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Methylenetetrahydrofolate reductase C677T variant in Moroccan patients with inflammatory bowel disease☆

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ABSTRACT

Background: The association of genetic polymorphisms related to metabolism of homocysteine and folate with inflammatory bowel disease has been evidenced. Several studies have identified genetic variants of *MTHFR* as significant susceptibility loci for Crohn's disease (CD) and ulcerative colitis (UC). The C677T genetic polymorphism in the *MTHFR* gene is found to be associated with a thermolabile variant enzyme that shows a reduced activity. Therefore, we investigated whether the C677T variant confers genetic susceptibility to CD or UC and evaluated the genotype–phenotype associations in the Moroccan population.
Methods: The present study included 96 inflammatory bowel disease patients (68 patients with CD and 28 with UC) and 182 healthy controls. DNA samples were genotyped for the *MTHFR* (C677T) mutation by the PCR-RFLP method. Statistical analyzes were performed using MedCalc software, Chi square test and Fisher test.
Results: The respective odds ratio for CD, UC and control group were, 1.55 (CI 95%: 0.53–4.53, P = 0.52); 0.50 (CI 95%: 0.06–4.15, P = 0.52) and 0.50 (CI 95%: 0.06–4.15, P = 0.52). Thus, no statistically significant association with the disease was observed in frequency of the TT variant in comparison to healthy controls. Stratification of IBD patients on the basis of CD or UC showed that individuals carrying at least one T allele are not protected against Crohn's disease. Furthermore, clinical features of the disease did not show any significant association.
Conclusion: In conclusion, the present study indicates that the genetic risk for IBD is not modulated by *MTHFR* C677T polymorphism in Moroccan population.

1. Introduction

The 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) enzyme plays a crucial role in the metabolism of homocysteine and folate (Ueland and Rozen, 2005). It catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate reductase (5,10-MTHF) into 5-methyltetrahydrofolate (5-MTHF) (Goyette et al., 1994), which acts as a carbon donor for the re-methylation of homocysteine to methionine. The derivative of methionine, the S-adenosyl methionine (SAM) serves as the main methyl donor in humans (Kutzbach and Stokstad, 1971; Ma et al., 1997). Thus the MTHFR enzyme may modulate levels of homocysteine, methionine and SAM, thereby it is involved in protein synthesis and *DNA* methylation. It can also indirectly influence the rate of nucleotide synthesis given that its substrate, the 5,10-MTHF is used in the synthesis of thymidine and can also be converted to other folate derivatives involved in the synthesis of purines.

The *MTHFR* gene is located on chromosome 1 (1p36.3) and has 11 exons spanning 22 kb (Goyette et al., 1998). The association of *MTHFR* with altered phenotypes was first reported by Mudd et al. (1972) He identified a major functional impairment of this enzyme in patients with homocystinuria. In 1988, a common variant of the enzyme which corresponds to the thermolabile form was characterized (Kang et al., 1988), the *MTHFR* 677C → T (A222V). This polymorphism is the most common genetic cause of hyperhomocysteinemia. Its involvement in several diseases/disorders has been demonstrated, such as: cancers (Chen et al., 1996; Heijmans et al., 2003; Suzuki et al., 2008), cardiovascular diseases (Frosst et al., 1995; They-They et al., 2010), thromboembolism

Abbreviation: °C, Celsius; 5-MTHF, 5-Methyltetrahydrofolate; CC, Wild type MTHFR C677T; CD, Crohn's disease; CI, Confidence interval; CT, MTHFR C677T heterozygous variant; DNA, Deoxyribonucleic acid; EIM, Extra intestinal manifestations; FSC, Familial similar cases; IBD, Inflammatory bowel disease; Kb, Kilobase; MTHFR, Methylenetetrahydrofolate reductase; N, Total number; OR, Odds ratio; P, P value; PCR, Polymerase Chain Reaction; PCR-RFLP, Polymerase Chain Reaction Restriction Fragment Length Polymorphism; SAM, S-adenosyl methionine; TT, MTHFR C677T homozygous variant; UC, Ulcerative colitis; µl, Microlitre; χ^2, Chi square.

