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**Patrizia De Marco · Maria Grazia Calevo · Anna Moroni
Lorenza Arata · Elisa Merello · Richard H. Finnell
Huiping Zhu · Luciano Andreussi · Armando Cama
Valeria Capra**

Study of MTHFR and MS polymorphisms as risk factors for NTD in the Italian population

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Abstract Homozygosity for the C677T mutation in the methylenetetrahydrofolate reductase (*MTHFR*) gene is a risk factor for neural tube defects (NTDs) in many populations, including Italians. Another common mutation on the *MTHFR* gene, A1298C, has also been described as a risk mutation. Furthermore, several studies have suggested that a defective methionine synthase (*MS*) enzyme could be a critical defect in folate-related NTDs. An A-to-G transition at bp 2756 on the *MS* gene has also been reported. In this case-control study, we studied the frequencies of these two polymorphisms in 203 Italian probands with non-syndromic NTDs: 98 mothers, 67 fathers, and 210 control individuals. Although the A1298C polymorphism is common in the Italian population (0.25), the allelic frequency was significantly higher among NTD cases and their parents. Heterozygous patients and mothers have an odds ratio (OR) of 1.98 and 2.11, respectively. The risk associated with the 1298CC genotype was higher for cases (OR = 3.67), for fathers (OR = 3.28), and, above all, for mothers (OR = 6.23). The prevalence of the A2756G polymorphism of the *MS* gene was determined (0.15). No increased prevalence of the mutated G allele was found in NTD families. This study shows that the *MTHFR* A1298C polymorphism is a genetic determinant for NTD risk in Italy. No association between the *MS* A2756G and NTD susceptibility was found.

Key words Neural tube defects · *MTHFR* gene · *MTHFR* A1298C · *MS* gene · *MS* A2756G

P. De Marco · A. Moroni · L. Arata · E. Merello · L. Andreussi · A. Cama · V. Capra (✉)
Istituto G. Gaslini, Largo G. Gaslini, 5 16148 Genova, Italy
Tel. +39-010-5636712; Fax +39-010-3993159
e-mail: capraval@tin.it; valeriacapra@ospedale-gaslini.ge.it

M. Grazia Calevo
Servizio di Epidemiologia e Biostatistica, Istituto G. Gaslini, Genova, Italy

R.H. Finnell · H. Zhu
Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX, USA

Introduction

Methylenetetrahydrofolate reductase (*MTHFR*) plays a central role in folate-dependent homocysteine metabolism. Mutations in *MTHFR* have been reported as causes of hyperhomocysteinemia (Frosst et al. 1995; Jacques et al. 1996). The best-characterized *MTHFR* genetic polymorphism is a 677C→T transition that affects the predicted catalytic domain of the *MTHFR* protein. This mutation, resulting in decreased enzyme activity, is associated with mildly elevated plasma homocysteine levels and a redistribution of folates, namely, elevated red cell folate and lowered plasma folate (van der Put et al. 1995, 1996). Homozygosity for the 677T mutation predisposes individuals to the development of hyperhomocysteinemia, especially during times of folate insufficiency (Frosst et al. 1995; van der Put et al. 1998). Recently, a second common polymorphism in the *MTHFR* gene (1298A→C) was discovered, leading to a glutamate-to-alanine substitution in the presumed regulatory domain of the protein. Individuals who are either heterozygous 1298AC or homozygous 1298CC showed decreased enzyme activity, although their plasma homocysteine levels were not altered significantly (van der Put et al. 1998; Weisberg et al. 1998).

Given its key role in folate metabolism, it has been suggested that *MTHFR* is an attractive candidate in the etiology of neural tube defects (NTDs) because periconceptional folic acid supplementation is known to reduce the occurrence and recurrence risk for women to give birth to an NTD child (MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992). Moreover, significantly elevated plasma homocysteine levels and reduced folate levels were found in mothers of NTD-affected children (Mills et al. 1995; Whitehead et al. 1995). Therefore, the two *MTHFR* polymorphisms (677C→T and 1298A→C) have been widely investigated as genetic risk factors for NTDs. Although the *MTHFR* C677T mutation is considered a genetic risk factor for NTD by some (Kirke et al. 1993; Mills et al. 1995; van der Put et al. 1995, 1996, 1997a; Whitehead et al. 1995; Ou et al. 1996), there are studies that could not

confirm this mutation as a major determinant for NTD (Papapetrou et al. 1996; Mornet et al. 1997; Speer et al. 1997; Boduroglu et al. 1998; Koch et al. 1998; Weitkamp et al. 1998; Shaw et al. 1999). The C677T mutation was studied in the Italian population, which has a relatively low prevalence of NTDs. In this study, an increased NTD risk was found for the T/T genotype (odds ratio [OR] = 1.73), and the corresponding attributable fraction was 10.8% (de Franchis et al. 1998).

