Disease Markers 26 (2009) 155–161
DOI 10.3233/DMA-2009-0626
IOS Press

Investigation of *CBS*, *MTR*, *RFC*-1 and *TC* polymorphisms as maternal risk factors for Down syndrome

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Abstract. Recent evidence shows that almost 92% of the DS children are born from young mothers, suggesting that other risk factors than advanced maternal age must be involved. In this context, some studies demonstrated a possible link between DS and maternal polymorphisms in genes involved in folate metabolism. These polymorphisms, as well as low intake of folate could generate genomic instability, DNA hypomethylation and abnormal segregation, leading to trisomy 21. We compared the frequency of *CBS* 844ins68, *MTR* 2756A>G, *RFC*-1 80G>A and *TC* 776C>G polymorphisms among 114 case mothers and 110 matched controls, in order to observe whether these variants act as risk factors for DS. The genotype distributions revealed that there were not significant differences between both samples. However, when we proceed the multiplicative interaction analyses between the four polymorphisms described above together with the previously studied *MTHFR* 677C>T, *MTHFR* 1298A>C and *MTRR* 66A>G polymorphisms, our results show that the combined genotype *TC* 776CC / *MTHFR* 677TT and *TC* 776CC / *MTR* 2756AG were significantly higher in the control sample. Nevertheless, there was no significant association after Bonferroni correction. Our results suggest that maternal folate-related polymorphisms studied here have no influence on trisomy 21 susceptibility in subjects of Brazilian population.

Keywords: Down syndrome, folate, polymorphisms, *TC*

1. Introduction

Trisomy 21 is the most common genetic cause of mental retardation and occurs with a prevalence of 1 in 800 live births [15]. Despite the fact that Down syndrome (DS) was described almost 150 years ago, little is known about the mechanisms that lead to trisomy of chromosome 21. Several reasons have contributed to the difficulty of understanding the pathogenesis of DS, including the nature of the genetic defect that leads to the syndrome, the multiplicity of systems involved and the high degree of phenotype variability [1].

In 95% of cases, DS is caused by a meiotic error occurring at meiosis I or II, mainly of maternal origin [7]. Despite the fact that advanced maternal age (>35 years) is a major risk factor for DS, recent evidence shows that almost 92% of children with this disease are born from mothers at a young age (<35 years) [12,25]. This fact suggests the existence of other predisposition factors in these mothers than the aging of their eggs.

Folate plays an important role in complex and essential metabolic pathways, as biosynthesis of nucleotides, DNA repair and cellular methylation reactions (Fig. 1) [11]. The donation of carbon atoms for maintenance of DNA methylation patterns involves an integrated action of many gene products and other important micronutrients also obtained through the diet, like vitamin B12, vitamin B6, zinc and methionine. All these elements are directly or indirectly required for the conversion of homocysteine to methionine, which is the immediate precursor of S-adenosyl methionine (SAM),

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ISSN 0278-0240/09/$17.00

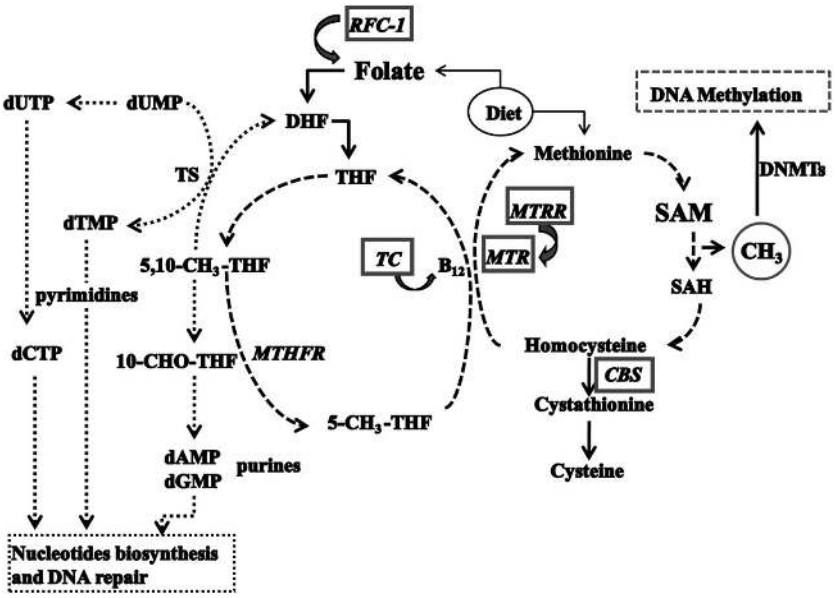

Fig. 1. Folate metabolism, illustrating the essential metabolic pathways, such as biosynthesis of nucleotides, used in the synthesis and repair of DNA and cellular methylation reactions.

