PRENATAL DIAGNOSIS
Prenat Diagn 2005; **25**: 1196–1199.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/pd.1296

Methylenetetrahydrofolate reductase gene polymorphisms and their association with trisomy 21

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Objectives To verify whether the frequencies of 5,10-methylenotetrahydrofolate reductase (MTHFR) polymorphisms at positions 677 and 1298 are higher in women with children affected by trisomy 21 than in those with chromosomally normal offspring.

Methods A case-control study was carried out with 70 women whose children had trisomy 21 and 88 controls whose children were chromosomally normal. The frequencies of polymorphisms of points C677T and A1298C of MTHFR gene coding were studied in these two groups. Odds ratios (OR) for having a child affected by trisomy 21 were estimated for homozygosis, heterozygosis or the absence of the above-mentioned MTHFR polymorphisms. Logistic regression models were used to control for the effect of confounding variables on these odds ratios.

Results The frequency of joint heterozygotic polymorphism (677 and 1298) was significantly higher in women with children affected by trisomy 21 than in those with chromosomally normal offspring (OR: 5.7).

Conclusions The presence of joint heterozygotic polymorphism in the codifying gene for MTHFR was a risk factor for having a child with trisomy 21. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: chromosomal abnormalities; DNA polymorphisms; folic acid; Down syndrome; MTHFR

INTRODUCTION

Trisomy 21 is the most frequent chromosomal disorder in newborns. In the majority of cases (95%), it is the result of an extra chromosome originating from maternal meiotic nondisjunction during meiosis I (Thompson *et al*., 1993). Although common, the cellular and molecular mechanisms involved in this meiotic nondisjunction remain unknown. Folic acid ingestion and polymorphisms on genes coding for enzymes involved in its metabolism are thought to interfere with meiotic disjunction. Folic acid, like other vitamins, is necessary in small quantities. Current knowledge suggests that individuals who carry these genetic variants require extra folic acid to overcome the inefficiency of the mutated enzyme (Chaney, 1998). Several studies indicate the relationship between a deficiency in folic acid and certain types of colon cancer, leukemia, myeloproliferative diseases, certain chronic skin conditions and other chronic debilitating diseases (Czeize and Dudas, 1992; Chaney, 1998).

One of the enzymes involved in the metabolism of folic acid is 5,10-methylenetetrahydrofolate reductase (MTHFR), which is essential to the biochemical process that results in production of the methyl groups required for DNA methylation. The gene codifying for MTHFR in humans is located in the short arm of chromosome 1, position 1p36.3, and it contains 11 exons (Goyette *et al*., 1994). The most common MTHFR gene polymorphisms reported are C677T (Frosst *et al*., 1995) and A1298C (Van der Put *et al*., 1998). These polymorphisms represent a risk factor for the birth of children with neural tube closure defects (NTDs) (Bjørke-Monsen *et al*., 1997; Eskes, 1997; Van der Put *et al*., 1998; Botto and Yang, 2000; Wenstrom *et al*., 2000). Other studies have correlated C677T polymorphism with the occurrence of facial clefts, cardiac malformation (Wenstrom *et al*., 2001), recurrent abortions (Wouters *et al*., 1993; Couto *et al*., 2005) and limb defects (Shashi *et al*., 2001). The isolated A1298C MTHFR gene polymorphism has not been associated with increased risk for NTDs, although combined heterozygosis for both polymorphisms (C677T and A1298C) has an effect equivalent to that found in patients with C677T homozygosis (Van der Put *et al*., 1998).

Some articles have suggested that chromosomal instabilities and aneuploidies observed in human tumors are related to genomic DNA hypomethylation (Rosenblatt and Erbe, 1977; Pogribny *et al*., 1997). Hypothesizing that DNA hypomethylation may interfere with chromosomal segregation, James *et al*. (1999) compared the frequency of MTHFR C677T polymorphism in mothers of Down syndrome children with those in a control group. These authors observed a higher frequency of MTHFR 677 polymorphism in the study group, increasing the odds of having a child affected by trisomy 21 by a factor of 2.6. The objective of this study was to

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Received: 1 February 2005
Revised: 19 July 2005
Accepted: 20 July 2005

evaluate the frequency of 677 and 1298 polymorphisms of the MTHFR gene and to verify whether the occurrence of these two polymorphisms is associated with trisomy 21.