☆ Conflict of interest: The authors declare that they have no competing interests.

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(Bernstein et al., 2001; Irving et al., 2005; Solem et al., 2004), complications of pregnancy (spontaneous abortion, preeclampsia) (Ueland and Rozen, 2005) and neural tube defects (Van der Put et al., 1998).

MTHFR polymorphisms have been studied in inflammatory bowel disease (IBD) and the results remain controversial. An association of *MTHFR* mutations with IBD has been reported in a series of studies (Magro et al., 2003; Mahmud et al., 1999; Nielsen et al., 2000; Yilmaz et al., 2006).

Chen et al. also showed that the variant C677T may predict ulcerative pancolitis in central China (Chen et al., 2008). However, other studies found no association of this polymorphism with IBD (Bjerregaard et al., 2002; Guédon et al., 2001; Papa et al., 2001; Vecchi et al., 2000).

We aimed to explore the association of the C677T variant of the *MTHFR* gene in IBD (Crohn's Disease and ulcerative colitis) patients. This is the first study (case – control) conducted in the Moroccan population.

2. Materials and methods

2.1. Subjects

This prospective study included 96 IBD patients (68 CD; 28 UC) recruited at Ibn Rochd University Hospital of Casablanca, and 182 unrelated healthy controls. The diagnosis of CD or UC was determined by standard clinical, radiological, endoscopic and histological criteria (Kornbluth et al., 1993).

CD was classified according to the Montreal classification (Silverberg et al., 2005). Other clinical features such as disease location, age at diagnosis and presence of extra intestinal manifestations were also recorded.

Moreover, written consent was obtained from each participant.

2.2. DNA extraction & PCR analysis

Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure (Miller et al., 1988). Spectrophotometry was used to quantify DNA.

The (C677T) polymorphism of the *MTHFR* gene was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (RFLP) as described by Frosst et al. (1995). Reactions were performed in a final volume of 25 μl. PCR products were cleaved overnight with *HinfI* (Biolabs) at 37 °C and electrophored on a 3% agarose gel in the presence of a molecular weight marker ladder 100 (New England Biolabs Ipswich, UK). After staining with ethidium bromide, ultraviolet was used on a transilluminator to visualize *DNA* bands.

The mutant allele (TT) contains a *HinfI* restriction site allowing RFLP analysis of the digested products. Digestion of the amplified fragment at the *HinfI* site yields fragments of 175 and 23 bp. The wild-type allele remains uncut.

2.3. Statistical analysis

Statistical analyses were performed using MedCalc 11.6 software. The statistical significance of non-association between different variables was performed with the Chi square test or Fisher test. Hardy–Weinberg equilibrium test was performed separately among the disease and the control groups for the *MTHFR* (C677T) polymorphism.

The χ^2 test or Fisher test was used to correlate the C677T *MTHFR* polymorphism and clinical parameters. The average age was determined by the rank sum test. Associations between genotypes and risk of IBD were estimated by calculating odds ratio (OR) with confidence

Table 1
Genotypic frequencies of C677T polymorphism in patients with Crohn's disease according to clinical parameters.

