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Hyperhomocysteinemia and methylenetetrahydrofolate reductase 677C→T and 1298A→C mutations in patients with inflammatory bowel disease

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ABSTRACT

Background: hyperhomocysteinemia has been recently described in patients with inflammatory bowel disease (IBD), that could be related to the increased risk for thrombosis that exists in this disease. The aim of this study was the assessment of hyperhomocysteinemia in patients with IBD and its relation among vitamin B_{12} and folate levels, and methylenetetrahydrofolate reductase (MTHFR) 677C→ T and 1298A→C mutations.

Patients and methods: fifty two consecutive patients with IBD were studied (29 women and 23 men); age: mean (standard deviation 41.7 [11.9] years) and 186 controls with no difference in age and gender. Hyperhomocysteinemia was considered as homocysteine levels higher than mean plus two standard deviations of the control group (≥ 13 μmol/l).

Results: patients had an elevated prevalence of hyperhomocysteinemia (17.3 *vs.* 3.7%; p = 0.002) and lower folate (7.6 [4.1] *vs.* 8.9 [3.7] ng/ml; p = 0.01) and B_{12} vitamin levels (499 [287] *vs.* 603 [231] pg/ml; p = 0.003). Homocysteinemia was higher (14.3 [5.8] *vs.* 9.1 [3.9] μmol/l; p = 0.006) in 6 patients (11.5%) that had suffered thromboembolism. Frequency of MTHFR 677C→T (13.5 *vs.* 11.3%; p = 0.66) and 1298A→C (7.8 *vs.* 7.0%; p = 0.76) mutations was not increased in patients. Odds ratio (OR) for IBD in hyperhomocysteinemic patient was 5.51, 95% confidence interval (CI), 1.81-16.76; p = 0.002). Hyperhomocysteinemia was negatively associated with feminine gender (OR 0.08, 95% CI 0.01-0.49; p = 0.006) and folate levels (OR 0.04, 95%CI: 0.007-0.20; p < 0.001).

Conclusions: hyperhomocysteinemia is associated with IBD and low folate levels, and could be involved in development of thromboembolism. MTHFR 677C→T and 1298A→C mutations are not related with the disease.

Key words: Inflammatory bowel disease. Homocysteine. Hyperhomocysteinemia. Thromboembolism. Folate. Vitamin B_{12}. Methylenetetrahydrofolate reductase.

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INTRODUCTION

Patients with inflammatory bowel disease (IBD) have an increased incidence of thromboembolic events that seem to be related to a hypercoagulability state. These episodes frequently occur in the active disease (1,2), although they may happen in the inactive phase (3,4). Venous and/or arterial thrombosis have been described in 1,2 and 7,1% of patients with IBD (5,6), rising to an incidence of 39% in autopsy studies (7).

The moderate elevation of homocysteine serum concentrations represents a risk factor for atherothrombosis (8,9). Hyperhomocysteinemia has been described in patients with IBD over the last few years, and it has been associated with the higher risk of thromboembolism in these patients (10-14). The increase of homocysteine in the IBD has been ascribed to low levels of folate and B_{12} vitamin that could be related to a decreased intake and absorption of these vitamins, as well as the antifolate effect of some drugs used in IBD (15-17).

The homozygous mutation in the gene encoding the enzyme involved in the remethylation of homocysteine, the methylenetetrahydrofolate reductase (MTHFR) 677C variant, consists of the replacement of cytosine by thymine at nucleotide 677 leading to alanine to valine substitution. This mutation (677C→T) decreases the enzyme activity and in cases of folate deficiency is associ-

ated with an increase in homocysteine levels and a higher incidence of thromboembolic events (8,18). Recently, a second mutation in the MTHFR gene, the 1298A→C, has been described. It consists of an adenosine to cytosine transition at nucleotide 1298, leading to a glutamate to alanine substitution. This mutation results in the reduced enzyme activity, to a lesser extent than in the case of the MTHFR 677C→T mutation (19,20). The prevalence of the 677C→T mutation and its association with hyperhomocysteinemia has been evaluated in two studies, with discordant results (14,21). No studies to date have analyzed the prevalence of the 1298A→C mutation in patients with IBD.

