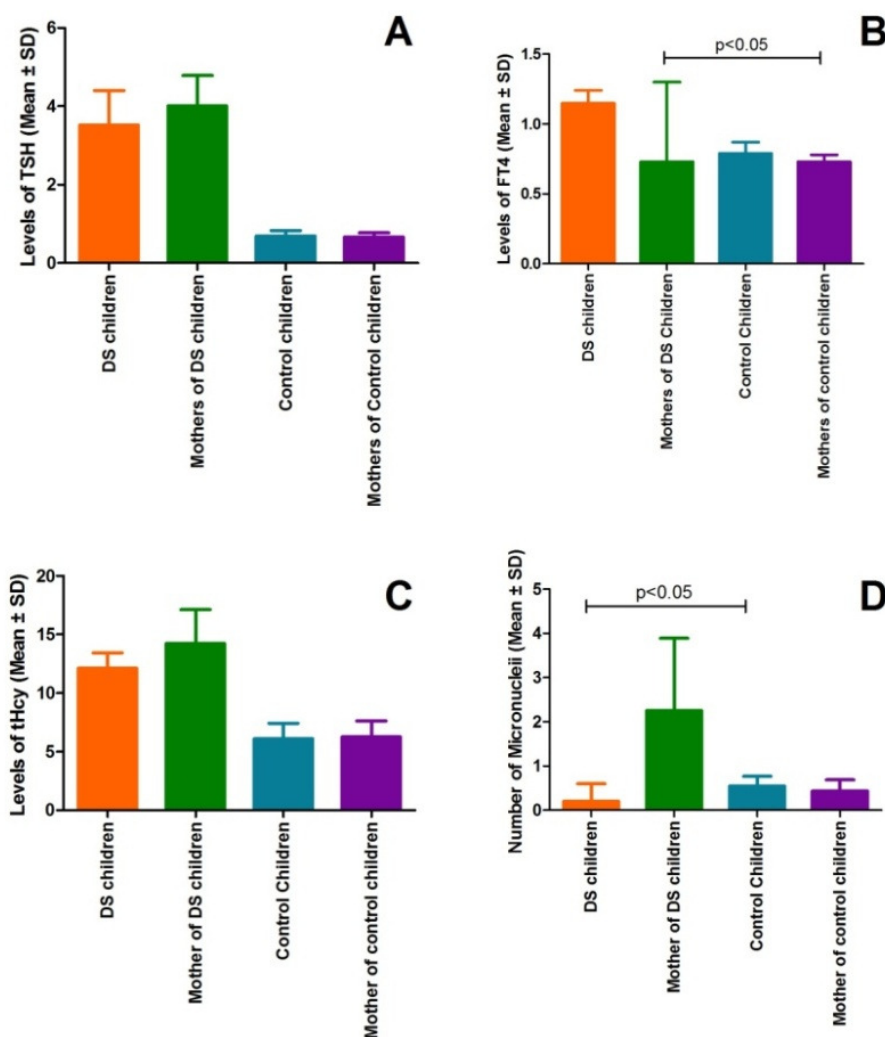


Figure 2. Comparative profile of thyroid hormone (A, B), homocysteine levels (C) and micronucleii (D) among selected Down's syndrome patients and controls.

Table 3. Frequency of MTRR (A66G) variants in MDS, DS children and Controls.

Genotype	DS children (n=30)	MDS (n= 30)	Control child (n=20)	Control mother (n=20)
AA	4 (13%)	1 (3%)	3 (15%)	3(15%)
AG	14 (47%)	14 (47%)	11(55%)	10 (50%)
GG	12 (40%)	15 (50%)	6 (30%)	7 (35%)
GG+AG	(87%)	(98%)	(84%)	(80%)

Thyroid dysfunction, especially hypothyroidism, is more prevalent in people with DS at all ages. Hypothyroidism can be either congenital or acquired at any age after birth. The estimated lifetime prevalence rate of thyroid dysfunction in

DS varied widely in different studies (between 3% and 54% in adult patients), depending on variations in population size, age, laboratory assays and definitions of thyroid dysfunction used [26]. However, what is clear from the literature

available to date is that the incidence of congenital hypothyroidism in DS cases is definitely 30-fold higher than that of the general population. There are consistent reports demonstrating thyroid status as an important determinant of the plasma/serum concentration of tHcy [27,28,29] which has been proposed as an independent risk factor for vascular occlusive disease [30].

