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The A677V Methylenetetrahydrofolate Reductase Gene Polymorphism and Carotid Atherosclerosis

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Background and Purpose—The alanine/valine (A/V) polymorphism at codon 677 of the 5,10 methylenetetrahydrofolate reductase (*MTHFR*) gene correlates with elevated levels of plasma homocysteine and with an increased risk of atherosclerotic cardiovascular disease. Our study was designed to assess the frequency of the A and V alleles in patients with asymptomatic severe carotid artery stenosis (CAS) assessed by extracranial duplex examination in comparison with age- and sex-matched subjects without carotid atherosclerosis.

Methods—Consecutive patients (n=48; 28 men, mean±SD age 67.1±11.4 years) with asymptomatic severe (>75%) CAS were compared with subjects without CAS (n=26; 15 men, aged 61.2±11.5). The *MTHFR* genotype was analyzed by polymerase chain reaction followed by *HinfI* digestion. The χ^2 analysis and *t* test were used to compare the groups.

Results—The frequency of V alleles was significantly higher in the CAS group (0.47) compared with control subjects (0.27, χ^2 test; OR 2.4 [95% CI 1.1 to 5.3]; *P*<0.02).

Conclusions—Our results indicate that the *MTHFR* A677V allele is significantly associated with severe CAS. (*Stroke*. 1999;30:2180-2182.)

Key Words: amine oxidoreductases ■ atherosclerosis ■ carotid stenosis ■ genetics ■ ultrasonography, Doppler

Homocyst(e)ine [H(e)] denotes homocysteine and its oxidized forms, homocystine and cysteine-H(e) disulfide, which are derived from the intracellular metabolism of methionine and circulate bound to proteins in the plasma.¹ Serum concentrations of H(e) are increased in 15% to 40% of patients with coronary, cerebral, or peripheral arterial diseases.²⁻⁷ Recent reports have suggested that the risk of carotid artery stenosis is increased in subjects with even slightly elevated H(e) concentrations, previously considered to be in the normal range.³ H(e) can be transsulfurated to form cysteine or remethylated to form methionine. The latter reaction uses 5-methyltetrahydrofolate as a carbon donor: 5-methyltetrahydrofolate is synthesized from 5,10-methylenetetrahydrofolate through the action of the methylenetetrahydrofolate reductase (*MTHFR*). Abnormalities of *MTHFR* are an important cause of hyperhomocyst(e)inemia. A polymorphism of *MTHFR* consisting of an alanine-to-valine substitution at codon 677 (A677V) generates a thermolabile form of the enzyme.⁴ The plasma H(e) levels in carriers of this polymorphism, especially homozygous individuals, are significantly higher than those of other individuals, which suggests that this mutation may be a risk factor for coronary artery disease (CAD) and may also influence the severity of the disease.⁵⁻⁷ In the present study, we assessed the frequency of the alanine and valine alleles in patients with

severe (>75%) carotid artery stenosis (CAS) compared with age- and sex-matched subjects without CAS. We hypothesized that the A677V allele would constitute a risk factor for CAS.

Subjects and Methods

Patient Selection

A total of 74 subjects referred to our Doppler laboratory for assessment of either asymptomatic carotid bruit or for nonspecific symptoms such as dizziness or headache were included in the study. Forty-eight consecutive patients (28 men, mean±SD age 67.1±11.4 years) with asymptomatic severe (75% to 99%) CAS were compared with 26 control subjects (15 men, aged 61.21±1.5 years) who were found to have nonsignificant (<25%) carotid stenosis. All subjects were of Israeli Jewish origin. Vascular risk factors were recorded and defined according to our stroke registry as previously described.⁸

Carotid artery disease was assessed and defined by duplex sonography (Diasonics Gateway2D) in all subjects according to validated criteria.⁹ Genomic DNA was isolated from peripheral blood cells by standard procedures.

