Association of Methylenetetrahydrofolate Reductase Gene Polymorphism With Carotid Arterial Wall Thickening and Myocardial Infarction Risk in NIDDM

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Hyperhomocysteinemia has been identified as an independent risk factor for arteriosclerotic diseases such as myocardial infarction (MI), peripheral arterial occlusive disease, and cerebrovascular disease (1-4). In contrast to the very low prevalence of homocysteinuria (a hereditary disease characterized by severe hyperhomocysteinemia, homocystinuria, and early-onset atherosclerotic diseases), individuals with mild homocysteinemia are frequently observed. Recently, a significant portion of these individuals was shown to have thermolabile 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR is an enzyme that catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and is thereby involved in the remethylation of homocysteine to methionine (5,6). The thermolability seems to be caused by a C->T substitution at nucleotide 677 (Ala->Val substitution) of the MTHFR-encoding gene (7). As compared with individuals homozygous for the 677C allele, those homozygous for the 677C->T mutation, which account for >10% of a French-Canadian population, revealed ~70% decrease of the MTHFR activity (7,8). Those individuals also revealed a 30-50% increase of plasma homocysteine levels when the folate level was below the median value (8).

As a step to understanding the pathophysiological role of the 677C->T mutation in the MTHFR gene in the development of vascular disease in diabetes, we examined the effect of the mutation on carotid atherosclerosis assessed by high-resolution ultrasonic B-mode imaging. The subjects in this study were 222 Japanese unrelated NIDDM subjects (163 male and 59 female, age 59.8 ± 8.4 years (40-75) (mean ± SD [range]). Those subjects with liver disease, renal dysfunction, and/or collagen disease were excluded. Fasting blood samples were drawn from the subjects, and serum total and HDL cholesterol, triglyceride, uric acid, creatinine, plasma glucose, and HbA1c levels were determined by the Clinical Research Center in Osaka University Hospital following standard laboratory protocols. In some of the subjects, serum homocysteine levels were also measured as total homocysteine by high-performance liquid chromatography (HPLC) with fluorescence detection as previously described (9). Blood pressure data were means of three determinations. All cases of MI had been diagnosed according to the symptoms plus either enzyme elevations or diagnostic ECG changes. Each subject gave informed consent for being enrolled in this study, which was approved by the Ethical Committee for Human Studies at Osaka University School of Medicine.

Ultrasoundographic scanning of the carotid arteries was performed using an echotomographic system (EUB-450, Hitachi Medico, Tokyo, Japan) with an electrical linear transducer (midfrequency of 7.5 MHz). Scanning of the extracranial carotid arteries in the neck (from bulb to bifurcation region) was performed bilaterally in three different longitudinal projections (i.e., anterior-oblique, lateral and posterior oblique) as well as the transverse projections used for screening. From the photographed images, the intimal plus medial thickness (IMT) was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line of the far and near walls. The average IMT was calculated as described previously (10,11). At each longitudinal projection, three determinations of IMT were conducted at the site of the greatest thickness and at two points, 1 cm upstream and 1 cm downstream, from the site of the greatest thickness. The greatest value among the six averaged IMT (three different longitudinal projections from each side) was used as the representative value for each individual.

Genomic DNA was isolated from peripheral blood cells, and the MTHFR genotypes were determined by polymerase chain reaction (PCR) and HinfI digestion, essentially as described by Frosst et al. (7). Amplification by PCR was performed using the forward and reverse primer in the initial denaturation step for 5 min at 96°C, 35 cycles of denaturation for 50 s at 93°C, primer annealing for 50 s at 55°C, and primer extension for 30 s at 72°C, followed by a final extension step for 7 min at 72°C. After digestion with HinfI, the samples were separated on 3% agarose gel and visualized with ethidium bromide.
TABLE 1
IMT and other clinical manifestations in MTHFR genotype groups

| Genotype          | 677T/677T | 677T/677C | 677C/677C |
|-------------------|-----------|-----------|-----------|
| n                 | 39 (15.8%)| 94 (43.6%)| 89 (40.6%)|
| IMT (mm)          | 1.58 ± 0.54| 1.35 ± 0.35| 1.41 ± 0.31| 0.043* |
| Sex (M/F)         | 29/10     | 72/22     | 62/27     | 0.56†  |
| Age (years)       | 60.6 ± 8.6| 60.6 ± 8.3| 58.8 ± 8.3| 0.37*  |
| Diabetes duration (years) | 13.7 ± 9.9| 13.5 ± 9.9| 12.2 ± 9.7| 0.60*  |
| HbA1c (%)         | 7.6 ± 1.6 | 8.0 ± 1.7 | 7.6 ± 1.4 | 0.33*  |
| BMI (kg/m²)       | 23.0 ± 3.1| 23.1 ± 2.9| 23.5 ± 3.5| 0.84*  |
| Total cholesterol (mg/dl) | 202.2 ± 37.6| 199.9 ± 36.5| 207.1 ± 37.6| 0.35*  |
| Triglycerides (mg/dl) | 143.4 ± 70.7| 132.4 ± 66.9| 140.4 ± 71.2| 0.73*  |
| HDL cholesterol (mg/dl) | 49.8 ± 13.4| 50.9 ± 13.7| 50.5 ± 16.3| 0.80*  |
| Uric acid (mg/dl) | 4.74 ± 0.99| 5.29 ± 1.40| 5.28 ± 1.41| 0.14*  |
| Serum creatinine (mg/dl) | 0.90 ± 0.20| 0.97 ± 0.31| 0.92 ± 0.29| 0.49*  |
| Systolic blood pressure (mmHg) | 130.8 ± 18.9| 133.4 ± 13.2| 137.5 ± 15.2| 0.29*  |
| Diastolic blood pressure (mmHg) | 77.9 ± 10.4| 75.9 ± 8.3| 77.4 ± 9.7| 0.59*  |
| Smoker/nonsmoker  | 16/23     | 26/68     | 30/59     | 0.31†  |