		MTHFR C677T				Chi-square test
	N	CC (%)	CT (%)	TT (%)	P value	
Age of onset	68				0.62	2.64
<17 years	12	4 (33.3)	7 (58.3)	1 (8.3)		
17–40	48	25 (52.1)	18 (37.5)	5 (10.4)		
>40 years	8	4 (50.0)	4 (50.0)	–		
Sex	68				0.46	1.56
Woman	25	14 (56.0)	10 (40.0)	1 (4.0)		
Man	43	19 (44.2)	19 (44.2)	5 (11.6)		
Type	68				0.37	6.52
Fistulizing	27	10 (37.0)	13 (48.2)	4 (14.8)		
Non fistulizing non stenosing	22	14 (63.6)	8 (36.4)	–		
Stenosing	13	6 (46.1)	5 (38.5)	2 (15.4)		
Fistulizing stenosing	6	3 (50.0)	3 (50.0)	–		
Localization	68				0.75	5.06
L1	20	10 (50.0)	7 (35.0)	3 (25.0)		
L2	22	11 (50.0)	8 (36.4)	3 (13.6)		
L3	16	8 (50.0)	8 (50.0)	–		
L4	1	–	1 (100.0)	–		
L4 + L2	3	2 (66.7)	1 (33.3)	–		
SFC	68				0.52	1.30
Presence	4	3 (75.0)	1 (25.0)	–		
Absence	64	30 (46.9)	28 (43.7)	6 (9.4)		
Smoking	68				0.34	2.15
Presence	27	13 (48.1)	10 (37.0)	4 (14.8)		
Absence	41	20 (48.8)	19 (46.3)	2 (4.9)		
Appendectomy	68				0.91	0.20
Presence	8	4 (50.0)	3 (37.5)	1 (12.5)		
Absence	60	29 (48.3)	26 (43.3)	5 (8.3)		
EIM	68				0.25	2.78
Presence	39	20 (51.3)	14 (35.9)	5 (12.8)		
Absence	29	13 (44.8)	15 (51.7)	1 (3.4)		
Surgery	68				0.85	0.33
Presence	29	13 (44.8)	13(44.8)	3 (10.3)		
Absence	39	20 (51.3)	16(40.0)	3 (7.7)		

SFC: similar familial cases (number of cases affected by IBD in the same family); EIM: extra intestinal manifestations; N: total number; CC: wild type MTHFR C677T, CT: MTHFR C677T heterozygous variant, TT: MTHFR C677T homozygous variant.

Table 2
Genotypic frequencies of C677T polymorphism in patients with ulcerative colitis according to clinical parameters.

		C677T MTHFR				Chi-square Test
	N	CC (%)	CT (%)	TT (%)	P value	
Age of onset	28				0.71	0.68
17–40	17	10 (58.8)	6 (35.3)	1 (5.9)		
>40 years	11	7 (63.6)	4 (36.4)	–		
Sex	28				0.59	1.06
Woman	14	8 (57.1)	5 (35.7)	1 (7.1)		
Man	14	9 (64.3)	5 (35.7)	–		
Localization	28				0.96	1.42
Left colitis	8	5 (62.5)	3 (37.5)	–		
Extensive colitis	5	3 (60.0)	2 (40.0)	–		
Pancolitis	12	7 (58.3)	4 (33.3)	1 (8.3)		
Proctitis	3	2 (66.7)	1 (33.3)			
SFC	28				0.93	0.15
Presence	3	2 (66.7)	1 (33.3)	–		
Absence	25	15 (60.0)	9 (36.0)	1 (4.0)		
Smoking	28				0.73	0.64
Presence	7	5 (71.4)	2 (28.6)	–		
Absence	21	12 (57.1)	8 (38.1)	1 (4.8)		
EIM	28				0.41	1.80
Presence	15	10 (66.7)	4 (26.7)	1 (6.7)		
Absence	13	7 (53.8)	6 (46.2)	–		
Surgery	28				0.77	0.52
Presence	4	2 (50.0)	2 (50.0)	–		
Absence	24	15 (62.5)	8 (33.3)	1 (4.2)		

SFC: similar familial cases (number of cases affected by IBD in the same family); EIM: extra intestinal manifestations; N: total number; CC: wild type MTHFR C677T, CT: MTHFR C677T heterozygous variant, TT: MTHFR C677T homozygous variant.

interval of 95% (CI). P values less than 0.05 were considered significant in all tests.