the major intracellular methyl donor [29]. The presence of genetic polymorphisms that modify the functionality of key molecules and enzymes required for the folate cycle steps could predispose to abnormal DNA methylation, DNA strand breaks, altered chromosome recombination and aberrant chromosome segregation [22,36]. Therefore, it is clear that the effects of these variants will be closely dependent on the individual folate nutritional status, as a high intake of folate and associated nutrients effectively could modulate the negative biochemical consequences of the polymorphisms [27].

On the basis of this evidence, James and colleagues [17] suggested the possibility that gene-nutrient interactions associated with abnormal folate metabolism and DNA hypomethylation might increase the risk of chromosome non-disjunction leading to DS. The most common folate pathway genes studied in different populations include the 5-methylenetetrahydrofolate reductase gene (*MTHFR*), whose product catalyzes the synthesis of methyltetrahydrofolate, the methyl donor of homocysteine, and methionine synthase reductase gene (*MTRR*), which codifies an enzyme that reduces the cobalt of cobalamin, maintaining methionine synthase in an active state and making possible the folate and vitamin B12 dependent synthesis of methionine by remethylation of homocysteine [20]. We investigated the relationship of cystathionine-beta-synthase (*CBS*) 844ins68, methionine synthase (*MTR*) 2756A>G, reduced folate carrier (*RFC*-1) 80G>A and transcobalamin (*TC*) 776C>G polymorphisms on the risk of having a DS child, as well as, the interaction between these polymorphisms and the *MTHFR* 677C>T, *MTHFR* 1298A>C and *MTRR* 66A>G polymorphisms, previously studied by our group [23].

2. Materials and methods

Peripheral blood samples were collected from 114 mothers (18–35 years during conception) of full trisomy 21 children and 110 age-matched mothers who had children with normal chromosomes and no history of abnormal pregnancies or miscarriages. All the selected women lived in Rio de Janeiro (Brazil) and descended from European (43.5%), African (15%), Amerindians (1.5%) or are ethically mixed (Brazilian mulattos, 40%). The regional Ethics Committee previously approved all the research protocols.

Genomic DNA was isolated from peripheral blood by GFX Genomic Blood DNA purification Kit (GE Healthcare) and analyzed by polymerase chain reaction. For *CBS* 844ins68 genotype, analysis conditions were performed according to Tsai and colleagues [28], whereas the *MTR* 2756 and *RFC*-1 80 genotypes were evaluated by PCR-RFLP according to the protocols described by van der Put et al. [31] and Winkelmayer et al. [33], respectively. The digested PCR products were

Table 1
Genotype and allele frequencies, odds ratios and p value of *CBS* 844ins68, *MTR* 2756A> G, *RFC*-1 80G> A and *TC* 776C> G polymorphisms in case and control mothers

Polymorphism	Genotypes	Case ($n = 114$) n [%]	Control ($n = 110$) n [%]	Odds ratio (CI 95%)	p value
CBS 844ins68	SS	80 [70.2]	85 [77.3]	1.0	reference
	SP	31 [27.2]	21 [19.1]	0.64 (0.34–1.20)	0.20
	PP	3 [2.6]	4 [3.5]	1.26 (0.27–5.79)	1.00
	SS+SP	34 [29.8]	25 [22.7]	0.69 (0.38–1.26)	0.29
	Frequency of ins68	0,16	0,13	1.38(0.82–2.32)	0.24
MTR 2756A>G	AA	79 [69.3]	71 [64.6]	1.0	reference
	AG	28 [24.6]	37 [33.6]	1.47 (0.82–2.64)	0.24
	GG	7 [6.1]	2 [1.8]	0.32 (0.06–1.58)	0.18
	AG+GG	35 [30.7]	39 [35.5]	1.24 (0.71–2.17)	0.48
	Frequency of G	0,18	0,19	1.01(0.63–1.63)	1.00
RFC-1 80G>A	GG	25 [21.9]	26 [23.6]	1.0	reference
	GA	64 [56.2]	55 [50]	1.12 (0.59–2.14)	0.74
	AA	25 [21.9]	29 [26.4]	0.76 (0.35–1.65)	0.56
	GA+AA	89 [78.1]	84 [7.8]	1.02 (0.55–1.89)	1.00
	Frequency of A	0,50	0,51	0.95(0.65–0.37)	0.78
TC 776C>G	CC	46 [40.4]	39 [35.5]	1.0	reference
	CG	51 [44.7]	48 [43.6]	1.11 (0.62–1.98)	0.77
	GG	17 [14.9]	23 [20.9]	1.60 (0.75–3.41)	0.25
	CG+GG	68 [59.6]	71 [64.5]	1.23 (0.72–2.12)	0.49
	Frequency of G	0,37	0,43	0.96 (0.66–1.39)	0.85

