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Genetic polymorphisms involved in folate metabolism and elevated plasma concentrations of homocysteine: maternal risk factors for Down syndrome in Brazil

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ABSTRACT. The aim of the present study was to investigate the effect of polymorphisms C677T and A1298C in the methylenetetrahydrofolate reductase (*MTHFR*) gene, A2756G in methionine synthase reductase (*MTR*) gene and A80G in reduced folate carrier 1 (*RFC1*) gene, and plasma homocysteine (Hcy), on the maternal risk for Down syndrome (DS). Seventy-two DS mothers and 194 mothers who had no children with DS were evaluated. The investigation of the *MTHFR* C677T, *MTR* A2756G and *RFC1* A80G polymorphisms was performed by polymerase chain reaction and enzyme digestion and the *MTHFR* A1298C polymorphism by allele-specific polymerase chain reaction. Hcy quantification was carried out by liquid chromatography-tandem mass spectrometry. The median number of polymorphic alleles for the four loci tested was greater in DS mothers compared to the control group, and the presence of three or more polymorphic alleles increased the risk for having a child with DS 1.74 times. Elevated maternal risk for DS was also observed when plasma Hcy

concentration was higher than 4.99 μmol/L. In conclusion, the presence of three or more polymorphic alleles for *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *RFC1* A80G, and plasma Hcy concentrations higher than 4.99 μmol/L are maternal risk factors for DS.

Key words: Trisomy 21; Down syndrome; Nondisjunction; Genetic polymorphism

INTRODUCTION

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) (Jyothy et al., 2001). Some studies suggest that genomic DNA hypomethylation may be associated with chromosomal instability and abnormal segregation (James et al., 2003; Sciandrello et al., 2004).

Folate plays an important role in cellular methylation reactions (Fenech, 2002). Its metabolism is responsible, in one pathway, for the synthesis of S-adenosylmethionine (SAM), the main methyl group donor for methylation reactions of DNA, proteins and lipids. Some enzymes play important roles in the maintenance of SAM concentrations. The enzyme methionine synthase reductase (MTR) catalyzes the remethylation of homocysteine (Hcy) to methionine, and this reaction results in SAM formation. The enzyme MTR requires 5-methyltetrahydrofolate as a methyl group donor for the remethylation of Hcy to methionine, and the formation of this radical depends on the action of the enzyme methylenetetrahydrofolate reductase (MTHFR) (Finkelstein, 1998).

Folate-transporting proteins are also important in the maintenance of DNA methylation, since they are responsible for the amount of folate available in the cells. The reduced folate carrier 1 (RFC1) protein is located in the intestinal mucosa membrane and plays a role in folic acid absorption, transporting 5-methyltetrahydrofolate into the cells (Nguyen et al., 1997).

Several studies have associated polymorphisms in genes involved in folate metabolism, such as *MTHFR*, *MTR* and *RFC1*, to an elevated maternal risk for DS (James et al., 1999; Grillo et al., 2002; Acácio et al., 2005; da Silva et al., 2005; Scala et al., 2006; Coppede et al., 2006). The observation of significantly higher Hcy concentrations in DS mothers compared to control mothers is another evidence that folate metabolism may be abnormal in these women (Bosco et al., 2003; Takamura et al., 2004; da Silva et al., 2005).

The aim of this study was to analyze the effect of four genetic polymorphisms, *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *RFC1* A80G, and plasma Hcy concentrations on the maternal risk for DS.

METHODS

The study protocol was approved by the Research Ethics Committee of the São José do Rio Preto Medical School (CEP-FAMERP), in the State of São Paulo, and by the National Research Commission (CONEP), Brazil. In this study, 72 women who had given birth to children with full trisomy 21 were included (DS mothers), recruited from the Genetics Outpatient Service of Hospital de Base (HB) of the São José do Rio Preto Medical School (FAMERP), São Paulo, Brazil. Control

mothers (N = 194) who had no children with DS were enrolled at the FAMERP Campus and at the HB Clinical Analysis Laboratory. Maternal age was calculated considering the age of the mother at the birth of the DS child for DS mothers, and the age at the birth of the last child for the control group. Mothers of DS individuals with translocation or mosaicism were excluded from the study. The exclusion criterion for the control group was the presence of a child with a genetic syndrome.