In our study, the increase in Hcy concentrations in cases with hypothyroidism may be explained by changes in folate status and also by modifications in enzymes involved in homocysteine metabolism, distribution or clearance, and/or by concurrent changes in renal function [27]. Transient hypothyroidism is the most common form of thyroid dysfunction observed in DS patients. Gibson et al. [31] observed that 47% of subclinical hypothyroid DS patients were subsequently found to have normal TSH levels after a gap of four to six years.

Folate and tHcy status and related polymorphisms have been considered maternal risk factors for DS [32]. Folic acid deficiency has been extensively associated with an increase in DNA damage in children and adults. Polymorphisms in folic acid metabolism genes in mothers have been shown to increase the risk of DS in offspring. It has been speculated whether increased tHcy degradation explains the low frequency of atherosclerosis in patients with DS [33]. Furthermore, a low tHcy concentration may also decrease the methionine synthase reaction, which in turn may trap 5-methyltetrahydrofolate and thereby impair folate function.

The *MTRR* enzyme catalyzes the remethylation of homocysteine to methionine via a cobalamin- and folate-dependent reaction. *MTRR* has a crucial role in maintaining cobalamin in an active form and is an important determinant of homocysteine concentration in plasma. The *MTRR* gene is located at 5p15.3-p15.2, and a common polymorphism in the *MTRR* gene is 66G>A (formerly *MTRR* 66A>G), which involves an amino acid substitution from methionine to isoleucine at codon 22 (p.M22I) [34]. The *MTRR* 66AA genotype seems to contribute to elevated homocysteine and low folate levels when compared with the *MTRR* 66GG genotype [35]. In healthy individuals, plasma homocysteine level and metabolism are well regulated, but various environmental or genetic factors determine an elevation of the levels of homocysteine [36].

We found a bi-allelic polymorphism caused by the A→G substitution at nucleotide 66 which abolishes the *NdeI* restriction site. The PCR fragment of 66 bp remained uncut in the presence of the G (methionine) allele but was digested into fragments of 44 and 22 bp in the presence of the A (isoleucine) allele.

The frequencies of the *MTRR* genotypes (AA, AG, and GG) among controls were 15%, 55%, and 30% in children and 15%, 50%, 35% in mothers (**Table 3**). The corresponding frequencies among the patients were 13%, 47%, and 40% in

DS and 3%, 48% and 50% in MDS. A combination of the heterozygous and homozygous *MTRR* variant genotypes (AG+GG) showed a very significant increase in the prevalence of the A66G mutation in MDS (98%). The prevalence of the GG homozygous variant genotype alone was significantly higher in MDS compared to controls (50%).

The possibility for non-dysfunction has been indicated when the mothers of children with DS have a high frequency of the T allele [37,38]. In addition, Kohli et al. (39) observed the lack of a relationship in their study on north Indian DS mothers, while a case-control study on Indian mothers of DS children from a north-eastern state showed a 7.6-fold increase in the frequency of the TT genotype in the case mothers compared with the controls. [8,40]. Nor was there preferential transmission of the T allele. In contrast, Devi et al. [41] found a significant difference in T allele frequency between case and control parents. The minor allele frequencies in case mothers and fathers were 4.17% and 6.94%, respectively. However, females were found to have a higher T allele frequency than males in a large study. Meguid et al. [38] also observed that the mutant genotype was significantly more common in case mothers than in controls, indicating a greater genetic impact of this polymorphism. Angeline et al. [42] found the frequency of 677TT among Tamilians to be 1.38% (1/72) and that of 677CT heterozygotes to be 18.1%, while the frequencies of 1298AC and 1298CC genotypes were 47.2% and 15.3%, respectively. The frequencies of the T allele of C677T and the C allele of A1298C in 30 control individuals were 0.17 and 0.45, respectively. James et al. [43] described that micronutrient intake was suggested to influence the effects of polymorphisms in genes involved in the folate pathway.

CONCLUSION

Cytogenetic assessment is essential for the confirmation of clinical diagnosis. In DS, karyotyping is also useful for prevention in successive pregnancies and genetic counseling. Folic acid and thyroid metabolism are also determinant in the phenotypic profile of DS cases and their mothers. Consequently, a pharmacogenomic assessment of DS cases and their parents is highly recommended for the prevention and treatment of these traits which influence brain function.

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