SS: wild homozygote without the insertion of 68 bp; SP: heterozygote; PP: polymorphic homozygote with the insertion of 68 bp.

visualized by 6% non-denaturing polyacrylamide gels stained by silver. *TC* 776C>G polymorphism analysis was performed by an amplification-refractory mutation system (PCR-ARMS), according to the method of Namour et al. [19]. For quality control procedures, a negative control reaction (all reagents except DNA) was processed for each PCR and a positive control reaction (DNA from an individual with a known genotype susceptible to endonuclease cleavage) was used for restriction reactions.

Hardy-Weinberg equilibrium for each polymorphism in cases and controls was tested by χ^2 test. Statistical comparisons were performed using the Graph Pad Instat Software (version 3.0, San Diego, USA). Allele frequencies were calculated for each genotype and the differences between case and control mothers were determined using the χ^2 test. Odds ratios were calculated for each genotype separately and for potential intragenic or gene-gene interactions. The relationship between the number of polymorphic alleles for the seven (*CBS* 844ins68, *MTR* 2756A>G, *RFC*-1 80G>A, *TC* 776C>G, *MTHFR* 677C>T, *MTHFR* 1298A>C and *MTRR* 66A>G) loci tested per case or control mother and the maternal risk for DS was analyzed by the Mann-Whitney test followed by logistic regression analyses. A Bonferroni correction was applied to account for multiple testing.

3. Results

The average age of case mothers when given birth to babies with DS was 28.36 ± 6.06 years compared with 29.54 ± 5.6 years for control mothers. The distributions of *CBS*, *MTR*, *RFC*-1 and *TC* genotypes in both populations were found to be in Hardy-Weinberg equilibrium.

Allele and genotype frequencies of *CBS* 844ins68, *MTR* 2756A>G, *RFC*-1 80G>A and *TC* 776C>G genotypes were similar among DS and control groups, even in the combination of heterozygous and homozygous variant genotypes (Table 1). However, the multiplicative interaction analysis between the four polymorphisms described above together with the previous studied *MTHFR* 677C>T, *MTHFR* 1298A>C and *MTRR* 66A>G polymorphisms [23] showed that the combined genotypes *TC* 776CC / *MTHFR* 677TT ($p = 0.038$) and *TC* 776CC / *MTR* 2756AG ($p = 0.03$) were significantly higher in control sample. However, neither association retained statistical significance after Bonferroni correction ($p = 0.002$; Table 2).

4. Discussion

The focus of this study was to identify risk factors for DS in mothers who had an affected child, since meiotic

Table 2
Gene-gene interaction analysis for *MTHFR*677/*TC*776 and *MTR*2756/*TC*776 in case and control mothers, odds ratio and p values

