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Maternal gene polymorphisms involved in folate metabolism as risk factors for Down syndrome offspring in Southern Brazil

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Abstract. This study aimed to investigate the role of maternal polymorphisms, as well as their risk genotypes combinations of *MTR* A2756G, *MTRR* A66G, *CBS* 844ins68, and *RFC* A80G, involved in folate/homocysteine metabolism, as possible risk factors for Down syndrome (DS) in Southern Brazil. A case-control study was conducted with 239 mothers of DS children and 197 control mothers. The investigation of polymorphisms was performed by PCR and PCR-RFLP. The distribution of genotypic variants was similar in both groups when they were analyzed separately. An investigation of combined risk genotypes showed that the risk of having a DS child for one, two or three risk genotypes was 6.23, 6.96 and 5.84 (95%CI 1.48–26.26; 1.69–28.66; 1.37–24.86), respectively. The combined *MTRR* 66G and *MTHFR* 677T alleles were significantly more common among mothers of children with DS than among control mothers (OR 1.55; IC 95% 1.03–2.35). The results show that individual polymorphisms studied in this work are not associated with DS; however, the effects of the combined risk genotypes among *MTR*, *MTRR*, *CBS* and *RFC* genes are considered maternal risk factors for DS offspring in our population.

Keywords: Down syndrome, folate, MTR, MTRR, CBS, RFC

1. Introduction

First described in 1866, Down syndrome (DS) is the most common genetic cause of mental retardation, with a prevalence of one in 700–800 live-born children [3]. It is characterized by trisomy of chromosome 21, which in 95% of cases is due to non-disjunction in maternal meiosis [20]. The factors responsible for trisomy 21 are under intense investigation, but the only established etiological factor related to chromosome nondisjunction in humans is advanced maternal age at conception [1]. Maternal polymorphisms linked to folate metabolism have been studied as a risk factor for DS. James and colleagues [4] hypothesized that aberrant DNA methylation patterns resulting from abnormal folate metabolism, due to polymorphisms in enzymes involved in this metabolic pathway, may increase hypomethylation of centromeric and pericentromeric regions, affecting non-disjunction rates. Decreased amounts of folate and methyl donor groups may result in DNA hypomethylation, DNA breaks, and abnormal chromosome segregation [4,16].

The folate metabolism begins with the intake of folic acid through diet. It is rapidly reduced to its active form, tetrahydrofolate, which converts to 5,10-methylenetetrahydrofolate by the enzyme methylenetetrahydrofolate reductase, encoded by the *MTHFR* gene [14]. This substrate is vital for nucleic acids metabolism, including those necessary for synthesis of

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nucleotides and consequent cell division [2]. The product of this reaction is methyl groups used for the synthesis of methionine, necessary for DNA methylation. Subsequently, the enzyme methionine synthase, encoded by the *MTR* gene, catalyzes the remethylation of homocysteine to methionine, necessary for the production of S-adenosylmethionine, the universal methyl donor. Vitamin B_{12} acts as a cofactor for methylation [22]. It becomes oxidized over time and the enzyme methionine synthase is inactivated. Functional regeneration of this methionine synthase requires the participation of another enzyme, methionine synthase reductase, which is encoded by the *MTRR* gene [23]. Cystathionine-beta-synthase (*CBS*) acts in the transulfuration of homocysteine to cystathionine, playing a critical role in linking the folate and methionine cycles in regulating homocysteine levels [9]. Another important protein involved in this metabolism is folate-transporting protein (*RFC-1*), transporting 5-methyltetrahydrofolate into the cells [26].

Polymorphisms involved in this metabolism have been studied as maternal risk factors for DS. Conflicting results have emerged in studies when evaluating the association between single polymorphisms in genes involved in folate metabolism and risk for DS. It is possible that the combined presence of two or more polymorphisms in the genome could increase the maternal DS risk [3,6,12,15,24,30,31].