To date, few studies have evaluated the frequency of the 1298A/C alleles in a normal population and in NTD families (van der Put et al. 1998; Weisberg et al. 1998; Friedman et al. 1999; Rady et al. 1999; Stegmann et al. 1999; Volcik et al. 2000; Dekou et al. 2001; Song et al. 2001). In two of these studies, combined heterozygosity of the two *MTHFR* variants appeared to be increased in NTD-affected children when compared with the controls (van der Put et al. 1998; Dekou et al. 2001). Therefore, additional investigations are needed to establish whether the A1298C polymorphism is an additional risk factor for folate-sensitive NTD.

Several studies have suggested that a defective methionine synthase (*MS*) enzyme could be a critical defect in folate-related NTDs (Kirke et al. 1993; Scott and Weir 1994; Steegers-Theunissen et al. 1994; Mills et al. 1995). In fact, *MS*, a vitamin B_{12}-dependent enzyme that catalyzes the remethylation of homocysteine to methionine, is essential for mantaining adequate intracellular methionine and tetrahydrofolate pools. An A-to-G transition at bp 2756, which converts an aspartic acid to glycine, has been investigated in NTD patients. No increased prevalence of the 2756GG and 2756AG genotypes was found in patients, and no correlation was observed between these two genotypes and homocysteine levels (Morrison et al. 1997; van der Put et al. 1997b; Shaw et al. 1999). Additional studies on *MS* variants are required to address the role of this variant on NTD risk.

In the present case-control study, we examined the genotype frequencies of the *MTHFR* A1298C polymorphism as well as the *MS* A2756G variant in the Italian population, and evaluated their impact on NTD individuals and their relatives. Our data show that the A1298C polymorphism is a contributing risk factor for NTDs.

Subjects and methods

Probands and controls

This study included 203 (100 male and 103 female) unrelated Italian probands with non-syndromic NTD recruited from the Spina Bifida Center of the Gaslini Hospital, Genoa; they were all alive with an age range between 1 month and 10 years, and all of them were the first occurrence in the nuclear family. Ninety-eight mothers and 67 fathers were also included in the investigation. All parents were young adults with a mean age of 35 years. NTD diagnoses were made according to the Tortori-Donati et al. (2000) classification. One hundred seventy-two cases were affected by open spinal dysraphisms (OSDs) (84.7%) and 31 by closed spinal dysraphisms (CSDs) (15.3%). Seventy-five percent of children with OSD presented with Chiari II malformation. Although the open lesions were limited to myelomeningocele ($N = 172$), the closed lesions included meningocele ($N = 5$), lipoma ($N = 9$), lipomyieloschisis ($N = 12$), dermal sinus ($N = 3$), and tight filum terminalis ($N = 2$). Two hundred ten unrelated healthy Italian volunteers (103 male and 107 female) were used as control group. They were young adults with an age range between 20 and 45 years; none of them suffered from NTDs or delivered an NTD-affected child. Informed consent was obtained from patients, parents, and control individuals.

Genetic analysis

DNA was isolated from peripheral leukocytes obtained in blood draws using standard procedures. The detection of the A1298C mutation was carried out by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) screening, using two PCR primers to generate a 241-bp fragment: a forward primer, 5′-ATGTGGGGGGAG GAGCTGAC-3′, and the intronic reverse primer, 5′-GTCTCCCAACTTACCCTTCTCCC-3′. If the individual is homozygous wild type (1298AA), then the *Mbo*II RFLP results in two fragments, 204 bp and 37 bp in length. For the homozygous mutant *MTHFR* genotype (1298CC), only the 241-bp fragment is produced, and the heterozygous genotype generates all three fragments (van der Put and Blom 2000).