METHODS

This is a case-control study. Sample size was calculated on the basis of the case-control study carried out by James *et al*. (1999), in which the odds ratio found was calculated at 2.6. Using the 95% confidence coefficient ($\alpha = 5\%$) 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 70 cases and 70 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 and ethnic group (white or nonwhite) 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. These were studied by polymerase chain reaction (PCR) (Saiki *et al*., 1989) followed by specific enzymatic digestion of DNA extracted from peripheral maternal blood (Woodhead *et al*., 1986). Amplification of the MTHFR gene fragment was based on the PCR technique (Saiki, 1989).

677 C → T polymorphism

Specific sense primers (5′-TGAAGGAGAAGGTGTCT GCGGGA-3′) and antisense primers (5′-AGGACGGTG CGGTGAGAGTG-3′) promoted the amplification of a fragment of 198 bp. The reaction was carried out using 54 mM Tris-HCl, 5.4 mM $MgCl_2$, 13.3 mM $(NH_4)2SO_4$, 0.8 mM of each triphosphate nucleoside, 400 ng of each primer, genomic DNA and 2U of Taq Polymerase, involving 30 incubation cycles at 94 °C (1 min), 55 °C (1 min) and 72 °C (2 min). Using 15 μL of the PCR product in a digestion reaction with 0.5 U of Hinf I, electrophoresis was then performed in 7% polyacrylamide gel stained with ethidium bromide, in which the polymorphic gene was split into two fragments (175 bp and 23 bp) and the normal allele remained at 198 bp.

1298 A → C polymorphism

This polymorphism destroys an MboII restriction site. The primers used were sense (5′-CTTTGGGGAGCTG AAGGACTACTA-3′) and antisense (5′-CACTTTGTGA CCATTCCGGTTTG-3′). The reaction was obtained using 200 uM dNTP, 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$ and 1 unit of Taq Polymerase with initial denaturation at 92 °C (2 min), followed by 35 cycles of 92 °C (60 s), 51 °C (60 s) and 72 °C (30 s). The final extension was performed at 72 °C (7 min). The amplified fragment is 163 bp and when it contains the 1298 polymorphism, it is digested by MboII endonuclease and split into four fragments of 84, 31, 30 and 18 bp (Van der Put *et al*., 1998). The normal allele is split into five fragments of 56, 31, 30, 28 and 18 bp. The analysis of these fragments is carried out following staining with ethidium bromide using electrophoresis in 20% polyacrylamide gel.

Age and ethnic group were described in the two groups and both were studied to verify whether they were in Hardy–Weinberg equilibrium (Beiguelman, 1994) concerning genotypical distribution of MTHFR, the gene coding in positions 677 and 1298. Odds ratios (OR) for having a child affected by trisomy 21 were estimated (with a 95% confidence interval) for homozygosis, heterozygosis or absence of the above-mentioned MTHFR polymorphisms. Logistical regression models were used to control the effect of age and ethnic group as well as to describe odds ratios generated by interaction of variables. Statistical analysis was carried out using SAS 8.0 statistical software package (SAS Institute Inc, Cary, NC, USA, 1999). The study was approved by the State University of Campinas Ethics Committee and a written informed consent was obtained from all participants.

RESULTS

A total of 158 women were enrolled in the study, divided into 70 cases and 88 controls. Maternal age was 25.3 years in cases and 31.3 years in controls ($p < 0.05$). The control group contained 51 white and 37 non-white women while there were 56 whites and 14 nonwhites in the cases group.

Both groups were in Hardy–Weinberg equilibrium for genotypical distribution of MTHFR. The frequencies of isolated MTHFR polymorphisms either at position 677 or 1298 (Table 1) were not significantly higher in cases than in controls. The heterozygotic polymorphism at both positions (677 and 1298) was significantly more frequent in cases (27.1%) than in controls (5.7%), with an odds ratio (OR) of 5.70 (CI 1.73–18.83) (Table 1).