Detection of the A677V Polymorphism

The primers for analysis and polymerase chain reaction (PCR) conditions were based on the methods described by Frosst et al.¹⁰ The PCR reaction generated a fragment of 198 bp that contains codon 677. The point substitution of T for C at codon 677 creates a *HinfI* recognition sequence with resulting 175- and 23-bp fragments. Alanine-coding alleles therefore produced a 198-bp fragment that

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TABLE 1. Clinical Characteristics of Study Patients With CAS and Control Subjects Without CAS

	CAS Cases (n=48)	Controls (n=26)
Mean±SD age, y	67.1±11.4	61.2±11.5
Male gender, n	27 (56%)	14 (54%)
Smoking, n	30 (62%)*	7 (27%)
Coronary artery disease, n	17 (36%)	4 (15%)
Periphery artery disease, n	10 (21%)	0 (0%)
Hypertension, n	22 (46%)	12 (46%)
Hyperlipidemia, n	12 (25%)	14 (54%)

The number of patients (% of total group) in the CAS and control groups are presented for each vascular risk factor.

* $P<0.005$ vs controls, χ^2 test.

was easily distinguished from the 175-bp fragment generated by valine alleles. The fragments were separated on 4% metaphor (FMC BioProducts) agarose gels and visualized with ethidium bromide.

Statistical Analysis

The χ^2 analysis and t test were used to compare the different parameters between the groups. The Mantel-Haenszel analysis was used post hoc to examine interaction between the *MTHFR* genotype and other risk factors.

Results

The clinical characteristics of the 48 subjects with CAS and the 26 subjects without significant carotid stenosis are shown in Table 1. The frequency of hypertension, hyperlipidemia, and peripheral artery disease (PAD) were similar in both groups. Mean age, the prevalence of smoking, and the prevalence of coronary artery disease (CAD) were higher in the study group than in the control group, although only the effect of smoking was statistically significant ($P<0.005$, χ^2 test).

The distribution of the *MTHFR* codon 677 A and V alleles in both groups is presented in Table 2. As can be seen, the frequency of V alleles was significantly higher in the CAS group (0.47) compared with controls (0.27, $P<0.02$, OR=2.4 [95% CI 1.1 to 5.3]). Examination of the distribution of AA, AV, and VV genotypes in the CAS and controls groups (Table 2) revealed that there was a significantly higher frequency of both heterozygous and homozygous V allele carriers in patients with CAS.

To exclude risk factors known to be associated with CAS that may interact with the effect of *MTHFR*, we used 2×2 tables and further analyzed the interaction of the *MTHFR* mutations with age, gender, PAD, CAD, smoking, hyperten-

TABLE 2. *MTHFR* Codon 677 Genotypes in CAS Patients and Control Subjects Without CAS

	CAS Cases (n=48)	Controls (n=26)
V allele frequency	47%*	27%
AA	12 (25%)*	15 (58%)
AV	27 (56%)*	8 (31%)
VV	9 (19%)*	3 (11%)

* $P<0.02$, χ^2 test.

TABLE 3. Interaction of *MTHFR* Codon 677 Genotypes and Vascular Disease

	CAS	Controls	Total
CAD/PAD positive	15/19 (79%)	2/4 (50%)	17/23 (74%)
CAD/PAD negative	21/29 (72%)	9/22 (41%)	30/51 (59%)

The proportion of patients with the *MTHFR* A677V allele relative to the total number of patients with either CAD or PAD or both (CAD/PAD positive) or neither (CAD/PAD negative) is subdivided into those with CAS or controls. Although the A677V allele was more frequent in the CAS patients than in the controls for either group, only in the CAD/PAD-negative patients did the association of the A677V allele with CAS reach significance ($P=0.023$, χ^2 test).

sion, and hyperlipidemia. We found no statistically significant relationship between *MTHFR* genotype and any of these vascular risk factors. Because the factors most influenced by *MTHFR* genotype are CAD and PAD, we examined the effect of the V allele on CAS in patients with neither CAD nor PAD. The results of this analysis are presented in Table 3. As can be seen, the *MTHFR* A677V allele is significantly associated with CAS in patients with neither CAD or PAD whereas this association, although qualitatively similar, failed to achieve statistical significance in patients with either of these risk factors, possibly due to the small number of cases in some categories.