The enzyme 5,10-methylenetetrahydrofolate plays an important role in folate metabolism; some studies have shown that polymorphisms in the *MTHFR* gene could be a factor contributing to increased chance of having a DS child [15,17,25,29,31,32]. Our recent work suggested an association between the C677T *MTHFR* polymorphism and the risk of having a child with DS [7]. In the present study, we have extended our previous work to analyze polymorphisms in genes encoding for enzymes other than *MTHFR* which are also involved in the folate metabolic pathway. We analyzed the role of *MTR* A2756G, *MTRR* A66G, *CBS* 844ins68, and *RFC* A80G polymorphisms as maternal risk factors for DS, as well as their gene-gene combination effect between these polymorphisms and *MTHFR* C677T, previously studied by our group [7].

2. Subjects and methods

2.1. Subjects

All DS cases were identified and ascertained through the Genetics Service of HCPA (Clinical Hospital of Porto Alegre) and local DS support-groups (APAEs). The control group consisted of healthy children's mothers that were randomly selected to participate in the study during blood collection for routine laboratory analyses at HCPA. The case-control study was conducted with 239 case mothers and 197 control mothers, all euro-descendent. More details of case and control selection can be found in a previous study developed by our group [7].

This study was approved by the Research Ethics Committee of HCPA. Informed consent was obtained from all mothers who participated in the study. After signature of informed consent, 5 ml peripheral blood was collected in EDTA tubes for genetic analyses. Samples were collected from June 2005 to May 2007.

2.2. Analysis of polymorphisms

DNA was extracted from blood samples as described by Lahiri and Nurnberger [21]. *MTR* A2756G, *MTRR* A66G, *CBS* 844ins68, and *RFC* A80G polymorphisms were analyzed by polymerase chain reaction (PCR), with primers and protocols already described by Van der Put and cols, Brown and cols, Kluijtmans and cols, Winkelmaver and cols, respectively [8,19,33,34]. The amplified fragments of genes *MTR*, *MTRR* and *RFC* were cleaved with HaeIII, NdeI and CfoI, respectively, and visualized on 6% polyacrylamide gel. The PCR fragments relative to polymorphisms of the *CBS* gene were visualized on 2% agarose gel.

2.3. Use of statistics

Analyses were performed with SPSS 10.0 statistical package for Windows. The Chi-square (χ^2) was used to test for deviation from Hardy-Weinberg equilibrium and to compare allelic and genotypic frequencies between groups. The odds ratio (OR) and 95% confidence intervals (CI) were determined to measure possible effects of having one, two, three, or four risk genotypes. Logistic regression models were used to control the effect of maternal age at the time of having a DS. A dichotomous variable was used (< 35 and $\geqslant 35$ year) because of its stronger effect on Down syndrome prevalence among women over 35 years of age. A Bonferroni correction was applied for multiple testing. The significance level was considered for $p < 0.05$ values.

Table 1
Distribution of genotypic and allelic frequencies in case and control groups

Genotype	Case		Control		p	p*
	n	%	n	%		
*MTR***A2756G**						
AA	159	66.5	130	66	0.890	0.838
AG	71	29.7	61	31		
GG	9	3.8	6	3		
G frequency	0.18		0.19			
*MTRR***A66G**						
AA	42	17.6	42	21.3	0.562	0.271
AG	137	57.3	111	56.3		
GG	60	25.1	44	22.4		
G frequency	0.46		0.49			
*RFC***A80G**						
AA	73	30.5	64	32.5	0.363	0.750
AG	101	42.3	91	46.2		
GG	65	27.2	42	21.3		
G frequency	0.48		0.44			
CBS						
Ins−/−	207	86.6	169	85.8	0.947	0.190
Ins +/−	28	11.7	25	12.7		
Ins +/+	4	1.7	3	1.5		
+ frequency	0.08		0.08			

*p was adjusted for age by logistic regression.

3. Results

A total of 239 case mothers and 197 control mothers participated in the study. Maternal age, as expected, was higher in the case group, with a higher incidence of mothers older than 35 years (case = 121; control = 29; $p = 0.00001$).