3. Results

We studied 68 subjects with CD and 28 subjects with UC recruited at the gastroenterology department of Ibn Rochd University Hospital of Casablanca, and 182 healthy controls from the DNA bank of general population available in the Laboratory of Genetics and Molecular Pathology. The clinical characteristics of patients according to *MTHFR* gene polymorphism are summarized in (Tables 1 and 2).

Statistical analysis showed that the distribution frequency of the CC, TT and CT genotypes ($p2 + 2pq + q2 = 1$) and C, T alleles ($p + q = 1$) were in Hardy–Weinberg equilibrium ($x^2 = 1.56$, $P < 0.46$; $x^2 = 1.06$, $P = 0.59$; $x^2 = 0.69$, $P < 0.71$) respectively in the three groups (CD, UC and controls). The average age of diagnosis was 33.5 ± 2.85 for patients with CD and 35.14 ± 5.46 for subjects with UC.

No correlation was observed between *MTHFR* polymorphism and clinical parameters in both cases (CD; UC) either in the age of diagnosis, disease location and behavior, family history, smoking, appendectomy or extra-intestinal manifestations and surgery.

The allelic and genotypic frequencies were respectively 48.5% CC, 42.6% CT, 8.8% TT; 69.9% C, 30.1% T for subjects with CD (Table 3) and 60.7% CC, 35.7% CT, 3.6% TT; 78% C, 21.4% T for UC subjects (Table 4).

The allelic and genotypic frequencies in controls were 51.6% CC, 42.83% CT, 6.0% TT and 72.8% C and 27.2% T (Tables 3 and 4).

In our study, no statistically significant difference was observed in both cases (CD and UC) compared to control group.

For subjects with Crohn's disease, the respective odds ratio of heterozygous CT, homozygous TT and individuals carrying the T allele are 1.07 (confidence interval 95% = 0.60–1.92, P = 0.81), 1.55 (CI 95%: 0.53–4.53, P = 0.52) and 1.16 (CI 95%: 0.75–1.78) (Table 3). Therefore, individuals carrying at least one T allele are not protected against Crohn's disease.

Additionally, the *MTHFR* C677T polymorphism is not associated with ulcerative colitis in the Moroccan population [with respective odds ratio 0.72 for the heterozygous CT, 0.50 for mutated homozygous TT and 0.73 for the mutated allele T (Table 4)].

4. Discussion

In this study, we determined heterozygote and homozygote *MTHFR* C677T variant respectively in a total of 40.6% (39/96) and 7.2% (7/96) of IBD patients. We found no evidence of a significant association of the *MTHFR* 677TT or 677T allele with the risk of IBD. When patients were stratified according to CD and UC, we found that homozygosity for the *MTHFR* C677T variant (TT) was present in 8.8% (6/68) of patients

Table 3
Genotypic and allelic frequencies of the MTHFR C677T polymorphism of CD patients and controls.

Gene	Case (%) N = 68	Controls (%) N = 182	OR (95% CI)	P
MTHFR C677T				
CC	33 (48.5)	94 (51.6)	Réf	
CT	29 (42.6)	77 (42.83)	1.07 (0.60–1.92)	0.81
TT	6 (8.8)	11 (6.0)	1.55 (0.53–4.53)	0.42
C	95 (69.9)	265 (72.8)	Réf	
T	41 (30.1)	99 (27.2)	1.16 (0.75–1.78)	0.51

CC: wild type MTHFR C677T, CT: MTHFR C677T heterozygous variant, TT: MTHFR C677T homozygous variant; N: total number; OR: odd ratio; P: p value (P < 0.05).

Table 4
Genotypic and allelic frequencies of the MTHFR C677T polymorphism for UC patients and controls.

