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Prevalence of Methylenetetrahydrofolate Reductase Polymorphisms in Young Patients with Inflammatory Bowel Disease

GABRIELE STOCCO,* STEFANO MARTELOSSI, MD,† FRANCA SARTOR,‡ GIUSEPPE TOFFOLI, MD,‡ PAOLO LIONETTI, MD,§ ARRIGO BARABINO, MD,¶ MASSIMO FONTANA, MD,|| GIULIANA DECORTI, MD,* FIORA BARTOLI, MD,* TULLIO GIRALDI,* and ALESSANDRO VENTURA, MD†

Inflammatory bowel disease (IBD) has been related to mutations of methylenetetrahydrofolate reductase (MTHFR), a critical enzyme in the metabolism of folate and methionine, both of which are important factors in DNA methylation and synthesis. A mutated MTHFR genotype was associated with increased toxicity of methotrexate treatment. The objective of this study was to verify, in a population of young patients with IBD, the presence of an association among mutations in the MTHFR gene, the incidence of IBD, and the risk of adverse events during the treatment with thiopurines azathioprine (AZA) or 6-mercaptopurine (6MP). Ninety-two patients with IBD were enrolled; 63 were treated with thiopurines; patients and 130 controls were genotyped for MTHFR mutations by PCR-based methods. The incidence of mutations in the MTHFR gene was not different between patients with IBD and control subjects; the mutated genotype was not associated with an increased risk of toxicity during thiopurine treatment.

KEY WORDS: inflammatory bowel disease; methylenetetrahydrofolate reductase; genetic polymorphism; thiopurines.

MTHFR (5,10-methylenetetrahydrofolate reductase) is a central regulatory enzyme in the folate pathway; it catalyzes the reduction of 5,10-methylentetrahydrofolate to 5-methyltetrahydrofolate and directs available folate toward the methylation of homocysteine at the expense of nucleotide synthesis (1).

In the last years mutations in the coding region of the MTHFR gene, affecting the activity of the enzyme, have been discovered. The more interesting is a common transition in the MTHFR gene at nucleotide 677 (C677T) (1, 2); this mutation results in an alanine-to-valine substitution in the MTHFR protein and, as shown with in vitro studies using cell extracts, renders the enzyme less active and more susceptible to heat inactivation compared with the wild-type enzyme (2, 3). The biological consequence of this mutation is an imbalance in the folate pools that can influence different cellular activities, such as purine and pyrimidine synthesis (4) and DNA methylation and repair (5). This mutation also has metabolic effects, such as an increase in the concentration of plasma total homocysteine (tHcy) (6). The prevalence of this polymorphism is related to ethnicity, and the frequency of the homozygous form for the TT allele is about 10% in Caucasians, 20% in some Italian populations, and much lower in Afro-Americans.

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From the *Department of Biomedical Sciences, University of Trieste, and †Research Children's Hospital 'Burlo Garofolo,' Trieste, ‡Experimental and Clinical Pharmacology Unit, Division of Experimental Oncology, Centro di Riferimento Oncologico, Aviano, Pordenone, §Research Children's Hospital 'Meyer,' Florence, ¶Research Children's Hospital 'Gaslini,' Genoa, and ||Children's Hospital 'Buzzi,' Milan, Italy.

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Address for reprint requests: Gabriele Stocco, Department of Biomedical Sciences, University of Trieste, Via L. Giorgieri 7,9, Trieste 34127, Italy; stoccog@units.it.

The TT variant was reported to be associated with an increased risk of vascular disease (2), spina bifida (7), diabetic neuropathy (8), and human cancer (9, 10). In addition, a positive association between the MTHFR 677 TT genotype and inflammatory bowel disease (IBD) has been observed in both an Irish (11) and a Danish (12) population; however, the association was not confirmed in Italian populations (13, 14).

A second common polymorphisms in the MTHFR gene (A1298C) causes a glutamic acid to alanine substitution, which also results in decreased MTHFR activity (15). This polymorphism, however, seems to have a milder effect on folate pools and to be less relevant from the clinical point of view. It is possible, however, that under conditions of extreme folate depletion, even this polymorphism may become clinically relevant.