	Genotype interactions	Case mothers ($n = 114$)	Control mothers ($n = 110$)	Odds ratio (CI 95%)	p value
MTHFR 677 /	677CC/776CC	26	18	reference	–
TC 776	677CT/776CC	19	19	1.44 (0.60–3.47)	0.51
	677TT/776CC	0	4	12.89 (0.65-254.38)	0.038*
	677CT+TT/776CC	19	23	1.75 (0.74–4.11)	0.28
	677CC/776CG	21	21	1.44 (0.62–3.39)	0.52
	677CC/776GG	6	10	2.41 (0.74–7.81)	0.16
	677CC/776CG+GG	27	31	1.66 (0.75–3.66)	0.23
	677CT/776CG	21	21	1.44 (0.62–3.39)	0.52
	677CT/776GG	9	8	1.28 (0.42–3.96)	0.78
	677TT/776CG	7	5	1.03 (0.28–3.77)	1.00
	677CT+TT/776CG+GG	39	38	1.41 (0.67–2.98)	0.45
	677CC+CT/776CC	45	37	1.19 (0.57–2.49)	0.71
	677CC+CT/776CG	42	42	1.44 (0.69–3.02)	0.36
	677CC+CT/776GG	15	18	1.73 (0.70–4.31)	0.26
	677CC+CT/776CC+CG	87	79	1.48 (0.76–2.89)	0.31
MTR 2756 /	2756AA/776CC	36	24	reference	–
TC 776	2756AG/776CC	7	15	3.21 (1.14–9.05)	0.03*
	2756GG/776CC	3	1	0.50 (0.05–5.10)	1.00
	2756AG+GG/776CC	10	16	2.40 (0.93–6.17)	0.098
	2756AA/776CG	30	31	1.55 (0.75–3.19)	0.28
	2756AA/776GG	13	16	1.85 (0.75–4.52)	0.26
	2756AA/776CG+GG	43	47	1.64 (0.85–3.18)	0.18
	2756AG/776CG	18	16	1.33 (0.57–3.12)	0.52
	2756AG/776GG	3	6	3.00 (0.68–13.17)	0.16
	2756GG/776CG	3	0	0.21 (0.01–4.31)	0.28
	2756AG+GG/776CG+GG	25	23	1.38 (0.64–2.97)	0.44
	2756AA+AG/776CC	43	39	1.36 (0.69–2.67)	0.40
	2756AA+AG/776CG	48	48	1.50 (0.78–2.88)	0.25
	2756AA+AG/776GG	16	22	2.06 (0.90–4.71)	0.10
	2756AA+AG/776CC+CG	91	86	1.42 (0.78–2.57)	0.30

*Bonferroni-corrected p values = 0.002. The sum of different combinations differs from the total number of patients/controls due to amplification fails. Combinations not found were not demonstrated.

non-disjunction is of maternal origin in 95% of the cases. Our results did not show an individual or combined association of *CBS* 844ins68, *MTR* 2756A>G, *RFC*-1 80G>A and *TC* 776C>G, *MTHFR* 677C>T, *MTHFR* 1298A>C and *MTRR* 66A>G polymorphisms with the increased risk of non-disjunction of chromosome 21 and the emergence of DS.

To our knowledge, this is the second study that has analyzed the relationship between the 776C>G polymorphism in the transcobalamin gene (*TC*) and the birth risk of a DS child. Transcobalamin is a plasma protein that transports vitamin B12 to cells [5]. The polymorphism *TC* 776C>G results in the substitution of proline by arginine in codon 259 [13,19] and the allele 776G is associated with reduced levels of *TC* transcription, as well as changes in the protein conformation, which seems to affect affinity of the enzyme receiver for vitamin B12 [14]. In cell, vitamin B12 acts as a co-factor for a subset of enzymes, such as MTR [30] which is responsible for homocysteine to methionine remethylation. So, the lack of vitamin B12 can result in increased plasma homocysteine and, consequently, reduction in SAM production from methionine, which could lead to the DNA hypomethylation [32]. Recently, another Brazilian group (São Paulo City) failed to find an association between *TC* 776 C>G polymorphism and the maternal risk of bearing a DS child in a cross-sectional study with 67 case mothers [3]. Nevertheless, in contrast to our study, 41.8% of the women in their case group have more than 35 years and the mean maternal age was significantly lower in the control sample.

Vitamin B12 can be found in liver, kidneys, milk, eggs, fish, cheese and meat. As previously published by our group [23], an estimative of vitamin B12 consumption through a food-frequency questionnaire revealed no differences in the medians of intake between our DS and control mothers and both were in agreement with the recommended daily amount. However, due

to the absence of additional biochemical data and the small number of individuals per each transcobalamin genotype, the stratification analysis of vitamin B12 by genotype was not performed to avoid bias [23].

Despite the growing number of studies focusing folate-related polymorphisms, few of them have made efforts to understand the influence of *MTR, CBS* and *RFC*-1 polymorphisms on the maternal risk of bearing a DS child and the results are contradictory [2,3, 8–10,24]. *MTR* catalyzes the remethylation of homocysteine to methionine, the precursor of the methyl-donor SAM. *MTR* 2756A>G polymorphism changes an aspartic acid residue to a glycine [16] and was just taken as a significant individual risk factor for having a DS child in Sicilian DS mothers in addition to the double heterozygosity *MTR* 2756AG/*MTRR* 66AG genotypes [4]. It is still not clear whether the *MTR* 2756A>G polymorphism has functional consequences for enzyme activity. Since the variant is located in a potentially functional domain of the enzyme, it was hypothesized that the *MTR* 2756A>G variant might increase homocysteine levels [4].