Gene	Case (%) N = 28	Controls (%) N = 182	OR (95%CI)	P
MTHFR C677T				
CC	17 (60.7)	94 (51.6)	Réf	
CT	10 (35.7)	77 (42.83)	0.72 (0.31–1.66)	0.44
TT	1 (3.6)	11 (6.0)	0.50 (0.06–4.15)	0.52
C	44 (78.6)	265 (72.8)	Réf	
T	12 (21.4)	99 (27.2)	0.73 (0.37–1.44)	0.36

CC: wild type MTHFR C677T, CT: MTHFR C677T heterozygous variant, TT: MTHFR C677T homozygous variant; N: total number; OR: odd ratio; P: p value (P < 0.05).

with CD with an odds ratio higher than the reference population but statistically insignificant. Therefore, individuals carrying at least one T allele are not protected against Crohn's disease while 3.6% (1/28) of patients with ulcerative colitis presented homozygosity for the *MTHFR* C677T variant (TT), revealing a non-association of this polymorphism with UC disease.

Our results were in agreement with previously published studies (Bjerregaard et al., 2002; Guédon et al., 2001; Papa et al., 2001) where there was no significant association with IBD. The non association of the *MTHFR* 677TT or 677T allele with the risk of UC was also obtained by other authors (Chen et al., 2005).

By comparison, this association was significant in other studies reported (Nielsen et al., 2000; Yilmaz et al., 2006). Mahmud et al. (1999), found 17.2% (30/174) of homozygote variant (TT) against 7.3% (20/273) of control subjects ($P = 0.01$). Nielsen et al. (2000) confirmed these results, with 16.2% of patients with IBD with C677T homozygosity against 8.3% in the general population ($P < 0.009$).

Magro et al. also found that the allelic frequency of *MTHFR* C677T polymorphism was higher in Crohn's disease and ulcerative colitis ($P < 0.001$) than in the reference population (Chen et al., 2008).

Furthermore, Chen et al. investigated the association of *MTHFR* polymorphisms with UC patients. They found that the 677TT genotype was associated with pancolitis. The frequency of the TT genotype was 2.7-fold higher in UC individuals with pancolitis than in other UC cases, with respective percentages of 27.3 (95% CI 16.4–42.0) and 10.5 (95% CI 6.3–17.1) ($P = 0.0123$). The frequency of subjects who presented with either 677TT or the double heterozygous 677CT/1298AC genotype was also significantly different between subjects with pancolitis and those with left colitis or proctitis, with respective percentages of 43.2 (95% CI 29.6–43.2) and 20.2 (95% CI 14.1–28.1) ($P = 0.0048$) (Vecchi et al., 2000).

In addition to these contradictory results, the frequencies of the mutations that may increase the tendency to thromboembolism and hyperhomocysteinemia show differences between different regions of the world (Bernstein et al., 2007; Den Heijer et al., 2005; Fernández-Miranda et al., 2005; Frosst et al., 1995; Gudnason et al., 1998; Koutroubakis et al., 2007; Nakano et al., 2003; Papa et al., 2003; Phelip et al., 2008).

However, these results are difficult to interpret because the frequency of 677T allele varies according to geographical and ethnic factors. The genetic background and the small size of these different series might explain these discrepant results. Differences in the nutritional intake of folate or in the features related to the population investigated could be responsible in part for the controversy over the influence of *MTHFR* mutations on IBD. Studies on larger populations are needed to evaluate the possible association of *MTHFR* polymorphisms and IBD pathogenesis.

Nevertheless, for our patients we were not able to acquire data on folate and homocysteine status, either on the presence or absence of thromboembolic complications.

In conclusion, our preliminary study showed that the *MTHFR* C677T variant, associated with a decrease in enzyme activity, seemed not to be significantly associated with the primary risk of inflammatory bowel disease or with its phenotype in the Moroccan population. Nevertheless, individuals carrying at least one T allele are not protected against Crohn's disease. Our study can be considered as a first epidemiological basis; however we suggest that other variables should be investigated, such as folate and plasma homocysteine levels and the enzymatic activity of the *MTHFR*.

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