The aim of this study was to verify in a northern Italian pediatric population if there is an association between the two main forms of IBD, Crohn's disease (CD), and ulcerative colitis (UC) and the two most common mutations in the MTHFR gene.

Investigations suggesting a link between polymorphisms in the folate pathways and the adverse effects of antimetabolite drugs have been reported (16), in particular, for drugs that directly affect these metabolic pathways, like methotrexate (17, 18). A subset of the IBD patients examined in our study was treated with the immunosuppressive drug azathioprine (AZA), a well-established therapy for this kind of disease (19, 20); however, AZA caused a high incidence of adverse drug reactions such as bone marrow toxicity and hepatotoxicity. AZA acts in vivo through the liberation of 6-mercaptopurine (6MP) (21); this drug is an antimetabolite, which exerts its cytotoxic activity through the combination of two mechanisms, reduction of de novo purine synthesis and intercalation in the nucleic acid structure and consequent disruption of its function (22). AZA has no direct reported effect on enzymes of the folate pathways, however, the MTHFR mutated genotypes and the related modifications of folate pools, of DNA methylation and of purine synthesis could increase cells' sensibility to AZA treatment. The more affected tissues should be those with a high cell turnover, which have increased DNA synthesis and high requirement for nucleotides.

Thus we investigated the possible correlation between the mutated MTHFR genotypes and an increased incidence of AZA treatment-related side effects and, in particular, of bone marrow toxicity.

METHODS

Sample Collection. Patients were enrolled between June 2002 and June 2004 in the Children Research Hospitals of Trieste, Florence, Genoa, and Milan. The study included 92 subjects with IBD: 49 (53.3%) had Crohn's disease (CD) and 43 (46.7%) had ulcerative colitis (UC). Forty-nine (53.3%) patients were female; the median age was 15 years (range, 4–38 years). Sixty-three patients were treated with a thiopurine antimetabolite; 45 patients were treated with a median AZA dose of 1.94 mg/kg/day (range, 1–5 mg/kg/day), while 18 received 6MP at a median dose of 0.93 mg/kg/day (range, 0.6–1.2 mg/kg/day). The dose of the drug administered was adjusted in order to obtain the optimal clinical response: the administration was started at a dose of 1 mg/kg/day for AZA and 0.5 mg/kg/day for 6MP. At monthly follow-up visits the dose was increased in order to obtain the optimal clinical response; in the case of adverse effects, the dose was reduced or the treatment interrupted as required. The average length of the treatment with thiopurine medications was 22.0 months (range, 0.5–71.3 months). None of these patients was taking folate supplements that could influence intracellular folate pools.

Clinically significant thiopurine-related adverse effects were graded using the NCI criteria for reporting toxicity of drug treatment. According to this scale each toxic manifestation is divided into five grades; grades 1 and 2 are considered mild manifestations of toxicity, while grades 3 and 4 are considered severe toxicity. Grade 5 corresponds to drug-induced death (23).

For comparison, genotyping was performed even on 130 consecutive healthy blood donors.

Analysis of the MTHFR Genotype. Genomic DNA was extracted with conventional methods from peripheral blood lymphocytes. MTHFR 677 genotyping was performed using a 100 Programmable Thermal Controller (MJ Research, Inc., Geneco, Florence, Italy). Two hundred nanograms of human genomic DNA was amplified with 100 pmol each of forward primer 5′-GCACTTGAAGGAGAAGGTGTC-3′ and reverse primer 5′-AGGACGGTGCGGTGAGAGTG-3′, 1.5 mM $MgCl_2$, 5% formamide, 0.2 μM each deoxynucleotide triphosphate, and 1 unit Taq polymerase (Polimed, Florence, Italy) in a total volume of 100 μl. PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 51°C for 30 sec, and 72°C for 30 sec; the terminal elongation was performed at 72°C for 5 min. The PCR products were ethanol precipitated, digested with *Taq*I, and run on a 4% agarose gel. The variant allele is created by a C-to-T change at nucleotide 677, which introduces a *TaqI* site: homozygous individuals (TT) show two fragments, of 173 and 30 bp; heterozygotes (CT) show three fragments, of 203, 173, and 30 bp; and wild-type individuals (CC) show only one band, of 203 bp. DNA from three lymphoblastoid cell lines with 677 TT, 677 CT, and 677 CC genotypes was used as quality control. Data obtained with *Taq*I were also validated by comparing results obtained with *Hin*fI digestion according to methods described by Frosst *et al.* (24).