Table 1 presents the distribution of genotypes and allelic frequency for *MTR* A2756G, *MTRR* A66G, *CBS* 844ins68, and *RFC* A80G among case and control individuals. The distribution of genotypes for these polymorphisms was in Hardy–Weinberg equilibrium in controls. In the case group, the distribution of genotypes for polymorphisms in genes *MTRR* ($\chi^2_1 = 5.59$; $p = 0.02$), CBS ($\chi^2_1 = 6.03$; $p = 0.01$) and *RFC* ($\chi^2_1 = 5.66$; $p = 0.02$) was not in Hardy–Weinberg equilibrium.

The results did not show any individual association between these polymorphisms and DS. The genotypic frequencies of *MTR* 2756GG, *MTRR* 66GG, *CBS* 844ins68, and *RFC* 80GG polymorphisms were not significantly higher among mothers of DS children (3.8%; 25.1%; 1.7%; 27.2%, respectively) than among controls (3.0%; 22.4%; 1.5%; 21.3%, respectively), even after adjustment for maternal age.

The gene-gene effect was analyzed by the combination of risk genotypes (*MTR* AG+GG and/or *MTRR* AG+GG and/or *RFC* AG+GG and/or *CBS* Ins +/− +/+/+) observed in case and control groups by comparing the presence of one to four risk genotypes with those without any risk genotype. As shown in Table 2, the risk of having a child with DS was 4.62, 5.02, and 4.25-fold higher for case mothers with one, two, or three risk genotypes, respectively, than for control mothers ($p = 0.032$, 0.018 and 0.049, respectively). When combined risk genotypes were adjusted for age, the risk increased to 6.23, 6.96, and 5.84 ($p = 0.130$, 0.001 and 0.02, respectively).

Our previous published results of *MTHFR* C677T genotypes were included in a more complex model that analyzed the combined presence of the *MTHFR* 677T allele and other polymorphic alleles obtained in the present study (Table 3). The results indicated that the simultaneous presence of *MTHFR* 677T and *MTRR* 66G polymorphism conferred a 1.55 fold increased risk for having a child with DS when adjusted for maternal age (95%CI 1.03–2.35).

4. Discussion

Studies on the role of polymorphisms in folate/homocysteine metabolism as a risk factor for DS have obtained controversial results [13,35]. Most of these studies have investigated polymorphisms in the *MTHFR* gene. Little attention has been given to genes responsible for coding other enzymes involved in this metabolic pathway; in addition, it is possible that the

Table 2
Combined risk genotypes analysis of *MTR*, *MTRR*, *RFC*, and *CBS* polymorphisms in case and control groups

Genotype combinations	Case (%)	Control (%)	OR (95%CI)	adjusted OR*
Without any risk genotype	03 (1.25)	11 (5.6)	Ref	Ref
With one risk genotype[a]	63 (26.4)	50 (25.4)	4.62 (1.22–17.46)	6.23 (1.48–26.26)
With two risk genotypes[b]	115 (48.1)	84 (42.6)	5.02 (1.36–18.55)	6.96 (1.69–28.66)
With three risk genotypes[c]	51 (21.3)	44 (22.3)	4.25 (1.11–16.21)	5.84 (1.37–24.86)
With four risk genotypes[d]	07 (3.0)	08 (4.1)	3.21 (0.63–16.38)	4.81 (0.83–28.06)

[a] *MTR* (AG or GG), *MTR*R (AG or GG),*RFC* (AG or GG) or *CBS* (ins +/− or +/+);
[b] *MTR* (AG or GG)+*MTRR* (AG or GG), *MTR* (AG or GG)+*RFC* (AG or GG), *MTR* (AG or GG)+*CBS* (ins +/− or +/+), *MTRR* (AG or GG)+ *RFC* (AG or GG), *MTRR* (AG or GG)+ *CBS* (ins +/− or +/+) or *RFC* (AG or GG)+*CBS* (ins +/− or +/+);
[c] *MTR* (AG or GG)+*MTRR* (AG or GG)+ *RFC* (AG or GG), *MTR* (AG or GG)+*MTRR* (AG or GG)+ *CBS* (ins +/− or +/+) or *MTRR* (AG or GG)+*RFC* (AG or GG)+*CBS* (ins +/− or +/+);
[d] *MTR* (AG or GG)+*MTRR* (AG or GG)+*RFC* (AG or GG)+*CBS* (ins +/− or +/+).
*The OR was adjusted for age by logistic regression.