To screen for the *MS* mutation (A2756G), an appropriate region of genomic DNA was amplified by using two specific primers (5′-GGTGTGTTCCCAGCTGTTA GATG-3′ and 5′-GACACTGAAGACCTCTGATTTGA AC-3′) and digested with *Hae*III (van der Put et al. 1997b). A 2756AA genotype results in an uncut fragment of 265 bp, whereas a 2756AG genotype gives three fragments of 265, 180, and 85 bp. The homozygous 2756GG genotype produces two fragments of 180 and 85 bp. The digested PCR was separated on 3.5%–4% MetaPhor agarose (FMC BioProducts, Rockland, ME, USA) gel electrophoresis and stained by ethidium bromide. Direct sequencing of PCR fragments was used to confirm mutations. The sequencing reactions were manually performed using the Thermosequenase Cycle Sequencing Kit (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions. The forward PCR primer for the *MTHFR* A1298C and the reverse PCR primer for C677T mutation analyses were used as sequencing primers.

Statistical analysis

Allele frequencies in the NTD patients, mothers, fathers, and controls were determined by counting alleles and calculating proportions. The Hardy-Weinberg equilibrium analysis was calculated using the data from the control group and was tested using chi-square statistics for goodness of fit (1 degree of freedom). The OR with an associated 95% confidence interval (CI) was calculated to estimate the rela-

tive risk of the different genotype combinations. *MTHFR* and *MS* alleles frequencies were determined for the study and control groups, and were compared by χ^2 analysis. Exact methods were considered preferable whenever expected numbers in any cell were less than five, and any results reported herein are based on exact methods. *P* values ≤ 0.01 were considered statistically significant, and all *P* values were based on two-tailed tests.

The attributable fraction, representing the proportion of cases attributable to the presence of the mutation, was estimated using the equation proposed by Miettinen (1974).

The SPSS statistical software program was used for all analysis.

Results

Genotype distributions and allele frequencies

MTHFR and *MS* genotype distributions and allele frequencies for Italian NTD patients, parents, and controls are presented in Table 1. We found a genotype frequency distribution for the *MTHFR* A1298C mutation that was as expected according to the Hardy-Weinberg equilibrium ($\chi^2 = 0.0325$). A slight discordance was found for the genotype distribution of the *MS* A2756G mutation ($\chi^2 = 4.06$). The frequency of the C allele of the MTHFR A1298C mutation is 0.25 among control individuals. Thus, we have not revealed striking differences between the prevalence of the A1298C mutant allele among healthy Italian individuals and other populations that have already been studied (Weisberg et al. 1998; Rady et al. 1999). As shown in Table 1, we found that the 1298A/C heterozygous and 1298C/C homozygous genotypes were significantly more prevalent in NTD cases and parents than in controls. The A1298C frequencies were 0.39, 0.44, and 0.36 among patients, mothers, and fathers, respectively. The frequency of the G allele of the *MS* A2756G polymorphism was 0.15 in the control population, which was in agreement with the incidence reported by other investigators (van der Put et al. 1997b). No increased prevalence of this mutated allele was found in NTD families, although a slightly higher frequency was present in patients and mothers (0.11) and in fathers (0.13).

No differences in the allele frequencies were found when we considered the type of defect. In fact, we found that the frequency of the 1298C allele between NTD children with open dysraphisms is 0.389 and with closed dysraphisms 0.370. Similarly, no significantly increased prevalence of the 2756G allele was present in NTD cases affected by open (0.106) and closed (0.13) lesions.

Odds ratio and NTD risk

To evaluate the impact of the *MTHFR* A1298C variant on NTD risk in our sample, we calculated the OR and 95% CI associated with the 1298A/C and 1298C/C genotypes (Table 2). The estimated OR for heterozygous NTD cases was 1.98 (95% CI: 1.28–3.07; $P \leq 0.001$), and increased to 3.67 (95% CI: 1.67–8.18; $P \leq 0.0003$) for homozygous NTD cases. Furthermore, the NTD risk significantly increased if both homozygous and heterozygous NTD children were compared with control individuals (OR = 2.21; 95% CI 1.46–3.36; $P \leq 0.00008$). More sensitive risk estimates resulted for the mothers: an increased risk of 2.11 (95% CI: 1.19–3.75; $P \leq 0.006$) and 6.23 (95% CI: 2.58–15.35; $P \leq 0.00001$) was found for the prevalence of the 1298AC and 1298CC genotypes, respectively, and of 2.67 if both heterozygous and homozygous mothers were compared with controls. There was no increased risk for the heterozygous A1298C genotype of the fathers (OR = 1.45; 95% CI: 0.77–2.74;

Table 1. Genotype distribution and allele frequencies of *MTHFR* A1298C and *MS* A2756G polymorphisms in Italian NTD families and controls