The presence of the MTHFR 1298 polymorphism was analyzed using the method of Skibola *et al.* (25) with slight modifications. DNA was amplified with the forward primer 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′ and the reverse primer 5′-CACTTTGTGACCATTCCGGTTTG-3′. Amplification and PCR conditions were the same as for MTHFR 677. The variant allele is created by an A-to-C change at nucleotide 1298, which abolishes a *Mbo*II site; wild-type individuals (AA) show five bands, of 56, 31, 30, 28, and 18 bp, whereas homozygous variant individuals (CC) show four bands, of 84, 31, 30 and 18 bp. The restricted product was analyzed by electrophoresis in a 4% agarose gel stained with ethidium bromide.

TABLE 1. PATIENT CHARACTERISTICS ACCORDING TO MTHFR 677 GENOTYPE

	CC	*CT*	*TT*	P *value**	*Odds ratio (95% CI)**	*Total*
Age (yr), median (range)	9 (3–26)	17.5 (8–38)	11.5 (6–26)			
Sex (F:M)	12:12	29:24	8:7			
IBD						
CD	10 (20.4%)	32 (65.3%)	7 (14.3%)	0.40	0.64 (0.26–1.57)	49
UC	14 (32.6%)	21 (48.8%)	8 (18.6%)	0.83	0.87 (0.36–2.10)	43
Total	24 (26.1%)	53 (57.6%)	15 (16.3%)	0.49	0.74 (0.37–1.49)	92
Controls	38 (29.2%)	65 (50.0%)	27 (20.7%)			130

*Odds ratios, with 95% confidence intervals (CI), and *P* values of Fisher's exact test refer to the comparison of the frequencies of mutated subjects (TT) in the sample population versus the control population.

Statistical Analysis. The distribution of individual characteristics was evaluated using simple descriptive statistics. Differences among distributions of selected variables were evaluated using Fisher's exact test for categorical data.

RESULTS

Ninety-two subjects with IBD entered the study; 49 (53.3%) patients had CD and 43 (46.7%) had UC. Patients characteristics in relation to MTHFR genotype at position 677 are summarized in Table 1. Data were compared with those obtained in a series of 130 consecutive blood donors used as control. The prevalence of homozygotes for the C677T variant (TT) of MTHFR among cases of IBD (15 of 92; 16.3%) was not significantly different from that in controls (27 of 130; 20.7%; $P = 0.49$, Fisher's exact test).

Patients' characteristics in relation to MTHFR 1298 genotype are summarized in Table 2. The prevalence of MTHFR A1298C mutation among cases of IBD (9 of 92; 9.8%) was not significantly different from that in controls 130 (10 of 130; 7.7%; $P = 0.63$, Fisher's exact test).

Sixty-seven of 92 subjects were treated with thiopurine antimetabolites (AZA or 6MP) for at least 3 months, and 30 of these 67 developed treatment-related side effects. The most frequent toxicity related to thiopurine treatment was bone marrow toxicity, which occurred in 19 of the 30 patients who developed side effects during the treatment with AZA. Details about toxicity are given in Table 3.

The relation between the outcome of thiopurine treatment and the MTHFR 677 genotype is reported in Table 4. Grade 3–4 toxicity was observed in 2 of 16 (12.5%) patients with the CC genotype, 5 of 39 (12.8%) patients with the CT genotype, and 1 of 12 (8.3%) patients with the TT genotype. Patients with a mutated TT MTHFR 677 genotype during treatment with AZA did not present an increased risk of adverse events of either grades 1–2 (OR, 0.95; $P = 1.00$, Fisher's exact test) or grades 3–4 (OR, 0.61; $P = 1.00$).

The relation between AZA treatment and MTHFR 1298 genotype is shown in Table 5. Grade 3–4 adverse events were developed by 5 of 34 (14.7%) patients with the CC genotype, 3 of 27 (11.1%) patients with the CT genotype, and 0 of 6 patients with the TT genotype. Patients with a CC mutated MTHFR 1298 genotype during treatment with AZA did not present an increased risk of adverse events of either grades 1–2 (OR, 1.79; $P = 0.66$, Fisher's exact test) or grades 3–4 (OR, 0.58; $P = 1.00$).