Table 3
Combined *MTHFR* 677 (CT+TT) genotypes and other gene polymorphisms genotypes

Polymorphisms	*MTHFR*677T: presence of allele T			OR (95%IC)	adjusted OR
	Allele	Genotypic Frequency n (%)			
		Case	Control		
*MTHFR*A1298C	A	198 (82.8)	156 (79.2)	Ref	Ref
	C	41 (17.2)	41 (20.8)	0.79 (0.48–1.27)	0.74 (0.44–1.25)
*MTR*A2756G	A	192 (80.3)	160 (81.2)	Ref	Ref
	G	47 (19.7)	37 (18.8)	1.06 (0.66–1.71)	1.22 (0.73–2.04)
*MTRR*A66G	A	115 (48.5)	86 (43.6)	Ref	Ref
	G	124 (51.5)	111 (56.3)	1.39 (0.95–2.03)	1.55 (1.03–2.35)*
*CBS*844ins68	−	218 (91.2)	180 (91.4)	Ref	Ref
	+	21 (8.8)	17 (8.6)	1.02 (0.52–1.99)	1.03 (0.50–2.11)
*RFC-1*A80G	A	139 (58.1)	126 (63.9)	Ref	Ref
	G	100 (41.8)	71 (36.1)	1.27 (0.87–1.88)	1.40 (0.92–2.13)

*Adjusted $p = 0.03$.

interaction between different polymorphisms may totally modify their individual effects [24].

The present study showed that maternal polymorphisms in genes *MTR*, *MTRR*, *RFC* and *CBS* are not associated with increased risk of DS children when independently analyzed. The combination of one or more of these polymorphisms, however, results in an increased risk for DS, suggesting the existence of a synergistic relationship among them in a multifactorial way. This analysis showed that mothers of DS children have a tendency to present more risk genotypes than mothers without DS children. Furthermore, increasing the number of risk genotypes seems to result in a proportional increase in risk for DS, mainly when adjusted for age. It is important to stress that the size of the sample did not allow for detection of a possible association for mothers with four risk genotypes.

Several studies have analyzed SNPs related to folate metabolism as maternal DS risk factors, yet few of them utilized multivariate analysis to determine if those mutations and their interactions increase the risk for DS. Hobbs and colleagues, in 2000, reported an association between polymorphisms of genes *MTHFR* and *MTRR* as a risk factor for DS when genotypes were analyzed independently (ORs = 1.91 and 2.57; 95%CI 1.19–3.05 and 1.33–4.99) or combined (OR = 4.08; IC 95% = 1.98–8.56) [15]. A similar study showed that risk for DS was higher when polymorphisms of *MTHFR* and *MTRR* genes were combined (OR = 2.98; 95%IC 1.19–7.46) but not for individual polymorphisms [27]. Furthermore, the association between altered folate metabolism and DNA hypomethylation supports the hypothesis that increased frequency of the *MTHFR* and *MTRR* combination observed in this study may be associated with chromosomal nondisjunction and DS. The variants of these enzymes could alter downstream methylation reactions leading to DNA hypomethylation. The reduction of methyl groups could influence chromosomal segregation and lead to the occurrence of trisomy of 21 [21,27]. In the present study we have observed that the same combinations could have different effects between cas-

es and controls, confirming that the interaction between these polymorphisms may modify their individual effect, and that some of these effects may be different in mothers of DS children.

Recent investigations into other polymorphisms in folate/homocysteine metabolism have been conducted. Chango and colleagues [11], analyzed the role of polymorphisms in genes *MTHFR*, *MTR*, *MTRR*, *CBS*, and *RFC*, and did not show statistically significant differences for the distribution of these genotypes among control and case groups. The role of the combined genotypes as risk factors for DS was also investigated, with negative results. The same polymorphisms were also studied by another research group [30], but only for the *MTHFR* and *RFC* genes. The results showed evidence of independent and combined associations as risk factors for DS. Likewise, another study observed an association between the *MTRR* A66G polymorphism as a maternal risk factor for DS (OR 2.21; 95% CI 1.11–4.40) [28].