Genomic DNA was extracted from blood leukocytes (Abdel-Rahman et al., 1994). Polymorphisms at four loci were evaluated, two of them in the *MTHFR* gene (C677T and A1298C), one in the *MTR* gene (A2756G), and one in the *RFC1* gene (A80G). The *MTHFR* A1298C polymorphism was determined by allele-specific polymerase chain reaction, using primers to separately amplify wild-type and mutated alleles (allele A: forward 5'- GGA GCT GAC CAG TGA AGA -3' and reverse 5'- TGT GAC CAT TCC GGT TTG -3' to amplify a 77-bp fragment; allele C: forward 5'- CTT TGG GGA GCT GAA GGA -3' and reverse 5'- AAG ACT TCA AAG ACA CTT G -3' to amplify a 120-bp fragment). Primers flanking the 677 polymorphic region of the *MTHFR* gene, described by Frosst et al. (1995), were used as positive controls of gene amplification, resulting in a 198-bp fragment. This strategy makes it possible to determine the *MTHFR* C677T genotype, after digestion of this fragment by enzyme *Hin*fI which recognizes the allele T. Genotyping for the *MTR* A2756G polymorphism was carried out by polymerase chain reaction, using specific primers (forward 5'- CCA GGG TGC CAG GTA TAC AG -3'; reverse 5'- GCC TTT TAC ACT CCT CAA AAC C -3') to amplify a 498-bp fragment, followed by digestion with enzyme *Hae*III which digests the fragment in the presence of the mutated G allele. The *RFC1* A80G polymorphism was determined using primer sequences described by Födinger et al. (2003) and digestion with enzyme *Cfo*I for recognizing the G allele.

Hcy quantification was possible only for 58 DS mothers and 49 control mothers. Plasma Hcy concentrations were determined by liquid chromatography-tandem mass spectrometry as previously described (Haddad et al., 2001; Vellasco et al., 2002; de Andrade et al., 2006), in overnight fasted mothers.

Statistical analysis

Data are reported as means ± standard deviation, number or frequency. To compare the maternal ages in the two groups, the Student *t*-test and logistic regression with ages grouped into quartiles were used.

Hardy-Weinberg equilibrium was tested by the chi-square test, using the BioEstat program, and genotype frequencies in the DS and control mothers were compared by the likelihood ratio test and logistic regression. The relationship between the number of polymorphic alleles for the four loci tested per case or control mother and the maternal risk for DS was analyzed by the Mann-Whitney test followed by logistic regression analysis. For this analysis, the samples were divided into subsets (0-2 and 3-5 alleles). *MTHFR* haplotypes were inferred using the Phase program (version 2.1), which creates population frequency estimates of the haplotypes. The distribution of combined *MTHFR* genotypes (677/1298) in the two groups was analyzed by the likelihood ratio test and logistic regression, excluding the only woman with the genotype 677CT/1298CC. In the logistic regression analysis, the 677CC/1298AA genotype was considered as reference.

The comparison between groups regarding the Hcy concentrations was performed using the Mood median test followed by logistic regression analysis of Hcy grouped into quartiles. The distribution of Hcy concentrations according to the genotypes was carried out

by ANOVA, using the logarithm scale of Hcy concentrations. For the combined *MTHFR* genotype analysis, we excluded the only woman with the genotype 677CT/1298CC. The computer-assisted statistical analyses were carried out with the Minitab for Windows (Release 12.22) program. For statistical analysis, values of $P \leq 0.05$ were considered to be significant.

RESULTS

Characterization of groups in relation to maternal age

The mean maternal age of DS mothers was 31.7 ± 8.4 years (range: 13-46), and in the control group it was 27.1 ± 5.8 years (range: 15-41). As expected, there was a prevalence of older mothers in the case group ($P < 0.0005$), and an increased risk for DS was observed above the age of 32.4 years (OR = 4.61; CI95% = 2.53-8.39; $P < 0.0005$). For women older than 35 years, the risk for DS was even higher (OR = 10.18; CI95% = 4.90-21.15; $P < 0.0005$).

