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GENETIC POLYMORPHISMS OF MTHFR (677T AND 1298C) AND HOMOCYSTEINE METABOLISM AS MATERNAL RISK FACTOR FOR DOWN 'S SYNDROME PATIENTS IN NORTH INDIAN POPULATION.

SANJEEV KUMAR PANDEY, PANKAJ KUMAR MOHANTY, SUNIL KUMAR POLIPALLI AND SEEMA KAPOOR

Pediatric Genetic Research Laboratory, Department of Pediatrics, Maulana Azad Medical College & Lok Nayak Hospital, New Delhi-110002.

ABSTRACT

Background &Aim: Down syndrome (DS) is caused by the presence of three copies of chromosome 21, in most cases due to the failure of chromosomal segregation during maternal meiosis (meiotic nondisjunction). Despite substantial research, the molecular mechanism underlying non-disjunction of trisomy 21 are poorly understood, as there was scanty data available from India the study was designed to investigate the MTHFR C677T and A1298C polymorphisms, with homocysteine as a maternal risk factor for DS. Materials and Methods**:** Eighty mothers of individuals with confirmed full trisomy 21, and 100 control mothers of the north Indian population were evaluated. Molecular analysis of MTHFR C677T and A1298C polymorphisms was performed by PCR-RFLP method. Biochemical correlation of total serum homocysteine was evaluated. Results**:** The frequency of genotypes of *MTHFR were* 677CC (82.6%), 677CT (15.4%) and 677TT (2.0%) in the patients with Down's syndrome, and among the 100 individuals of the control group, genotypes 677CC (92.2%), 677 CT (6.12%) and 677TT (1.6%) were found. As for polymorphism 1298C, the patients with Down's syndrome presented genotypes with frequencies 1298AA (15.0%), 1298AC (52.5%) and 1298CC (32.5%) respectively and in the control group the frequencies of genotypes were 1298AA (60.0%), 1298AC (22.0%) and 1298CC (17.0%) respectively. Homocysteine concentrations were significantly different in women with an *MTHFR* 1298CC genotype according to the group (case or control), being higher in DS mothers than in control mothers. Conclusion: No correlation was observed in *MTHFR* gene polymorphism 677T in DS. However, the *MTHFR* gene polymorphism at position 1298, mainly genotype 1298CC that reduces the enzyme efficiency, was more frequent in the group of Down's Syndrome patients, suggesting its involvement in mechanisms related to chromosomal imbalances.

KEY WORDS: Down syndrome, serum and RBC folate, serum homocysteine, MTHFR gene,CHD

SEEMA KAPOOR
Pediatric Genetic Research Laboratory, Department of Pediatrics, Maulana Azad Medical College & Lok Nayak Hospital, New Delhi-110002.

INTRODUCTION

Down syndrome, being most common aneuploidy at birth and most commonly recognized cause of mental retardation is caused by the presence of three copies of chromosome 21.[1] The extra chromosome is maternal in 95% of cases and is due to failure of normal chromosomal segregation during meiosis. Both clinical and experimental studies have shown that genomic DNA hypomethylation is associated with chromosomal in stability and abnormal segregation. Folate plays an important role being responsible for synthesis of S-adenosylmethionine (SAM), the main methyl group for methylation reaction. MTHFR act as critical metabolic juncture catalyzing the conversion of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate and it donates methyl group for remethylation of homocysteine to methionine.[2] Folate deficiency results in DNA strand breaks, DNA hypomethylation, abnormal gene expression and chromosomal segregation[3] which can be estimated by determination of RBC folate level being a more sensitive marker for remote folate status of the mother.[4] The genotype C677T and A1298C polymorphism affect this pathway by reducing enzyme activity.[5-6]Homocysteine being a key branch-point intermediate in methionine cycle, generates methyl group for all kinds of transmethylation reaction including DNA methylation which is essential for life. The plasma homocysteine level is affected by folate, vitamin B12, and by genetic polymorphism MTHFR enzyme of one- carbon metabolism.[7] MTHFR is partly controlling the folate metabolism through catalyzing the reduction of 5, 10-methylenetetrahydrofolate to 5- methyltetrahydrofolate;[8] a co substrate for the conversion of homocysteine to methionine; thus resulting into a state of hyperhomocysteinemia;[9-10-11] particularly the thermolabile variant of MTHFR enzyme with reduced activity that leads to elevated plasma homocysteine concentration. [12] Folic acid also plays an important role during embryological development including development of cardiovascular system but the result being in conclusive, environmental factors appear to play a role in such defects. Several studies have associated genetic polymorphism in folate metabolism to an elevated maternal risk factor of DS [5,13]. In some other studies, they failed to get any association [14-15]. It has also been observed that low thiols, is indicative of low protein intake can be a cause of hyperhomocysteinemia.Hyperhomocysteinemia, whether caused by a genetic mutation or diet, alters the abundance of several liver proteins involved in homocysteine/methionine metabolism, and antioxidant defense.[16] The association of the C677T polymorphism of the MTHFR gene has been studied in Indian Gujrati mothers and East Indian mothers [17-18]. Both reported a significant correlation with homozygous TT genotype. However, its simultaneous correlation with the biochemical parameters in the Indian subcontinent has not been investigated so far. The practice implications of these findings may answer the intriguing question as to whether folic acid supplementation in women around conception can be effective in reducing risk of Down's syndrome. In our present study we analyzed genetic polymorphism of MTHFR C677T, MTHFR A1298C in association with serum and RBC folate and serum homocysteine on maternal risk factor of Down syndrome.