In Brazil, Silva and colleagues [31] investigated polymorphisms in genes *MTHFR*, *MTR*, *MTRR*, and *CBS* among 154 mothers with DS children and obtained results similar to ours. Only the polymorphism C677T of the *MTHFR* gene was associated with increased risk for DS; yet when the five polymorphisms were combined, a significantly higher number of polymorphic alleles were seen in mothers with DS children than in control mothers, corroborating with our data. Another Brazilian research group analyzed polymorphisms in genes *MTHFR*, *MTR*, *CBS*, *RFC-1*, and *TC* through a case-control study (114 DS mothers) and did not find any influence of these polymorphisms on DS susceptibility. Although our findings are similar to that of Silva and colleagues, some factors may have contributed to the inconsistencies between these Brazilian studies and ours, namely: the smaller sample size, control selection, and ethnic mixture of our study population. Despite these differences, data from these and others studies are important to provide information for meta-analyses to reveal the importance of these polymorphisms, even when they are combined.

Since advanced maternal age is an important risk factor for DS, we also analyzed the genotypic distribution of these polymorphisms according to mothers' age at childbirth (< 35 or $\geqslant 35$ years old), totalizing 118 case mothers and 168 control mothers. The results did not show significant differences in the distribution of genotypes among case and control mothers (data not shown), supporting the adjustment for age results (Table 1). According to Zintzaras [35], maternal age may represent an independent risk factor associated with increased incidence of DS. The lower frequency of DS in children of younger mothers, however, does not exclude the possibility that they may have a DS child when they are older. A meta-analysis considering the role of age in the association of polymorphisms of genes involved in folate metabolism may provide more conclusive results.

Some limitations of our study must be acknowledged. First, our study population was restricted to white women because of known differences in the distribution of genotypes according to ethnicity [11,15, 27]. Thus, our findings may not be generalized to women of other ethnicities or populations given the evidence of discordant conclusions in studies of different geographic regions. The discrepant results observed in different populations may also reflect the interactions existing between genetic and environmental factors implicated in folate metabolism [18]. Second, gene-environment interactions were not analyzed in this study. Risk factors may depend on genetic polymorphisms or on the gene-environment interaction represented by genotypes and dietary habits, in particular on the use of folic acid, which may be crucial for maintaining the effects of these polymorphisms [24]. Thus, the meaning of *MTHFR* and *MTRR* genotypes interaction found in this study in terms of specific metabolic biomarkers such as plasma homocysteine, folate, and B_{12} levels would increase the power to detect its significant impact on DS risk.

Based on these results we can conclude that: a) The polymorphisms studied cannot be considered as risk factors by themselves, corroborating with the multifactorial trait concerning the etiology of human nondisjunction. The current opinion is that the presence of a single polymorphism of a gene participating in folate metabolism would be insufficient to increase the risk of having a child with DS [3,6,12,15,24,30,31]. b) Our results are consistent with other studies showing an association between combined gene polymorphisms involved in folate metabolism and risk for DS; the combined presence of two or more polymorphisms in the genome increase the risk of DS in our population, showing the importance of their gene-gene interaction. c) An interaction of MTHFR and MTRR polymorphism is evident in our study; it is plausible since both genes work in the methylation pathway, disestablishing it and contributing to nondisjunction events. d) Data presented here will be important for future pooled analysis and meta-analysis to test additional gene-gene interactions with larger sample sizes and greater sta-

tistical power. Most reports are based on small samples. It is important to stress that the number of DS mothers included in the present study, with correspondent ethnic and socio-demographic background in case and control groups, is, to our knowledge, the largest reported until now. More rigorous studies investigating combinations between genetic and environmental factors are needed to establish the role of polymorphisms in folate/homocysteine metabolism genes as risk factor for non-disjunction.

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