SCIENTIFIC REPORT

5,10-Methylenetetrahydrofolate reductase C677T gene polymorphism in Behçet's patients with or without ocular involvement

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Br J Ophthalmol 2005;**89**:1634–1637. doi: 10.1136/bjo.2005.076836

Background: Increased serum levels of homocysteine (Hcy) have been reported in patients with Behçet's disease (BD) with an established risk factor for vascular involvement. Recently, the authors demonstrated that elevated Hcy levels are associated with ocular involvement in such patients. On the other hand, elevated levels of Hcy can result from genetic errors. Indeed, a mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR C677T) gene influences Hcy metabolism and, therefore, MTHFR C677T polymorphism provokes hyperhomocysteinaemia.

Aim: To investigate the possible genetic factor for the elevation of plasma Hcy level in patients with BD by examining gene interaction with the MTHFR C677T polymorphism, a crucial factor of the Hcy metabolism. In addition, the authors aimed to evaluate if there is an association between the C677T polymorphism and the presence of ocular involvement in such patients.

Method: A total of 59 patients with BD (25 men, 34 women) with a mean age of 34.9 years and 42 age and sex matched healthy control subjects (19 men, 23 women; mean age 32.2) were included in this investigation. MTHFR gene polymorphism was investigated by the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) of a genomic DNA fragment at nucleotide 677 in all subjects in both groups. The genetic equilibrium is assumed for the gene frequencies of the MTHFR polymorphism in both samples.

Results: The genotype of the MTHFR gene differed between the Behçet's patients and control subjects (TT: 11.9 *v* 2.4%; CT: 55.9 *v* 61.9%; CC: 32.2 *v* 35.7 %). TT homozygous genotype was more frequently in BD patients than the controls, though the difference was not significant ($p = 0.063$). In BD patients with ocular involvement, however, the frequencies of MTHFR TT homogenetic type (27.8%) were significantly and statistically higher than those in BD patients without ocular involvement (4.9%, $p = 0.022$, odds ratio = 7.5), or the controls (2.4%, $p = 0.003$, odds ratio = 20.0). TT homozygous genotype was associated with an increased risk for ocular involvement.

Conclusion: Elevated serum levels of Hcy seem to be a result of C677T polymorphism of the MTHFR gene, with increased TT individuals over CC and CT genotype BD patients. Although no association was shown between the MTHFR reductase C677T polymorphism and the increased risk of oral aphtahe or genital ulcers, a mutation in this gene was associated with an increased risk of ocular involvement, suggesting genetic instability with a potential initiation of Hcy lowering therapy in this patient group.

Methylenetetrahydrofolate reductase (MTHFR) is a crucial enzyme that regulates the metabolism of homocysteine (Hcy) and methionine by catalysing the reduction of 5,10-methylene THF to 5-methyl THF, the methyl donor for methionine synthesis from Hcy.[1] Genetic polymorphisms (mutation) of the C677T MTHFR are associated with reduced enzyme activity and, therefore, cause impaired remethylation of Hcy to methionine with subsequent hyperhomocysteinaemia.[2] Hyperhomocysteinaemia, in turn, describes a mild to moderate elevation of Hcy in blood or serum, resulting in a cascade of cytokine activation and lipid peroxidation with vascular endothelial injury, prothrombotic surface, atherothrombogenesis, thromboembolism, and systemic and retinal vascular occlusive disease.[3 4]

Behçet's disease (BD), first described in 1937 by a dermatologist Dr Hulusi Behçet from Istanbul, as a triad of symptom complex (oral aphthae, genital ulcers, hypopyon uveitis), is a chronic, relapsing, multisystemic idiopathic inflammatory disease characterised by an occlusive vasculitis.[5–9] This unique disorder is endemically higher, particularly in Turkey and Japan with a prevalence between 8/10 000 and 42/10 000, the population derived historically from the ancient Silk Road.[10 11] It occurs more commonly in men than in women and affects primarily subjects between the second and fourth decades of life, with a more aggressive course in young male adults.[4] The leading cause of chronic morbidity is high especially with ophthalmic inflammation, which can eventually result in blindness. Although various aetiopathogenic mechanisms have been suggested, the management of the disease with severe organ involvement is still unsatisfactory.[12]

Hcy is suggested to be a new risk factor in the hypercoagulability state and in thrombotic complications of BD patients. Indeed, we have recently demonstrated that serum levels of Hcy are increased and correlated with ocular disease.[13] This novel finding was supported by various independent investigations with genetic implications.[14–16] Because hyperhomocysteinaemia is assumed to be an independent and correctable risk factor for venous thrombosis in such patients, such recent evidence and our results on ocular BD gave us the unique opportunity to test further the hypothesis that polymorphisms of the MTHFR C677T gene may be the underlying variant for the demonstrated hyperhomocysteinaemia. Therefore, this study evaluated the association between the MTHFR C677T polymorphism and BD and the significance of ocular BD in relation to the gene polymorphism.