Genotype analysis and risk for Down syndrome

The allele frequencies of *MTHFR* 677T, *MTHFR* 1298C, *MTR* 2756G, and *RFC1* 80G are presented in Table 1. Genotype frequencies for the four polymorphisms were in Hardy-Weinberg equilibrium in both groups ($P > 0.05$) and showed no difference between DS mothers and control groups ($P = 0.85$ for *MTHFR* C677T; $P = 1.0$ for *MTHFR* A1298C; $P = 1.0$ for *MTR* A2756G; $P = 0.1$ for *RFC1* A80G). The distribution of combined *MTHFR* 677/1298 genotypes showed no differences between DS and control mothers ($P = 2.4$), and no association was found with the risk for DS ($P = 0.88$ for CC/AC; $P = 0.20$ for CT/AA; $P = 0.38$ for CC/CC; $P = 0.17$ for CT/AC; $P = 0.27$ for TT/AA). In addition, the logistic regression analysis for all polymorphisms also showed no association between the variant genotypes and the risk for DS (Table 2). We also evaluated the genotype frequencies between the groups considering only women with maternal ages below 35 years and also below 32.4 years. However, no differences were found between case and control mothers in these analyses. The distribution of the number of polymorphic alleles per individual (case or control) for the four loci tested is presented in Table 3. The median number of polymorphic alleles was higher in the group of DS mothers than in the control group (medians 3 and 2 for case and control groups, respectively; $P = 0.02$). The presence of three or more polymorphic alleles increases the risk of having a child with DS 1.74-fold (OR = 1.74; CI95% = 1-3.02; $P = 0.048$).

Table 1. Allele frequencies of the *MTHFR* C677T, A1298C, *MTR* A2756G, and *RFC1* A80G polymorphisms in Down syndrome (DS) and control mothers.

Polymorphism	Allele	DS mothers	Control mothers
MTHFR 677	T	0.3542	0.2861
MTHFR 1298	C	0.2569	0.2526
MTR 2756	G	0.2014	0.1881
RFC1 80	G	0.5417	0.5000

Table 2. Genotype frequencies of *MTHFR* C677T, A1298C, *MTR* A2756G, and *RFC1* A80G polymorphisms in Down syndrome (DS, N = 72) and control (N = 194) mothers.

Genotype	DS mothers N (%)	Control mothers N (%)	OR (95%CI)	P
MTHFR 677				
CC	29 (40.3)	100 (51.5)	reference	
CT	35 (48.6)	77 (39.7)	1.69 (0.92-3.08)	0.089
TT	8 (11.1)	17 (8.8)	1.70 (0.62-4.66)	0.305
MTHFR 1298				
AA	40 (55.6)	108 (55.7)	reference	
AC	27 (37.5)	74 (38.1)	1.07 (0.58-1.96)	0.832
CC	5 (6.9)	12 (6.2)	1.53 (0.48-4.95)	0.473
MTR 2756				
AA	47 (65.3)	129 (66.5)	reference	
AG	21 (29.2)	57 (29.4)	1.01 (0.55-1.87)	0.965
GG	4 (5.6)	8 (4.1)	1.63 (0.46-5.83)	0.451
RFC1 80				
AA	15 (20.8)	50 (25.8)	reference	
AG	36 (50.0)	94 (48.5)	1.25 (0.62-2.53)	0.533
GG	21 (29.2)	50 (25.8)	1.38 (0.63-3.03)	0.426

Table 3. Distribution of the *MTHFR* 677T, 1298C, *MTR* 2756G, and *RFC1* 80G alleles per individual in the two groups.

	Number of polymorphic alleles					
	0	1	2	3	4	5
Case (N, %)	1 (1.4)	12 (16.7)	14 (19.4)	26 (36.1)	14 (19.4)	5 (6.9)
Control (N, %)	9 (4.6)	32 (16.5)	58 (29.9)	64 (33.0)	23 (11.9)	8 (4.1)

Haplotype analysis for *MTHFR* C677T and A1298C

The haplotypes were inferred by the Phase program, and a higher frequency of the C-A haplotype was observed in both groups (0.390 and 0.468 in DS and control mothers, respectively). The frequency of haplotype T-A was 0.353 and 0.280, and of haplotype C-C 0.256 and 0.246, in DS and control mothers, respectively. The estimated frequency of haplotype T-C was 0.001 in DS mothers and 0.006 in control mothers. Thus, although haplotype T-C was present in our study population, its frequency was lower than the expected allele combination frequency calculated by the Phase program, confirming negative selection of this haplotype.