On the other hand, *CBS* is a key enzyme in the homocysteine to cystathionine transsulfuration step that plays a critical role in linking the folate and methionine cycles and in regulating homocysteine levels [6]. Recent evidence has demonstrated that *CBS* 844ins68 has no statistically significant effects on homocysteine and folate concentrations, but it appears to counterbalance the homocysteine-raising and folate-lowering effects of the *MTHFR* 677TT genotype, making it similar to that observed in *MTHFR* 677CT or CC individuals [26]. Therefore, like ours, until now none of the studies that investigated this polymorphism founded any independent or combined association with DS risk [8,10,24].

Finally, reduced folate carrier (*RFC*-1) is responsible for folate uptake from jejunum and the subsequent translocation of this vitamin across biological membranes in a variety of cells [18]. *RFC*-1 80G>A polymorphism leads to the replacement of a histidine by an arginine and has impact on folate status [34]. In Italy, in which folate-related polymorphisms had not been previously associated to trisomy 21 probably because of the Mediterranean enriched-folate diet [8], *RFC*-1 80G>A polymorphism was lately borderline associated to DS when combined to *MTHFR* genotypes (RFC-1 80GG/MTHFR 677TT) [9]. Also, the *RFC*-1 80 (GA or AA)/*MTHFR* 1298AA genotype was inversely associated with the risk of bearing a DS child [9]. Moreover, in a further Italian study, mothers older than 34 years with *RFC*-1 80G allele had a 2-fold increased risk of having a DS child [24]. Using the same statistical approach of the previous study [9], the results of this latter research were similar, indicating that the combination of the *RFC*-1 80G allele with the *MTHFR* 1298C allele increases DS risk, whereas the combination of the 1298A and 80A alleles (at one or two loci) could be protective [9].

Conversely to other studies focusing on folate-related polymorphisms in predominantly Caucasian populations [4,8,9,24], the Brazilian ethnic background is mainly characterized by extensive genetic admixture of Europeans, African immigrants and native Indigenous descents during five centuries of interethnic crosses [21]. Even though, we observed that our allele and genotype frequencies were quite similar to those seen from other groups [4,8,9,24]. In Brazil, two previous studies carried out in São Paulo City focused the issues of maternal folate pathway polymorphisms and risk of DS. Da Silva and colleagues and Biselli and colleagues showed a higher median number of polymorphic alleles in DS mothers compared to controls, but only da Silva and colleagues found an independent association of folate polymorphisms to DS (*MTHFR* 677T). Although the allele frequencies found by ours are close to those seen in São Paulo studies, we did not support the evidence of a greater number of polymorphic alleles per individual in our case mothers from Rio de Janeiro city. However, it must be also emphasized that the studies of da Silva and colleagues and Biselli and colleagues included a percentage of women over 35 years-old.

The discordant results found in distinct geographic populations may be explained by a range of factors, including distinct sizing of the samples, differences in case and control matching criteria (mainly mother's age at conception), as well as the differential contribution of geographic environmental factors, such as social and nutritional habits. In this sense, alcohol ingestion, which has not been evaluated in almost all the studies, has been shown to cleave folate, impair folate absorption, increase folate excretion, and interfere with methionine synthase activity [33]. Besides, extensive losses of folate can occur during cooking and preparation of foods, which may considerably reduce the amount of folate ingested by 50–80% in green vegetables after boiling [20]. In view of the complexity of the folate metabolism and the factors mentioned above, our results, as well as those of previous studies, should not be regarded as definitive. Although the studies performed until now in specific populations are of extreme importance for the understanding of potential interactions, it is consensus [9] that the results found so far in different

populations need to be analyzed together in order to clarify the real contribution of maternal gene polymorphisms reducing the folate bioavailability in the development of trisomy of chromosome 21. Besides, a recent study by Young and colleagues [35] showed that satisfactory intake of folate by non-smoking men is capable of reducing the probability of several aneuploidies, including the non-disjunction of chromosome 21. Thus, we urge the settling of an international consortium to analyze the importance of polymorphisms influencing folate metabolism and their relationship with the non-disjunction of chromosome 21 during female gametogenesis. Besides, the new generated data could serve as base to investigate this relationship also in spermatogenesis.

Acknowledgements

The authors thank the DS and control mothers who kindly participated in this study. This work was public funded by FAPERJ (E-26/171.288/2004; E-26/170.217/2006), CNPq and CEPUERJ.

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