MATERIALS AND METHODS

Our Study was conducted at Pediatrics Research and Genetic lab, Department of Paediatrics, Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi from February 2008 to January 2009. The study protocol was approved by the Institutional Ethics Committee. This is a case-control study. Sample size was calculated on the basis of the case-control study carried out by James *et al*.[3], in which the odds ratio found was calculated at 2.6. Using the 95% confidence coefficient (α = 5%) and a confidence interval for the odds ratio with an amplitude of d = 4.6, the sample size required for this study was calculated at a minimum of 80

cases and 100 controls.Mothers of children with trisomy 21 were included in the study as cases and women whose children were not affected by trisomy 21 and who had never suffered a miscarriage were enrolled as controls. The following variables were controlled by logistic regression—maternal age at delivery as self-referred by the patient and defined by studying the previous two generations of the family (heredogram). The dependent variable was the child's karyotype result and the independent variable was the occurrence of a polymorphism in the 677 and/or 1298 positions of the human gene codifying for MTHFR in the study sample. Proper informed consent was taken before enrolling the cases and control study group. Maternal age was calculated considering the age of the mother at the birth of the DS child for DS mothers.After obtaining informed consent 10 ml blood was collected in fasting state. Five ml aliquots were used for plasma homocysteine and RBC folate, serum folate estimation and 5 ml in EDTA tubes for PCR amplification for MTHFR gene in our study. Another 5 ml was collected from child with Down syndrome for karyotyping and thyroid function tests. Plasma homocysteine was measured from the mother's blood in fasting state using HPLC. RBC folate was measured by using in the Roche Elecsys Folate II Assay. For calculating the folate concentration in erythrocyte the dilution factor and the hematocrit value were taken into account. A value serum homocysteine >15 micromol/L was considered as hyperhomocysteinemia.DNA was extracted according to the method of Lahiri and Nurnberger [19]. C677T and A1298C polymorphisms of the MTHFR gene were analyzed by the polymerase chain reaction (PCR) with specific primers and protocols as described by Frosst et al.[20] and Van der Put et al. [21]. The PCR-amplified fragments were digested with endonucleases HinfI and MboII and analyzed by electrophoresis in 2% Agarose gel.

Statistical analysis

Results of continuous data e.g., serum homocysteine, RBC folate,serum folate were expressed as median with interquartile difference, number of frequencies. For comparison of maternal age in two groups, the Student t- test and logistic regression with age group into quartiles were used. Odds ratio and 95% confidence interval (95%CI) were calculated to estimate the risk of different genotypes. Comparisons of percentages between groups were evaluated with chi-square test corrected for continuity. Odds ratio for having a child affected by trisomy 21 was estimated for homozygous, heterozygous or absence of above mentioned MTHFR polymorphism and regression analysis obtained from interacting variables. Logistic regression models were used to control for the effect of maternal age at the time of having a child with Down syndrome. The data were analysed with SPSS software, version 16.0. P value was considered statistically significant if less than 0.05.