Homocysteine concentrations

The median Hcy concentrations were significantly higher in DS mothers (5.38 μmol/L) as compared to the control group (4.22 μmol/L) (P = 0.01), as shown in Figure 1, and a higher maternal risk for DS was observed when the Hcy concentrations were higher than 4.99 μmol/L (OR = 4.62; CI95% = 1.69-12.59; P = 0.003).

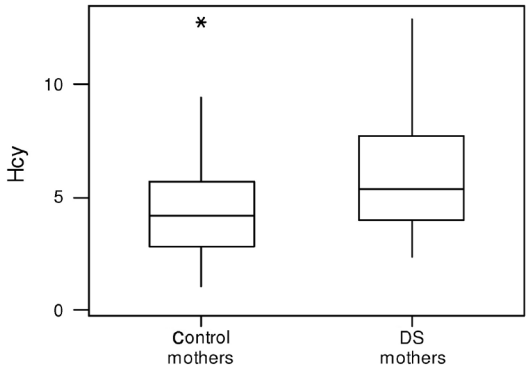

Figure 1. Distribution of plasma homocysteine (Hcy) concentrations (μmol/L) in the two groups. Control mothers: median 4.22 μmol/L (range: 1.1-12.8); Down syndrome (DS) mothers: median 5.38 μmol/L (1.4-12.9). *Only one individual of the control group presented high Hcy concentrations.

Plasma homocysteine concentrations and their relation to genotypes

The analysis of Hcy distribution by genotypes showed that in women with *MTHFR* 1298CC genotype the Hcy concentrations varied significantly according to the group (case or control) (P = 0.036). In DS mothers, the Hcy concentrations were higher in the presence of the 1298CC genotype, while in the control group these concentrations were lower (Figure 2).

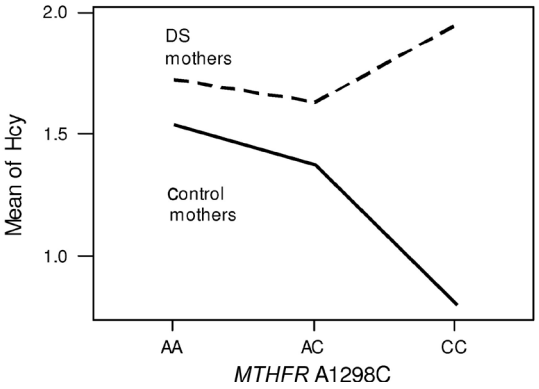

Figure 2. Plasma homocysteine (Hcy) concentrations according to *MTHFR* A1298C genotypes. DS = Down syndrome.

DISCUSSION

The association between advanced maternal age and the occurrence of trisomies has been well established. A Brazilian study conducted in the southeastern region of the country showed that the maternal age of women with DS children was significantly higher than that of women without DS children (Beiguelman et al., 1996), and our results confirm this finding.

The occurrence of DS independent of maternal age represents evidence of other risk factors for this syndrome. Several studies have correlated the maternal risk for DS to an abnor-

mal folate metabolism (James et al., 1999; Grillo et al., 2002; da Silva et al., 2005; Acácio et al., 2005; Scala et al., 2006; Coppede et al., 2006). The observation of an increased occurrence of abnormal chromosome 13 and 21 segregation events in peripheral lymphocytes of young mothers with DS children suggests a generalized susceptibility to chromosomal malsegregation events, taking place in both the meiotic and the mitotic process (Migliore et al., 2006).

Folate deficiency has been linked to chromosomal instability and chromosome 21 aneuploidy (Wang et al., 2004; Beetstra et al., 2005), and the genome-damaging effect of folate deficiency in cultured lymphocytes may be modulated by the *MTHFR* genotype (Kimura et al., 2004). Certain studies have associated the presence of one or two *MTHFR* 677T alleles with an elevated maternal risk for DS (James et al., 1999; Hobbs et al., 2000), some of them carried out in Brazilian populations (Grillo et al., 2002; da Silva et al., 2005). The *MTHFR* A1298C variant was associated with the maternal risk for DS when in heterozygosity and in combination with the 677CT genotype (Grillo et al., 2002; Acácio et al., 2005).