Data are n (%) or means ± SD. Based on Bonferroni’s comparison, P values are 0.03 and 0.015 for 677T/677T, 0.03 for 677T/677C, and 0.015 for 677C/677C. *Kruskal-Wallis test; †χ² test.

The Kruskal-Wallis test was used to evaluate differences among three MTHFR genotype groups, and the Bonferroni procedure was used for all combinations. The χ² test was used to assess deviation from the Hardy-Weinberg equilibrium and the difference in smoking habit history. Fisher’s exact test was used to assess the difference in the risk for MI among the three MTHFR genotype groups. These statistical analyses were performed using HALBAU statistical software (Gendai-Sugaku-sha, Kyoto, Japan) on a personal computer. The threshold of statistical significance was taken as P < 0.05.

As observed in recent studies with white subjects (7,8,12), the mutation was also common in the Japanese NIDDM population. The allele frequency for the mutation (677T) was 37.6%, which was comparable to that observed in the French-Canadian population (38%) (7). The frequencies for 677T/677T, 677T/677C, and 677C/677C were 15.8, 43.6, and 40.6%, respectively (Table 1), with the genotype distribution being in accordance with the Hardy-Weinberg equilibrium. A similar genotype distribution was observed for nondiabetic Japanese (K.A., Y.Y., unpublished observations).

To evaluate the effect of the 677C→T mutation on arterial wall thickening, the 222 NIDDM subjects were divided into three groups according to the genotype for the mutation. As shown in Table 1, there was no significant difference among the three MTHFR genotype groups in age, BMI, frequency of smoking, or duration of diabetes. Also, biochemical examinations revealed no differences in serum total and HDL cholesterol, triglyceride, uric acid, creatinine, plasma glucose, and HbA1c levels, as well as systolic and diastolic blood pressures.

Despite no differences in those background factors which can affect the development of atherosclerosis, the 677T/677T group displayed significantly higher average IMT values (1.58 ± 0.54 mm) than the 677T/677C group (1.35 ± 0.35 mm; P = 0.03) or the 677C/677C group (1.31 ± 0.31 mm; P = 0.015). The difference between the 677T/677C and 677C/677C groups was not significant (P = 0.68). Thus these observations suggest that the 677C→T mutation (Ala→Val substitution), at least when occurring in homozygotes in NIDDM patients, contributes to the development of atherosclerosis.

To investigate whether the 677C→T mutation contributes to the induction of hyperhomocysteinemia also in the Japanese population, we measured blood homocysteine levels in a total of 66 subjects that were randomly chosen from each genotype group. The results indicated that total homocysteine levels in the 677T homozygotes (19.8 ± 7.4 nmol/ml; n = 22) were higher than those in the 677C homozygotes (15.4 ± 5.7 nmol/ml, P = 0.01; n = 22). The difference between the 677T homozygotes and the 677T/677C heterozygotes (15.5 ± 3.1 nmol/ml, P = 0.29) did not reach statistical significance.

The prevalence of MI was also investigated and found to be significantly higher in the 677T/677T group (7/39 [17.9%]) than in the 677T/677C (6/94 [6.4%]; P = 0.047) or 677C/677C group (3/89 [3.4%]; P = 0.009). The 677T/677C group displayed no significant difference from the 677C/677C group.

To date, the influence of the 677C→T mutation on the thermolabile MTHFR on ischemic heart disease has been studied in various populations (6,12–15). Some studies have revealed a positive correlation between the mutation (or thermolability) and the MI risk (6,15), while others have not (12–14). This inconsistency may be related to the fact that the impact of the 677C→T mutation on plasma homocysteine levels seem to be dependent on diet patterns such as the levels of folate intake and consumption (13,14). Also, the effects of the 677C→T mutation in MTHFR gene may be amplified in subjects with certain risk factors such as diabetes. Evaluation of carotid artery thickening in populations with various risk factors should be helpful for understanding the clinical significance of the MTHFR mutation.

In conclusion, our present observations suggest that the 677C→T mutation (Ala→Val substitution) of the MTHFR gene, which is known to cause mild hyperhomocysteinemia, is a strong genetic factor for accelerating arterial wall thickening in NIDDM patients. This effect of the mutation is likely to play a primary role in increasing the risk for MI and other arteriosclerotic vascular diseases. Because hyperhomocys-
teinemia can be normalized by safe, simple treatment with vitamin B6, folic acid, and/or betaine (16), determining the genotype for the 677C→T mutation in the MTHFR gene in high-risk individuals such as NIDDM patients should be clinically useful for helping to prevent atherosclerotic disease.

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