Discussion

Our study was designed to assess the frequency of the *MTHFR* A677V allele in subjects with carotid atherosclerosis. We found that this allele is significantly associated with severe CAS. Several recent studies have shown that high H(e) levels are associated with the severity and progression of atherosclerotic disease.^{3-7,10-12} The association of the *MTHFR* A677V allele with high serum H(e) levels is well established and is more pronounced in homozygous carriers. In spite of this, a clear association between this allele and CAD is difficult to demonstrate.^{13,14} Interestingly, there was a trend toward an association of this allele with PAD and CAD independent of the presence of CAS in the present study. The many confounding risk factors for stroke may explain why a previous study that examined the association of cerebrovascular disease and the A677V allele failed to detect any significant interaction.¹³ More relevant to the present results is a previous report¹⁵ that did not find any relationship between the *MTHFR* gene polymorphism and CAS. This study differed from the present one in that all the study patients were symptomatic for cerebrovascular disease (not necessarily due to CAS), that patients with moderate (>50%) CAS were included (in contrast to the present study, which included only those with more severe CAS), and that the control group was not studied by duplex sonography and therefore did not exclude patients with asymptomatic CAS. These factors, especially the more homogeneous nature of the patients in the current study, may account for the discrepancies between the 2 studies.

Our present findings suggest that the A677V allele of *MTHFR* is a genetic risk factor for carotid artery stenosis. This effect is not more pronounced in homozygous carriers (Table 2). Whether this is linked to H(e) levels is not clear, since these levels were unavailable for study and the lack of

a gene dose effect would seem to argue against the effect of the A677V allele in CAS directly through H(e) levels. Significantly, the association of the V allele with CAS was also independent of the existence of CAD and PAD (Table 3). It is interesting to note that the allele frequency of the Jewish population in Israel is approximately 0.40,¹⁶ which is lower than the CAS group but higher than the control group in the present study. The controls in the present study were selected for the absence of CAS, which is significant in light of the 20% to 30% CAS expected in the general population of this age.¹⁷ These results are compatible with the *MTHFR* A677V allele being a risk factor for CAS, because its frequency in CAS-free controls is lower than in unselected controls and highest in patients with significant CAS.

H(e) may contribute to both atherosclerotic and thrombotic processes by modulating vascular cell proliferation and promoting prothrombotic activity in the vascular wall. These effects of H(e) may underlie any correlation between the A677V allele and atherosclerosis in the carotid artery and elsewhere. Aside from the significant association between the A677V allele and CAS, we found no other risk factors associated with CAS except smoking. This risk factor did not interact significantly with the *MTHFR* genotype and thus did not seem to affect the conclusions of the study. In contrast to reports by others,^{11,12,18} there were no significant differences between CAS and the control group in terms of high blood pressure and hyperlipidemia in our study. The cholesterol concentration was not found to be associated with concurrently determined carotid artery atherosclerosis. From the present relatively small study, it is not clear whether A677V is an independent risk factor for CAS. There was, however, no significant correlation between the *MTHFR* genotypes and the other examined vascular risk factors. It is especially relevant that the A677V allele was a risk factor for CAS in patients with no CAD or PAD, which themselves have been proposed to be associated with *MTHFR* polymorphisms. Although the sample size of the present study was sufficient to confirm the prestudy hypothesis that *MTHFR* genotype may influence CAS, a much larger study is needed to assess the interplay between genetic and environmental factors.

In conclusion, our results indicate that the *MTHFR* A677V allele is significantly associated with CAS. *MTHFR* 677 A/V genotyping may be of clinical importance as a prognostic and therapeutic marker, although further studies are needed to substantiate this hypothesis.

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