Adjusted OR (heterozygotic polymorphism at both positions) for ethnic group, maternal age and categorized age (<35 years) was, respectively, 6.07, 8.91 and 9.00 (Table 2).

DISCUSSION

This study found that the concomitant presence of C677T and A1298C polymorphisms was associated with a higher risk of having a child with trisomy 21. These data support the hypothesis that mutations in genes involved in the metabolism of folic acid represent an independent risk factor for chromosomal nondysjunction (James *et al*., 1999).

Table 1—Distribution of the different genotypes of the codifying genes for MTHFR at positions 677 and 1298 between cases and controls

MTHFR genotype			Case		Control			
677		1298	*n*	%	*N*	%	OR	(CI 95%)
0	and	0	14	20.0	21	23.9	Reference	—
0	and	1	18	25.7	27	30.7	1.00	(0.41–2.46)
0	and	2	3	4.3	6	6.8	0.75	(0.16–3.51)
1	and	0	11	15.7	20	22.7	0.82	(0.30–2.24)
1	and	1	19	27.1	5	5.7	5.70	(1.73–18.83)
2	and	0	5	7.1	9	10.2	0.83	(0.23–3.01)
Total			70	100.0	88	100.0	—	—

Cases and control were in Hardy–Weinberg equilibrium.
Fischer's Exact Test, $p = 0.0131$.
0 = Normal homozygote; 1 = Mutant heterozygote; 2 = Mutant homozygote.

Table 2—Distribution of the genotypes of genes codifying for MTHFR at positions 677 and 1298 in their compound heterozygote form between cases and controls following different adjustments

MTHFR genotype			Case		Control			
677		1298	*n*	%	*n*	%	Adjusted OR	(CI 95%)
1	and	1	19	27.1	5	5.7	6.07 (race)	(1.75–21.12)
1	and	1	19	27.1	5	5.7	8.91(age)	(2.49–31.93)
1	and	1	15	32.6	5	6.3	9.00 (<35 years)	(2.29–35.43)

1 = Mutant Heterozygote.

The studied groups comprised young patients in whom the risk of trisomy 21 was lower and causal factors other than age could be studied. Combined heterozygosis for C677T and A1298C polymorphisms is not infrequent. In this study, this was found in 5.7% of control and 27.1% of cases. In other populations, this combination was found in 15% of Canadians (Weisberg *et al*., 1998), 17% of North Americans (Trembath *et al*., 1999) and 20% of Dutch (Van der Put *et al*., 1998).

The first report on the association of the MTHFR polymorphism in the 677 and 1298 positions and trisomy 21 is a previous note of this study (Grillo *et al*., 2002).

The fact that both polymorphisms were needed for increasing the risk of Down syndrome in this study suggests that there is an underlying synergy between these two polymorphisms.

In fact, the presence of more than one mutation in genes involved in folic acid metabolism is known to considerably increase the association with the occurrence of NTDs. In a case-control study, the presence of combined C677T and beta-synthetase mutation increased five times the background risk of NTDs compared with two times for C677T alone (Botto and Mastroiacovo, 1998).

Likewise, in a study exploring the association between mutations in two genes and Down syndrome, the combined presence of two polymorphisms (C677T in MTHFR and A66G in methionine synthetase reductase) was associated with a greater risk of Down syndrome (odds ratio 4.08) than the presence of either alone (Hobbs *et al*., 2000). Further evidence to support the hypothesis of MTHFR polymorphisms as an independent risk factor for chromosomal nondysjunction is that a higher than expected prevalence of Down syndrome was found in families with a history of NTD (Barkai *et al*., 2003). Conversely, a higher than expected incidence of NTDs was found in families with a history of Down syndrome (Barkai *et al*., 2003). This reinforces the hypothesis that changes in the metabolism of folic acid may be involved in the etiology of both conditions. In conclusion, this study suggests that the association of the two polymorphisms studied substantially increased the risk of trisomy 21. Further studies are needed to confirm these findings and to address if folic acid supplementation would be beneficial, as it is for NTDs.

ACKNOWLEDGEMENTS

This project was partially supported by Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP), Brazil—Grant number 98/12403-0. We thank Javier Miguelez for his linguistic revision of the manuscript.

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