Abbreviations: BD, Behçet's disease; Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

MATERIALS AND METHODS

The local ethics committee of the Erciyes University Faculty of Medicine approved the initial research proposal. A total of 59 Turkish patients with BD (25 men, 34 women) with a mean age of 34.9 (SD 10.1) years and 42 age and sex matched healthy controls (19 men, 23 women; mean age 32.2 (8.6)) from a similar ethnic background were included in the present investigation. All patients were diagnosed according to the criteria of the International Study Group for Behçet's Disease.[17] After all patients and control subjects gave their informed consent to participation in this study, MTHFR gene polymorphism was investigated by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of a genomic DNA fragment in all subjects in both groups. The genetic equilibrium is assumed for the gene frequencies of the MTHFR polymorphism in both samples.

The details of the ocular BD patients were obtained from case notes and ocular examinations were performed by the experienced ophthalmologists using a standard procedure. In particular, evidence for retinal vascular occlusion was sought. BD patients with an end stage ocular disease (phthisical or completely blind) were assumed to have retinal vaso-occlusive disease if the posterior segment could not be visualised.

Genetic evaluation/MTHFR genotyping

Genomic DNA was isolated, using standard methods.[18] The DNA's sites were analysed by PCR based RFLP methods as described previously.[19] PCR was performed in a Perkin Elmer 9600 and the profile consisted of an initial melting step of 2 minutes at 94℃; followed by 35 cycles of 30 seconds at 94℃, 30 seconds at 61℃, and 30 seconds at 72℃; and a final elongation step of 7 minutes at 72℃. PCR primers (5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and 5′-AGG ACG GTG CGG TGA GAG TG-3′) were used to amplify a portion of the MTHFR gene from 100 ng of genomic DNA in a 50 µl reaction containing 5 µl of 10X PCR buffer, 0.15 mM dNTP, 1.5 mM $MgCl_2$, 0.6 pM each primers, and two units of Taq DNA polymerase.

After amplification, the 198 bp PCR product was digested with *Hinf I* in a 20 µl reaction solution containing 10 µl of PCR product, 2 µl of 10X buffer, and five units of *Hinf I* at 37℃ overnight.

The digestion products were separated on 3% agarose gels, and fragments stained with the ethidium bromide were photographed on a ultraviolet transilluminator. Wild type (CC) individuals were identified by only a 198 bp fragment, heterozygotes (CT) by both the 175/23 bp, and homozygote variants (TT) by the 175 bp.[19]

Statistical analysis

Results were given as the mean (standard deviation, SD). The software SPSS for Windows version 10.0 was used to perform statistical analysis. The χ^2 test was used to analyse differences between the patients and controls. Odds ratio (OR) and their 95% confidence intervals (CI) were used to estimate the risk for ocular involvement. A multiple logistic regression model was used to identify risk factors for ocular involvement in patients with BD.

RESULTS

The most frequent clinical symptoms in BD patients were oral apthea (100%), genital ulcers (91.5%), arthralgia (67%), papulopustular eruptions (62.7%), erythema nodosum (49.2%), ocular disease (30.5%), neurological findings (11.8%), and gastrointestinal symptoms (8.4%). No arterial or venous vascular disease was detected. A positive pathergy reaction was observed in 18 patients (30.5%). Thirteen of of 18 patients with ocular disease had panuveitis with occlusive vasculitis and the remaining five had only anterior uveitis.

There were three possibilities of genotypes, including TT, CT, and CC, about base variation of MTHFR gene at locus 677. The genotype of the MTHFR gene differed between the Behçet's patients and control subjects (TT 11.9 *v* 2.4%; CT 55.9 *v* 6 %; CC 32.2 *v* 35.7%). The distributions of the MTHFR genotypes in patients and controls are shown in table 1. Overall, the C677T polymorphism of the MTHFR gene was not significantly different in frequency in patients with BD and controls (67.8% *v* 64.3%; $p = 0.678$, odds ratio = 1.276). Although the frequency of TT homogenetic type was higher in BD patients than the controls (11.9 *v* 1.9%), the difference was not significant ($p = 0.063$; odds ratio = 7); the frequency of CT allele was not different in patients and controls either ($p = 0.702$). Similarly, the CT genotype was not significantly different in ocular BD patients compared with the non-ocular BD patients or controls ($p = 0.718$ and $p = 0.264$, respectively).

However, the frequencies of TT genotype were significantly higher in ocular BD patients (27.8%) than those in non-ocular BD patients (4.9%) (OR: 7.5, CI: 1.29 to 43.43, $p = 0.022$) or controls (OR: 20.0, CI: 2.14 to 186.3, $p = 0.003$; fig 1). The presence of the TT allele appeared to have a strong association with the development of ocular disease in Behçet's patients. Family history was elicited in four patients (6%), one of them had homozygous TT and the others had CT genotype. No correlation or relation was found between the MTHFR polymorphism with erythema nodosum ($p = 0.646$), papulopustular lesions ($p = 0.949$), arthralgia ($p = 0.728$), neurological involvement ($p = 0.986$), gastrointestinal symptoms ($p = 0.423$), and positive family history ($p = 0.182$).