RESULTS

A total of 80 case mothers and 100 control mothers were studied. The mean maternal age in case mothers and control mothers were 24.9±3.2 and 26.9±4.6 respectively. We have found a higher frequency of Down syndrome in age group more than 25years. Since we had very few mothers of age range more than 35 years, so we analyzed only two age groups both more and less than 25 years of age. (Table 1) The genotype distribution and allelic frequencies for MTHFRC677T and A1298C were depicted in Table 1. The heterozygous and homozygous genotype frequencies of MTHFR at position 677(CT and TT) among case mothers and controls were (14.8% vs. 9.1% and 2.55 vs. 3.03%, OR= 1.12, 95% CI 0.637-3.44, P=0.487) No significant statistical difference found between the two groups. Nevertheless we could find a significant association of A1298C in cases as compared to controls when adjusted for maternal age. The heterozygous and homozygous genotype frequencies at 1298 position (AC and CC) among case and control mothers were (38.3% vs. 22.2% and 28.4% vs. 17.25% respectively, OR= 3.10, 95% CI 1.6-

5.79, P=0.002), showing a statistically significant difference between two groups. Table 2 represents the risk factors associated in occurrence of Down syndrome. We found abnormal value of folate (< 3ng/ml) in 33.3% in cases vs. 8.0% in controls, which was significant (p value 0.0001). This actually reflected the recent folate status and would not have had correlation with folate levels at the time of conception Abnormal RBC folate levels; an important long term predictor of folate status, (< 160ng/ml) were found in 28.3 % of cases vs. 11.1 % in controls, which was significant (P=0.005) High serum homocysteine was found in 9.8% of cases vs. 2.0% in controls and the difference was statistically significant (P=0.04) (Figure:2). On regression analysis adjusted for maternal age significant statistically difference was found between case and controls (P=0.04). The median serum homocysteine in cases was 10.2(Q1-Q3, 8.2-12.0) vs. 7.6(Q1-Q3, 5.7-10.1) in controls, showing higher serum homocysteine in cases. We didn't find any significant association with the genotype .The emerging data also highlighted the impoverished nutritional status of both the case and control mothers which may have a significant impact on other fetal growth parameters.The combined low serum and RBC folate are statistically significant difference between case and control (OR=6.15, 95% CI 2.34-15.99, p value 0.0001). But combinations of above risk factor with high serum Homocysteine have no significant difference in between case and controls.

Table 1
MTHFR genotype distribution among mothers of children with Down syndrome and control mothers

MTHFRC677T	CC	CT	TT	P	Pa
Case	67(82.7%)	12(14.8%)	2(2.56%)	0.487	0.524
Control	87(87.9%)	9(9.01%)	3(3.03%)		
AGE <25YRS	CC	CT/TT		P	
Case	44(78.5%)	12(21.4%)		0.480	
Control	55(84.6%)	10(15.3%)			
AGE>25YRS					
Case	23(92%)	2(8%)		0.749	
Control	32(94.1%)	2(5.8%)			
MTHFRA1298C	AA	AC	CC	P	Pa
Case	27(33.3%)	31(38.3%)	23(28.4%)	0.001	0.002
Control	60(60.6%)	22(22.2%)	17(17.2%)		
AGE<30	AA	AC/ CC		P	
Case	13(52%)	12(48%)		0.22	
Control	23(67.6%)	11(32.4%)			
AGE>30					
Case	14(25%)	42(75%)		0.0001	
Control	37(56.9%)	28(43.1%)			

Table 2
The risk factors associated in occurrence of Down syndrome

Risk factors	Cases (%)	Controls (%)	OR (95%CI)	P	Pa
Maternal age					
< 30 yrs	25(42.4%)	34(57.6%)			
≥30 yrs	56(46.3%)	65(53.7%)	1.17(0.625-2.19)	0.621	
Low serum	27(77.1%)	8(22.9%)	5.68(2.41-13.41)	0.0001	0.0001
Folate					
Low RBC	23(67.6%)	11(32.4%)	3.17(1.43-6.99)	0.005	0.004
Folate					
High serum	8(80%)	2(20%)	5.31(1.09-25.77)	0.04	0.036
Homocysteine					
Combined low	23(79.3%)	6(20.6%)	6.15(2.34-15.92)	0.0001	0.0001
serum and RBC folate					