The aims of the present study in patients with IBD have been: a) to evaluate the association of hyperhomocysteinemia and MTHFR 677C→T and 1298A→C mutations with the disease; b) to determine if hyperhomocysteinemia correlates with folate and vitamin B_{12} concentrations.

PATIENTS AND METHODS

Fifty-two consecutive patients with the diagnosis of IBD, based on clinical, radiological, endoscopic and histological criteria, were enrolled from the outpatient department of gastroenterology between January 2002 and February 2003. Patients with active disease, malignant disease or those receiving B-complex vitamins were excluded. Inactive IBD was defined as the absence of gastrointestinal symptoms. Control group consisted of 186 healthy hospital personnel and friends, unrelated and similar in age and gender to the patients, who voluntarily participated in the study.

Plasma homocysteine concentrations were determined by fluorescence polarization immunoassay with an IMX analizer (Abbott laboratories. IL. USA). Blood samples were collected into EDTA-containing tubes, immediately put into ice and centrifuged within one hour. Plasma was separated and stored at 20 ºC until analysis. Hyperhomocysteinemia was defined as homocysteine levels higher than the mean plus two standard deviations of the control group (≥ 13 μmol/l), as they followed a normal ditribution. Vitamin B_{12} and folate concentrations were determined by radioimmunoassay (SimulTRAC-SNB; ICN Pharmaceuticals, Diagnostics Division, Orangeburg, New York) and creatinine concentration was measured by the Jaffé method (Boehringer Mannheim, Germany). Hemoglobin concentration and leukocytes were determined by an Advia 120 autoanalyzer (Bayer, In, USA), and C-Reactive proteine (CRP) by immunonephelometry (Immage, Beckman, Fullerton, USA). The C677T and A1298C MTHFR polymorphisms were investigated by real-time polymerase chain reaction, using hybridization probes on the Light Cycler System (Roche Applied Science, Mannheim, Germany).

The study was approved by the Hospital Ethical committee and all patients gave their informed consent to participate in the study.

Statistical analysis

We used Student´s t test to compare quantitative variables and the χ^2 test or Fisher exact test for qualitative variables. The differences were considered significant at $p < 0.05$. As vitamin B_{12} and folate concentrations followed a non-normal distribution a logarithmic transformation was applied before statistical analysis.

The association between hyperhomocysteinemia and IBD was determined in a first logistic regression analysis, after adjusting for age and gender. The correlation between hyperhomocysteinemia and the independent variables: age, gender and vitamin B_{12} and folate serum levels was investigated in a second analysis. The magnitude of the association was measured by the Odds ratio (OR), with a 95% confidence interval (CI). Statistical analysis was performed using the SAS system, version 12.1, CARY. NC. USA.

RESULTS

The clinical characteristics of the patients and controls are shown in table I.

Hyperhomocysteinemia was present in 9 patients and 7 controls (17.3 *vs.* 3.7%; p = 0,002). Homocysteine concentrations were higher in male patients than in female

Table I. Clinical characteristics of patients and controls

Patients (n = 52)	
Age (years)	41.7 (11.9)
Male/female (%)	23 (44.2)/29 (55.8)
Smoking (%)	18 (34.6)
Ulcerative colitis (%)	16 (30.8)
Crohn´s disease (%)	33 (63.5)
Indeterminate colitis (%)	3 (5.7)
Disease duration (years)	8.0 (6.2)
Ileal resection (%)	3 (5.7)
Corticosteroids (%)	21 (40.4)
5-ASA (%)	38 (73.1)
Azathioprine (%)	12 (23.1)
Thromboembolic event (%)	8 (15.3)
Creatinine (mg/dl)	0.7 (0.1)[a]
Hemoglobin (g/dl)	13.8 (1.8)
Leucocytes (mm^3)	7.840 (2.352)
Creactive protein (mg/dl)	0.65 (0.67)
Controls (n = 186)	
Age (years)	41.9 (10.1)
Male/female (%)	71 (38.2)/115 (61.8)
Creatinine (mg/dl)	0.8 (0.1)

Data are expressed as means with standard deviation of the mean (SD) or number of cases (%); [a]: $p < 0.05$ compared with controls.