MTHFR A1298C				
	NTD cases % (*N* = 203)	Mothers % (*N* = 98)	Fathers % (*N* = 67)	Controls % (*N* = 202)
A/A	36.9 (*N* = 75)	32.7 (*N* = 32)	43.3 (*N* = 29)	56.4 (*N* = 114)
A/C	48.8 (*N* = 99)	45.9 (*N* = 45)	41.8 (*N* = 28)	37.6 (*N* = 76)
C/C	14.3 (*N* = 29)	21.4 (*N* = 21)	14.9 (*N* = 10)	5.9 (*N* = 12)
1298A	0.61	0.56	0.64	0.75
1298C	0.39	0.44	0.36	0.25
MS A2756G				
	NTD cases % (*N* = 174)	Mothers % (*N* = 75)	Fathers % (*N* = 48)	Controls % (*N* = 210)
A/A	79.3 (*N* = 138)	82.7 (*N* = 62)	77.1 (*N* = 37)	70.5 (*N* = 148)
A/G	19.5 (*N* = 34)	12.0 (*N* = 9)	18.8 (*N* = 9)	29.0 (*N* = 61)
G/G	1.1 (*N* = 2)	5.3 (*N* = 4)	4.2 (*N* = 2)	0.5 (*N* = 1)
2756A	0.89	0.89	0.87	0.85
2756G	0.11	0.11	0.13	0.15

NTD, Neural tube defect

Table 2. Odds ratios with 95% confidence intervals of *MTHFR* A1298C genotype in NTD cases, mothers and fathers

MTHFR A1298C	Odds ratio (95% CI)	*P*
Controls-patients		
AA/CC	3.67 (1.67–8.18)	0.0003
AA/AC	1.98 (1.28–3.07)	0.001
AA/AC-CC	2.21 (1.46–3.36)	0.00008
Controls-mothers		
AA/CC	6.23 (2.58–15.35)	0.00001
AA/AC	2.11 (1.19–3.75)	0.006
AA/AC-CC	2.67 (1.57–4.59)	0.0001
Controls-fathers		
AA/CC	3.28 (1.17–9.16)	0.01
AA/AC	1.45 (0.77–2.74)	0.22*
AA/AC-CC	1.70 (0.94–3.08)	0.06*

NTD, Neural tube defect; CI, Confidence interval
*Not significant

$P = 0.22$ not significant), but there is a trend toward an increased risk for fathers that have a homozygous mutant genotype (OR = 3.28; 95% CI 1.17–9.16; $P \leq 0.01$). Thus, we estimate the attributable fraction of NTD cases due to the *MTHFR* C/C genotype in Italy to be 7.8% and 10.4%, depending on the comparison group used for the estimation of the risk (1298CC versus 1298AA, or 1298AC plus 1298CC, versus 1298AA).

Finally, the possible involvement of *MS* A2756G in the risk for NTD was investigated by analyzing the prevalence of the mutation in NTD patients and their parents. There was no significantly increased prevalence of 2756AG and 2756GG genotypes present in either the NTD patients or their parents when compared with the prevalence observed among the controls. Calculated OR indicates that neither the homozygous nor the heterozygous mutant genotype increased the NTD risk (data not shown), demonstrating that this polymorphism is not a risk factor for NTDs, but instead is most probably a benign polymorphism.