Linkage disequilibrium between *MTHFR* C677T and A1298C alleles has been reported (Stegmann et al., 1999; Shi et al., 2003). However, this linkage disequilibrium is not complete, since the presence of some individuals with the haplotype T-C was observed in some studies (Shi et al., 2003; Parle-McDermott et al., 2003; Scala et al., 2006). In our study, in a total sample of 266 women, only one with this haplotype was observed, confirming its negative selection.

Regarding *MTR* A2756G, Bosco et al. (2003) observed an elevated risk for DS in the presence of the *MTR* 2756G allele, in combination with elevated Hcy concentrations. However, the association of this polymorphism with the risk for DS was not confirmed by Chango et al. (2005). Few studies have evaluated the *RFC1* A80G polymorphism in DS mothers. Coppede et al. (2006) showed a role for the 80GG genotype combined with the *MTHFR* 1298AA genotype in the maternal risk for DS in an Italian population. Another study (Chango et al., 2005) found no association between the variant *RFC1* A80G and the risk for DS.

In the present study, even though the isolated analysis of the polymorphisms did not show an association with maternal risk for DS, we observed a 1.74-fold increase in maternal risk for DS in the presence of three or more polymorphic alleles ($P = 0.048$). The higher median number of polymorphic alleles observed in our DS mothers compared to the control mothers corroborates the findings of another Brazilian study (da Silva et al., 2005). The authors of the latter cited study investigated polymorphisms 844ins68 in the cystathionine β-synthase gene and A66G in the methionine synthase reductase gene, in addition to the polymorphisms in genes *MTHFR* and *MTR*, analyzed in our study. They observed a 1.259-fold increase in the chance of having a DS child associated with the presence of each one of the polymorphic alleles studied.

Another factor that may modulate the maternal risk for DS is plasma Hcy concentration. Hcy is an amino acid formed during folate metabolism, and an increased concentration of Hcy, indicative of alterations in this metabolic pathway, has been associated with an elevated risk for DS (James et al., 1999; Takamura et al., 2004; da Silva et al., 2005). Our results confirm this association, even though none of the women in either group showed hyperhomocysteinemia, characterized by a plasma Hcy concentration above 15 μmol/L (American Society of Human Genetics, 1998).

Several studies have shown the contribution of genetic polymorphisms to the increase in plasma Hcy concentrations. The contribution of the *MTHFR* 677T allele is well established (Takamura et al., 2004; da Silva et al., 2005; Ulvik et al., 2007), while the participation of the A1298C polymorphism is still controversial (Weisberg et al., 2001; Castro et al., 2003;

da Silva et al., 2005). Polymorphism *MTR* A2756G has also been associated with variations in Hcy concentrations. Some authors have associated elevated Hcy concentrations with the presence of the wild-type allele (A) (Harmon et al., 1999; Chen et al., 2001; Fillon-Emery et al., 2004), whereas others observed a correlation with the polymorphic allele (G) (Feix et al., 2001; Laraqui et al., 2006). An influence of the *RFC1* A80G polymorphism on Hcy concentrations of healthy individuals has been observed, but only when associated with the *MTHFR* C677T polymorphism (Chango et al., 2000).

Our results show that Hcy concentrations are 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. Martínez-Frías et al. (2006) also observed that Hcy concentrations varied significantly between DS and control mothers in the presence of some genotype combinations of polymorphisms *MTHFR* A1298C and *MTRR* A66G. Our observations may be attributed to the combinations of the *MTHFR* A1298C genotype with genotypes of other polymorphisms and/or to differences in environmental factors between the groups, such as nutritional status (Bailey et al., 2002). In addition, these results may be interpreted as a contribution of the elevated maternal Hcy concentration to the survival of a DS fetus, as proposed by Hobbs et al. (2002). According to these authors, a maternal-fetal genotype interaction of the *MTHFR* C677T polymorphism would provide a favorable balance of folate distribution between both DNA synthesis and cellular methylation in DS individuals, due to the increase in Hcy concentrations. It is possible that other polymorphic alleles involved in folate metabolism produce a similar effect, which could explain our findings.

In light of the results obtained, we conclude that three or more polymorphic alleles, *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *RFC1* A80G, and Hcy concentrations above 4.99 μmol/L are maternal risk factors for DS. This study confirms the association between abnormal folate metabolism and DS in Brazil.

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