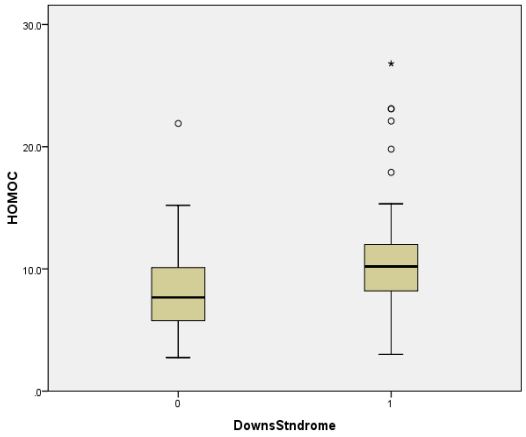

Figure 1

Distribution of plasma Homocysteine concentration (µmol/L) in two groups.down syndrome mothers median 10.2µmol/L (Range 8.2-12.0). Control mothers median 7.6µmol/L (Range 5.7-10.1). Zero (0) signifies control mothers and one (1) signifies DS case mother.

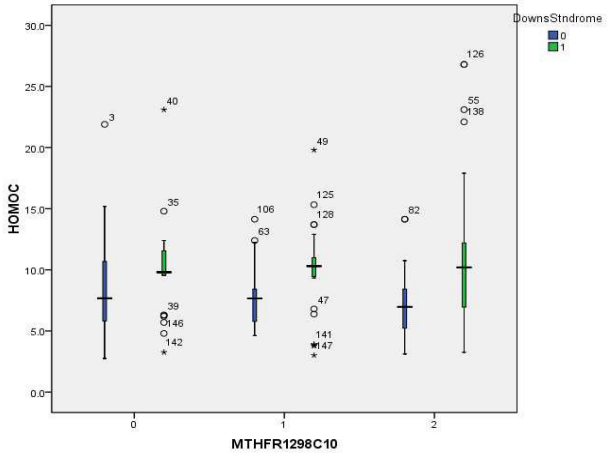

Figure 2

Demonstrates the distribution of Plasma Homocysteine concentrations according to MTHFR A1298C genotypes. Middle bar corresponds to 50th centile of the median. Zero, one two signifies allele AA, AC, CC respectively.

DISCUSSION

DOWN SYNDROME is the first clinically defined genetic disorder shown to be chromosomal in origin.[1] It is characterized by multiple somatic anomalies and cognitive impairment. Although common cellular and molecular mechanism involved in this meiotic nondisjunction remain unknown, folic acid and polymorphism on genes coding for enzymes involved in its metabolism are thought to influence meiotic disjunction.[3, 6] We studied serum levels of folate, RBC folate, homocysteine and MTHFR C677T and A1298C polymorphism in mothers of Down syndrome versus control mothers.[6, 17] The frequent genetic mutations may also modify the enzymatic activity of MTHFR gene. The enzymatic activity of MTHFR is low in heterozygote and in homozygote mutants. The cytosine to thymine transition mutation at position 677 within MTHFR gene (677C/T) causes alanine to valine substitution in MTHFR protein and reduced