Table II. Prevalence of hyperhomocysteinemia and homocysteine, folate and B_{12} vitamin concentrations in patients and controls

	Patients (n = 52)	*Controls (n = 186)*
Hyperhomocysteinemia (%)		
Overall	9 (17.3)[a]	7 (3.7)
Male	7 out of 23 (30.4)[a]	9 out of 71 (12.6)
Female	2 out of 29 (6.8)[a]	0 out of 115 (0)
Homocysteine (μmol/l)		
Overall	9.7 (4.4)	9.1 (1.9)
Male	11.1 (3.6)[b]	10.2 (2.0)[b]
Female	8.0 (3.6)	8.3 (1.6)
B_{12} vitamin (pg/ml)		
Overall	499 (287)[a]	603 (231)
Male	502 (284)	544 (214)
Female	497 (294)[a]	629 (231)
Folate (ng/ml)		
Overall	7.6 (4.1)[a]	8.9 (3.7)
Male	7.0 (3.8)	8.3 (3.4)
Female	8.2 (4.2)	9.2 (4.1)

Data are expressed as means with standard deviation of the mean (SD) or number of cases (%); [a]: $p < 0.05$ compared with controls; [b]: $p < 0.05$ compared with females of the same group.

Table III. Methylenetetrahydrofolate reductase (MTHFR) C677T A1298C polymorphisms in patients and controls: prevalence and homocysteine concentrations

	N° cases (%)	*Homocysteine (μmol/l)*
Patients (n = 52)		
Polymorphisms MTHFR C677T		
TT	7 (13.5)	10.4 (6.2)
CC/CT	45 (86.5)	9.2 (3.5)
Polymorphisms MTHFR A1298C		
CC	4 (7.8)	11.3 (3.5)
AA/AC	48 (92.3)	9.6 (4.5)
Controls (n = 186)		
Polymorphisms MTHFR C677T		
TT	21 (11.3)	9.8 (2.5)
CC/CT	165 (88.7)	9.0 (1.8)
Polymorphisms MTHFR A1298C		
CC	13 (7.0)	8.2(2.2)
AA/AC	173 (93)	9.2 (1.9)

patients (media [standard deviation]) (11.1 [3.6] *vs.* 8.0 [3.6] μmol/l; p = 0.003). Patients had lower folate (7.6 [4.1] *vs.* 8.9 [3.7] ng/ml; p = 0.01) and vitamin B_{12} (499 [287] *vs.* 603 [231] pg/ml; p = 0.003) concentrations compared with controls (Table II). Homocysteinemia was similar in ulcerative colitis and Crohn's disease (9.5 [2.9] *vs.* 9.4 [4.4] μmol/l; p = 0.95).

Patients with hyperhomocysteinemia had lower folate plasma levels (4.6 [2.5] *vs.* 8.2 [4,2] ng/ml), but not lower vitamin B_{12} levels (385 [126] *vs.* 523 [306] pg/ml; p = 0.29). We found no statistical association between hyperhomocysteinemia and smoking, IBD duration, serum creatinine, CRP, or treatment with 5-ASA, corticosteroids or azathioprine.

Six patients (11.5%) presented with a thromboembolic event (5 deep venous thrombosis of the legs and 1 cerebral venous thrombosis). In two cases the event ocurred at the time of diagnosis of IBD, and in the remaining cases subsequently during the active phase of the disease. In the 6 patients homocysteine levels were higher (14.3 [5.8] *vs.* 9.1 [3.9] μmol/l; p = 0.006) and hyperhomocysteinemia was more frequent (50 *vs.* 6.5%; p = 0.02) than in the rest of the patients.