DISCUSSION

MTHFR catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl donor in the remethylation of homocysteine to methionine and, therefore, has a crucial role in DNA methylation (5), synthesis, and repair (1). A C677T polymorphism in the MTHFR gene was reported in 1995

TABLE 2. PATIENT CHARACTERISTICS ACCORDING TO MTHFR 1298 GENOTYPE

	AA	*AC*	*CC*	P *value**	*Odds ratio (95% CI)**	*Total*
Age (yr), median (range)	15 (5–38)	11 (4–26)	13 (12–17)			
Sex (F:M)	25:17	21:20	3:6			
IBD						
CD	26 (53.1%)	20 (40.8%)	3 (6.1%)	1.00	1.28 (0.34–4.85)	49
UC	16 (37.2%)	21 (48.8%)	6 (14.0%)	0.23	1.95 (0.66–5.72)	43
Total	42 (45.7%)	41 (44.6%)	9 (9.8%)	0.63	1.30 (0.51–3.34)	92
Controls	62 (47.7%)	58 (44.6%)	10 (7.7%)			130

*Odds ratio, with 95% confidence intervals (CI), and *P* values of Fisher's exact test refer to the comparison of the frequencies of mutated subjects (CC) in the sample population versus the control population.

TABLE 3. GRADE OF TOXICITIES AFTER AZA TREATMENT

Toxicity	*Number of subjects (%)*	*Grade of toxicity*			
		1	*2*	*3*	*4*
Bone marrow*	19 (63.3%)	5	9	3	2
Pancreatic	5 (16.7%)	1	1	1	2
Hepatic	6 (20.0%)	2	4	0	0
Total	30				

*Bone marrow toxicity manifested as lymphopenia ($n = 11$), leukopenia ($n = 5$), anemia ($n = 1$), thrombocytopenia ($n = 1$), and pancytopenia ($n = 1$).

by Frosst *et al.* (2); the phenotype of this genetic variant is characterized by reduced catalytic activity and thermolability of the enzyme in vitro and elevated plasma homocysteine in subjects with low folate concentrations. This polymorphism is common in the general population and its prevalence is related to ethnicity: the frequency of homozygosity for the TT allele is about 10% in Caucasians, 20% in some Italian populations, and much lower in Afro-Americans.

Another common polymorphism in the MTHFR gene has been identified (26) at nucleotide 1298 (A1298C) and results in an alanine-to-glutamate substitution. Also, this variant is associated with reduced specific activity of the enzyme but its clinical implications are not clear and this mutation probably has a lesser effect on MTHFR activity than the MTHFR C677T variant (9, 26).

The 677 TT genotype has been recognized as a genetic cause of hyperhomocysteinemia (24) and this is an established risk factor for thromboembolic complications. Patients with IBD have a higher risk of developing thromboembolic complications that seem to be linked to elevated plasma total homocysteine. However, in these patients, hyperhomocysteinemia could be due not only to a MTHFR mutation, which produces a thermolabile enzyme, but also to other causes such as impaired absorption of dietary folates and vitamin B12, reduced alimentary intake due to malaise and anorexia, and excessive nutrient requirements due to bowel inflammation. In addition, sulfasalazine, which is widely used in the treatment of IBD, has been shown to impair the absorption of folic acid, polyglutamyl folate, and methyltetrahydrofolic acid, causing subclinical tissue depletion (27).

The pathogenesis of IBD is considered multifactorial, and it has been suggested that small vessel thrombosis leading to intestinal microvascular infarction and inflammation may predispose to the development of the disease (28), however, a recent study did not confirm this hypothesis (29), hence studies in children are of particular interest since they should be subject to fewer confounding variables. In adults, a positive association between the TT genotype and IBD has been observed in an Irish (11) and a Danish (12) population. On the contrary, the association was not observed in two studies performed on Italian populations (13, 14). Only two studies have been reported in children (30, 31), and the prevalence of MTHFR C677T mutations was not correlated with the prevalence of IBD. In none of these studies was the correlation with the MTHFR A1298G mutation investigated.