DISCUSSION

An activated haemostatic system with arterial and venous occlusive process or thrombus formation has been demonstrated during the course of BD.[20 21] Prothrombin gene G20210A mutations and increased levels of a mutant blood clotting factor of G1691A (factor V Leiden), especially in ocular BD, further supported this thrombotic tendency, indicating a systemic prothrombotic (hypercoagulable) state with endothelial cell activation in such patients.[22–26]

Although genetic thrombotic defects, impaired coagulation, defective fibrinolysis, and endothelial injury or dysfunction with many other immunoinflammatory molecules have all been proposed as contributors,[27–32] the underlying cause of such a thrombotic state in BD still remains to be identified. Recent studies have reported that the elevated levels of homocysteine are related to arterial and venous thromboembolism.[33–35] These studies suggest that homocysteine may only

Table 1 The distributions of MTHFR genotypes in patients and controls

Groups	No	CC	CT	TT
BD patients	59	19 (32.2%)	33 (55.9%)	7 (11.9%)
BD patients with ocular involvement	18	4 (22.2%)	18 (50.0%)	5 (27.8%)
BD patients without ocular involvement	41	15 (36.6%)	24 (58.5%)	2 (4.9%)
Control subjects	42	15 (37.5%)	26 (61.9%)	1 (2.4%)

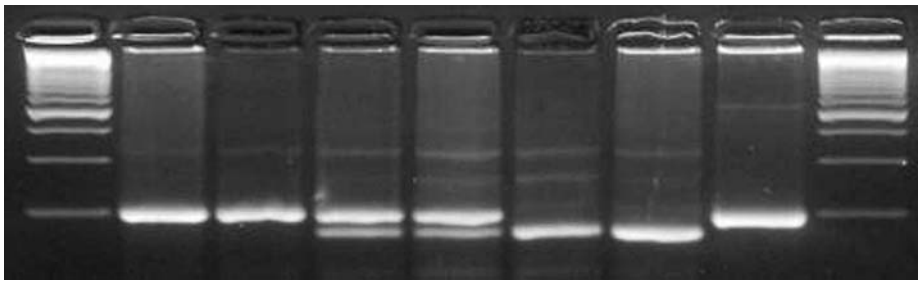


Figure 1 Gel photography of 5,10-methylenetetrahydrofolate reductase C677T gene polymorphism in ocular Behçet's disease. First and last columns (BM) = size markers; column 2–3 = homozygote normal; column 4–5 = heterozygote; column 6–7 = homozygote mutant; column 8 = undigested PCR product.

exert an effect on vascular risk in synergy with other risk factors. In addition, we have reported that serum levels of Hcy are increased and correlated with ocular disease in Behçet's patients.[13] Moreover, C677T polymorphism in MTHFR gene may lead to hyperhomocysteinaemia in such patients.[36 37] Heterozygous or homozygous individuals for mutations with an alanine to valine substitution have reduced enzyme activity and thermolability, resulting in elevated plasma Hcy caused by suboptimal intake of folate. This study aimed to investigate the MTHFR C677 polymorphism in patients with BD and evaluate if there was an association with ocular involvement.

We found equal frequencies of MTHFR C677T polymorphism overall in patients and healthy controls. Although the frequency of the TT homozygous genotype was higher than in the controls, the difference was statistically not different. On the other hand, TT homozygous genotype was significantly more frequent in patients with ocular BD. Therefore, it is possible to speculate that the MTHFR C677TT genotype and related hyperhomocysteinaemia might further increase the risk of ocular vascular involvement and related complications during the course of BD.

In the normal population, the frequency of MTHFR C677T polymorphism may differ from the country to country, and this mutation is not the unique factor that regulates the homocysteine levels.[2 38] Indeed, it has been hypothesised that the region linked to the MTHFR polymorphism is involved in folate binding and that the enzyme may be stabilised in the presence of sufficient levels of folate; the World Health Organization has proposed a lower limit of 13.6 nmol/l for folate concentrations.[39 40] It is plausible to speculate that the combination of the genetic defect and inadequate folate intake may cause elevated Hcy concentrations, and this elevation of Hcy could be corrected with folic acid supplements. However, such a speculation is open to further research.

Since hyperhomocysteinaemia could be caused by deficiencies of nutritional factors, dietary supplements of folic acid and vitamin B_{12} might reduce the elevated plasma Hcy levels. Moreover, another common polymorphism (1298A–C; glutamate to alanine) has recently been reported as an association with hyperhomocysteinaemia, but this polymorphism was suggested to increase homocysteine only in individuals who carried the bp677 variant.[38]

In conclusion, this study further supports our previous studies and demonstrates for the first time that the MTHFR C677T polymorphism (TT genotype, but not CT genotype) may represent as a genetic risk factor for BD, particularly for ocular vascular events. However, before definitive conclusions can be reached, long term, large scale interventional studies assessing both Hcy levels and folate status with related gene polymorphism in BD are needed. Similarly, clinical trials, especially in high risk populations, await further investigation before a novel therapeutic approach is formulated for this unique groups of patients.

ACKNOWLEDGEMENTS

The authors have no proprietary or commercial interest in any concept or product discussed in this article.

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Accepted for publication 8 August 2005

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