In 7 patients and in 21 controls the MTHRF 677C→T (homozygous TT) mutation was identified (13.5 *vs.* 11.3%; p = 0.66). Homocysteine concentration in subjects with this mutation was similar to that of subjects with other genotypes (CC/TT). The 1298A→C MTHRF (homozygous CC) mutation was present in 4 patients and 13 controls (7.8 *vs.* 7.0%; p = 0.76), and the homocysteine levels were not different from those with other polymorphisms (AA/AC) (Table III). Double heterozygosity for MTHFR C677T and A1298C variants was not more prevalent in patients (30.7 *vs.* 28.5%; p = 0.74) and did not associate higher homocysteine levels (9.1 [1.8] *vs.* 9.8 [3.5] μmol/l; p = 0.41).

Logistic regression analysis showed an OR for IBD in patients with hyperhomocysteinemia, adjusted for age and gender, of 5.51 with a confidence interval of 95%: 1.81-16.76; p = 0,002. Hiperhomocysteinemia was negatively associated with feminine gender (OR 0.08, 95% CI 0.01-0.49; p = 0.006) and folate levels (OR 0.04, 95% CI: 0.007-0.20; p < 0.001), and no correlation with B_{12} vitamin levels was found.

DISCUSSION

The present study shows a significantly higher prevalence of hyperhomocysteinemia in patients with IBD (17.3%) compared with controls. In other studies the prevalence ranges from 11 to 26% (10,11,13,14), although the definition of hyperhomocysteinemia is not the same in all of them. Furthermore we found an association between IBD and hyperhomocysteinemia. Since the difference in proportions of hyperhomocysteinemia between patients and controls was 13.6%, with a statystical power of 87%, we consider it sufficient to demonstrate the association with IBD.

Female patients in the study had lower levels of homocysteine than male, as reported in other studies (11,22). Creatinine concentrations, implicated in hyperhomocysteinemia when increased, were within the normal range in patients as well as in controls. A negative association between hyperhomocysteinemia and folate levels was found, as reported in other publications (10-

12,14). Some drugs used in the treatment of IBD, such as sulfasalazine and methotrexate (16,17) have an antifolate effect, but the patients in our study did not receive this medications, so low levels of folate could be attributed to a decreased intake and/or absorption of this vitamin (15). The low concentrations of vitamin B_{12} has been correlated to hyperhomocysteinemia in some studies of IBD (11,22), although it has not been confirmed in other publications (12,21), including the present study. Ileal resection in Crohn's disease is a cause of vitamin B_{12} deficiency, but there were just 3 cases in our study.

The 6 patients presenting with thromboembolic events had higher levels of homocysteine and increased frequency of hyperhomocysteinemia, so it could represent another thrombogenic factor in IBD, apart from those enumerated in previous publications (2).

The prevalence of the MTHFR 677C→T mutation was not higher in patients, which correlates with the results of a similar study published by Papa et al. (14). Although other authors identified a higher prevalence of homozygous TT among patients with IBD, this mutation did not represent the sole cause of hyperhomocysteinemia (21). It has been reported that homozygous TT carriers present higher levels of homocysteine (23), which differs from our data, probably due to the few patients with this mutation in our series.

The MTHRF 1298A→C mutation increases homocysteine levels, to a lesser extent than 677C→T (19), and is associated with an increased risk of thrombosis (24), although this result has not been confirmed in other studies (19,25). The prevalence of the MTHFR 1298A→C mutation, not evaluated in patients with IBD to date, was not increased in our study neither were homocysteine levels in patients with this mutation. Double heterozygosity for MTHFR C677T and A1298C polymorphisms have been associated with hyperhomocysteinemia in some cases (19) but not in others (26,27). In our series, the prevalence of this double heterozygosity was not increased and homocysteine levels were not higher in carriers.

Vitamin B_{12} an folate supplementation in patients with IBD significantly reduced homocysteine concentrations (10,21). Since IBD is relatively frequent in our country (28,29), and taking into account that thromboembolic complications are underdiagnosed, it would be convenient to consider thrombogenic factors, such as hyperhomocysteinemia associated to low folate levels, as demonstrated in the current study.

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