Discussion

In this study we investigated whether the *MTHFR* A1298C mutation, as reported for the C677T mutation, could have an impact on NTD in the Italian population. The 1298C allele frequency in Italy was determined to be 0.25. Previous studies on the prevalence of this polymorphism revealed that there is wide heterogeneity in the prevalence of the A1298C polymorphism throughout the world, as reported for the C677T mutation. We found the frequency of the 1298C allele in Italy to be similar to that reported for the Canadian (0.25) (Weisberg et al. 1998) and Ashkenazi Jewish population (0.28) (Rady et al. 1999), demonstrating that this polymorphism is common in the Italian population, although its rate is lower than the C677T mutation. The results of the present study indicate an increased prevalence of heterozygosity and homozygosity for the *MTHFR* A1298C polymorphism among NTD patients and mothers. The level of the conferred risk was minor for heterozygous NTD cases (OR = 1.98) and mothers (OR = 2.11) and was higher for homozygous mutant genotypes of patients (OR = 3.67) and mothers (OR = 6.23). The father's genotype appeared to be a significant risk factor if he has the homozygous 1298CC genotype (OR = 3.28). The results of this study, which had a relatively large number of subjects, reproducible genotyping methods, and significantly increased OR, are unlikely to be due to selection bias. The allelic frequencies among our controls are consistent with those derived by Hardy-Weinberg equilibrium and those reported previously in other European populations, demonstrating the randomness of our control selection. However, since case-control studies of a single polymorphism can produce conflicting data, our results will be confirmed by a family-based transmission disequilibrium test. Furthemore, our results are biologically plausible because the A1298C mutation results in diminished enzyme activity. A significant decreasing effect on *MTHFR* activity has been observed in the homozygous 1298CC as well as in the heterozygous 1298AC state (van der Put et al. 1998). Therefore, there seems to be an interaction between the C677T mutation and the A1298C mutation, because heterozygosity for both mutations is associated with lower activity than heterozygosity alone for either mutation, resulting in increased homocysteine levels (van der Put et al. 1998). A limitation of the present study is that we did not have access to plasma samples for evaluation of the impact of the A1298C variant on folate and homocysteine levels in our cases and mothers. Since the A1298C mutation influences enzyme activity and homocysteine and folate concentrations to a lesser extent than does the C677T mutation, it may be expected that the A1298C mutation is a risk factor for NTD, but with a smaller relative risk. On the contrary, we found that the A1298C polymorphism is an important risk factor for the Italian population with a risk associated with the 1298C/C genotype that is even higher than that reported for the homozygous mutant genotype of the C677T mutation (de Franchis et al. 1998). *MTHFR* nucleotide 1298 is located on the regulatory domain of the protein, where it may be involved in protein stabilization (Shan et al. 1999). We speculate that the effect of the A1298C variant results in the alteration of one-carbon metabolism within a cell rather than a simply generalized repression of *MTHFR* activity. This mutation could become of clinical importance, particulary under conditions of low intake of folate or high requirements of folate, such as occurs in pregnancy. The increased risk that we found in NTD-affected children and their mothers suggest that this mutation could be responsible for a proportion of NTD cases that is not explained by homozygosity for the C677T mutation.

Our finding of no association between the A2756G polymorphism in the *MS* gene and the occurrence of the NTD phenotype is in agreement with the findings of other groups who did not report a direct or significant role for this polymorphism in the etiology of NTD (Morrison et al. 1997; van der Put et al. 1997b; Shaw et al. 1999; Johanning et al. 2000). Given the importance of *MS* in homocysteine and folate metabolism, it is likely that during evolution, mutations of *MS* were so deleterious that they were lethal to the fetus

and were thus not propagated. The importance of this enzyme for early development has been recently demonstrated in mice by targeted disruption of the *MS* gene. In fact, homozygous knock-out embryos survive through implantation but die soon thereafter (Swanson et al. 2001). Potential interaction of *MTHFR* and *MS* variants may be of significance because *MS* and *MTHFR* are both key enzymes in homocysteine and folate metabolism. Recently, it has been reported that associations between the 677T mutation in *MTHFR* and the *MS* A2756G polymorphism slightly increased NTD risk (Johanning et al. 2000). Further research should explore the comorbidity of *MTHFR* and *MS* polymorphisms in a large population.

Spinal dysraphisms are categorized as open (OSD) and closed (CSD) (Tortori-Donati et al. 2000). OSD is characterized by exposure of the nervous tissue and/or meninges to the environment through a congenital bony defect. Conversely, CSD is covered by skin (there is no exposed neural tissue), although cutaneous stigmata usually betray its presence. CSD is more heterogeneous than OSD and a large number of malformations belong to this group. In our study, we categorized our patients by type of dysraphism and we attempted to correlate it with *MTHFR* A1298C and *MS* A2756G genotype distribution. No statistically significant heterogeneity was found among NTD cases in the allelic frequency, demonstrating that neither variants could be associated to the type of defect. Moreover, the level of the defect was not always available and therefore could not be considered in the present study.

In conclusion, in this study we identified a second genetic risk factor for Italian NTD cases, the *MTHFR* A1298C mutation, which, like the C677T mutation, affects enzyme activity. From the point of view of general health care, the estimated OR of the A1298C mutation is of great importance because of the high prevalence of homozygous and heterozygous individuals in the general population. Homozygosity of NTD children for the A1298C mutation can at most explain 10.4% of the observed protective effect of folate. Since periconceptional folate supplementation reduces the risk for 70% of the population, other defective genes of folate, vitamin B_{12}, or homocysteine metabolism may be associated with NTD risk.

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