enzyme activity.[1, 2] The A1298C polymorphism is located in exon 7 within presumptive regulatory domain. The A1298C mutation results in decreased MTHFR activity. We studied RBC folate in addition as it is preferred marker to assess long term folate status. Plasma folate is indicative about recent folate status.[3] In our study; we found isolated analysis of MTHFRC677T polymorphism did not show a significant association with maternal risk for Down syndrome though we observed a higher median number of polymorphic alleles in Down syndrome mothers (17.3%) compared to controls (12.1%). The present study showed that the frequencies of MTHFR 677C/T (heterozygotes) and 677T/T (homozygous) were 14.8% and 2.5% respectively than the control mothers 9.01% and 3.0. % respectively. The sum of both variant genotypes (CT plus TT) showed a marginal increase in cases (17.3%) as compared to control (12.1%) but the difference was not statistically significant. This is in contrast to the two Brazilian study (Acacio et al.[4], 2005; Silva et al[13].,2005). Acarcio and colleagues observed a 5.7 fold increase of DS risk when both polymorphisms were combined where as Silva et al compared 154 cases and 158 controls and found marginally significant higher frequency of 677T allele in cases group (OR=1.44; CI 1.00-2.062; P=0.049). Another study by Brandalize ABC et al. who analyzed 239 case mothers and 197 control mothers and after adjustment for maternal age , the genotype C677T frequency in cases versus controls reached statistical significance (OR= 1.36,95% CI 1.00-1.860; P< 0.05). Our study also being supported by Kohli et al [16](2004) who didn't find association of MTHFR C677T polymorphism with increased risk of Down syndrome in North Indian population. Studies by James et al. and Hobbs et al.[2] have shown higher frequencies of both the MTHFR CT and TT genotype in mothers of Down syndrome as compared to controls. But this was not reflected in our study, the difference in our result can be explained by smaller sample size, the control selection and the ethnic mixture of the study population in north Indian mothers. In a study by Rai et al.[18] in India, where 677T allele frequencies was quite low. They have shown a strong association of SNP (single nucleotide polymorphism), especially T homozygotes, with Down syndrome mothers. A study done in AIIMS by Kohli et al. [16](2004) showed 28.16% mutant allele against 15.38% in our case. They did not find any homozygous TT genotype in case mothers. In the present study regarding the MTHFR 1298A/C polymorphism, we found the frequency of homozygous genotype (CC) was significantly higher among case mothers than controls (28.4% vs. 17.2%). The sum of both variant genotypes (AC and CC) were higher among case groups compared to control group with significant between the two groups (66.7% vs. 39.4%). Our study was also supported by Frosst P et al., [20] who investigated 94 case mothers and 264 controls. They reported an increase risk of DS associated with MTHFR 1298AC allele and the MTHFR 1298CC genotype. The result of this initial study indicate that folate metabolism is abnormal in mothers of children with Down syndrome and this may be explained, in part, by the genetic susceptibility due to polymorphism in the MTHFR gene.[11,15] We also studied the association of serum folate, vitamin B12 and homocysteine levels with C677T, A1298C polymorphism of MTHFR gene. We found significant association of low serum and RBC folate and high serum homocysteine with C677T polymorphism (p value 0.0001, 0.005, 0.04 respectively). On regression analysis with respect to maternal age above value came out to be significant (P value 0.0001, 0.004, 0.036 respectively) respectively). The combined of low serum and RBC folate have 6 times more risk in causing MTHFR polymorphism and occurrence of Down syndrome (OR= 6.15, 95% CI 2.34-15.99, P value 0.0001). However combined value low serum and RBC folate and high homocysteine were not significant with respect to genetic polymorphism (MTHFR A1298C). The serum homocysteine value is higher in heterozygote cases (AC) in comparison to homozygous cases (CC), the reason may be due to less number homozygous obtained in MTHFRA1298C in our study as compared to heterozygotes. In India, few studies on folate

and homocystiene levels in general populations have revealed that the basal level homocystiene is higher where that of folate is lower than the recommended WHO standard [16-17]. James et al.[3]hypothesized the possibility that gene-nutrient interactions associated with abnormal folate metabolism and DNA hypomethylation might increase the risk of maternal chromosome non-disjunction and Trisomy 21.Our study reveled significant statistical association of polymorphism of MTHFR A1298C with occurrence of Down syndrome. We also a found statistically significant association of low serum and RBC folate with occurrence of Down syndrome and RBC folate is a more reliable marker. Our study also revealed higher amount of plasma homocysteine in Down syndrome mothers as compared to control mothers and the difference between two groups were significant. The increase in serum homocysteine can be lowered or prevented by folic acid, thus the metabolic imbalance with genetic mutation can be largely prevented. In light of our study results and comparing the literature available in this respect we may conclude that MTHFRA1298C polymorphism, low serum folate and RBC folate and high homocysteine are maternal risk factors for Down syndrome. Currently, we may suggest that a much larger multicentric study needs to look into the geo-ethnic variation in folate and polymorphism and evaluate its association in this era of nutrigenomics.

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