Our data confirm that, in a northern Italian population, there is no difference in the frequency of MTHFR 677 TT genotype between healthy controls and young patients with IBD, and demonstrate that the MTHFR 1298 CC mutation is not associated with a higher frequency of the disease.

Ethnic variations in the frequency of the C677T allele in the normal population probably account, at least in part, for the differences observed in various studies. Indeed, only 7.3 and 8.3% of controls were homozygous in the Irish (11) and Danish (12) studies, respectively, compared to about 20% observed in the Italian population (13, 14), including this study. In the study conducted in North America, only 4.5% of patients were homozygous for MTHFR, but there was no difference from controls (31).

The imbalance in the folate pools associated with MTHFR mutations is responsible for a decreased availability of folate metabolites for thymidine and purine synthesis. This may result in greater DNA damage and decreased ability to repair DNA (32), and a consequent association between MTHFR polymorphism and the efficacy and toxicity of antimetabolite drugs has been reported

TABLE 4. MTHFR 677 GENOTYPE AND TOXICITY OF AZA TREATMENT

*WHO toxicity**	*CC*	*CT*	*TT*	P *value*†	*Odds ratio (95% CI)*†	*Total*
No toxicity	8 (21.6%)	22 (59.4%)	7 (19.0%)			37 (55.2%)
Grades 1–2	6 (27.3%)	12 (54.5%)	4 (18.2%)	1.00	0.95 (0.24–3.71)	22 (32.8%)
Grades 3–4	2 (25.0%)	5 (62.5%)	1 (12.5%)	1.00	0.61 (0.064–5.82)	8 (11.9%)
Total	16 (23.9%)	39 (58.2%)	12 (17.9%)			67

*The highest grade of toxicity (hematologic or nonhematologic) observed in each patient after AZA.
†*P* value of Fisher's exact test and odds ratio, with 95% confidence intervals (CI), refer to the comparison of the frequencies of mutated subjects (CC) in the population with toxicity versus patients without toxicity.

TABLE 5. MTHFR A1298C GENOTYPE AND TOXICITY OF AZA TREATMENT

*WHO toxicity**	*AA*	*AC*	*CC*	P *value*†	*Odds ratio (95% CI)*†	*Total* (n = *67)*
No toxicity	19 (51.4%)	15 (40.5%)	3 (8.1%)			37 (55.2%)
Grades 1–2	10 (45.5%)	9 (40.9%)	3 (13.6%)	0.66	1.79 (0.33–9.76)	22 (32.8%)
Grades 3–4	5 (62.5%)	3 (37.5%)	0 (0.0%)	1.00	0.58 (0.027–12.3)	8 (11.9%)
Total	34 (50.7%)	27 (40.3%)	6 (9.0%)			67

*The highest grade of toxicity (hematologic or nonhematologic) observed in each patient after AZA.
†*P* value of Fisher's exact test and odds ratio, with 95% confidence intervals (CI), refer to the comparison of the frequencies of mutated subjects (CC) in the population with toxicity versus patients without toxicity.

(16–18, 33). The antimetabolite AZA has no reported effect on enzymes of the folate pathways: the active metabolites of AZA cause cytotoxicity through the inhibition of de novo purine synthesis and incorporation into DNA and RNA. The MTHFR mutated genotypes and the related modifications of folate pools, of DNA methylation, and of purine synthesis could increase cells' sensibility to AZA-induced inhibition of purine synthesis and DNA damage, in particular, in tissues with a high cell turnover such as the bone marrow. Our data show, however, that after the treatment of IBD patients with AZA, there is no difference in the incidence of bone marrow toxicity, or of other forms of side effects, in subjects with the 677 TT or 1298 CC genotype compared to wild-type and heterozygous subjects.

The folate antagonist methotrexate is increasingly used to treat IBD; further studies could be conducted to evaluate the influence of genetic variability in folate metabolizing enzymes, like MTHFR, on methotrexate sensitivity in IBD patients.

In conclusion, this study confirms that IBD is not associated with MTHFR mutated genotypes in an Italian pediatric population. Moreover, the presence of mutations in the MTHFR gene does not appear to correlate with the incidence of AZA-induced side effects.

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