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Methylenetetrahydrofolate Reductase Gene Polymorphism and Ischemic Stroke: Sex Difference in Japanese

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Moderately elevated plasma homocysteine levels have been established as independent risk factors in vascular disease, including ischemic stroke. Recently, a common mutation (C677T) in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene reducing the activity of MTHFR and increasing homocysteine levels in plasma was reported. The C677T MTHFR mutation may be a risk factor for ischemic stroke, but the results of previous studies have been conflicting. One possible explanation is that the association with the MTHFR genotype may be different according to gender. To investigate the association for ischemic stroke, we conducted a case-control study of 77 hospital cases (49 men and 28 women) with ischemic stroke and 229 (120 men and 109 women) control subjects in Japanese. The prevalence of conventional vascular risk factors and MTHFR genotypes were determined in case and controls. After adjustment by multiple analysis in all there was no statistical significance in MTHFR genotypes. The conventional vascular risk factors such as diabetes mellitus (adjusted odds ratio [OR], 17.21), hypertension (adjusted OR, 4.67), smoking habit (adjusted OR, 4.70), and hyperlipidemia (adjusted OR, 2.73) were identified independently associated with ischemic stroke. With a separate sex analysis it was identified that the relationship of the MTHFR T/T genotype was statistically significant in women (adjusted OR, 9.49; 95% CI, 1.75-51.47, $P=0.0091$). The relevance of the MTHFR T/T mutation appears to be restricted to women, suggesting a role of female hormones in the resistance to elevated homocysteine levels due to the MTHFR T/T mutation.

Moderate elevation of homocysteine in the plasma is an established risk factor in the development of coronary heart disease^{6,30)}, myocardial infarction^{2,32)}, ischemic stroke^{4,34)}, and venous thrombosis^{9,10)}. Several genetic and non-genetic factors are known to influence plasma homocysteine levels^{17,22,33)}. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is a folate-dependent enzyme catalyzing the rate-limiting step in the methylation of homocysteine to methionine. Kang¹⁸⁾ first showed a link between the moderately disturbed functions of MTHFR in lymphocytes, measured as an increased thermolability, and the moderate increases of homocysteine levels in the plasma of coronary artery disease patients. Following the cloning and sequencing of the gene for MTHFR¹³⁾, a C→T transition at 677, which results in the conversion of alanine to valine at amino acid 222, was reported and was associated with a decreased specific MTHFR activity and an elevation of the homocysteine levels in the T/T homozygous state¹¹⁾. Subsequent studies have reported that polymorphism of the MTHFR gene is one of the potential genetic risk factors for cardiovascular disease in many countries, although this is still unclear^{3, 5, 12, 27, 29, 31)}, and studies examining the

association between the MTHFR (C677T) mutation and ischemic stroke have also been conflicting^{25, 26)}.

One potential explanation for these inconsistent findings is that previous studies have correlated the MTHFR (C677T) mutation and ischemic stroke but not separating analysis for sex. Sex difference in the association of premature ischemic stroke with factor V Leiden²⁴⁾ has been found, and gender has also been reported to be independently associated with homocysteine levels^{19, 28)}. Therefore, we hypothesized that the association with the MTHFR genotype may be different according to gender. To explore this hypothesis, we undertook a case-control study of patients hospitalized with ischemic stroke and examined with a separate analysis for sex specifically whether there may be an association between the C677T MTHFR mutation and ischemic stroke according to gender.

MATERIALS AND METHODS

Subjects

A total of 77 documented ischemic stroke patients (49 men and 28 women; age 61.4 ± 6.8) were selected for the study from May 1998 to February 1999, having been identified by a clinical examination of a CT brain scan or MRI at the Rehabilitation Hospital of Kobe, Japan. The criteria of the National Institute for Neurological Disorders and Stroke were applied in this study¹⁾. Patients with cerebral hemorrhage were not included.

The control subjects ($n=229$, 120 men and 109 women, age 59.6 ± 6.9) without any history of ischemic stroke were recruited into the study. They underwent clinical examinations at Hyogo Health Service Association (Kobe, Japan).

The subjects with fasting blood glucose levels ≥ 140 mg/dl were classified as diabetes mellitus; hyperlipidemia was diagnosed by a total cholesterol level ≥ 220 mg/dl and hypertension was defined as a systolic blood pressure ≥ 140 mmHg and/or a diastolic blood pressure ≥ 90 mmHg in the sitting position on at least 3 different occasions at the time of admission. Relevant information on current smoking habit and alcohol consumption was obtained from all of the subjects. Informed consent was obtained from all the individuals after a full explanation of the study.

DNA analysis

DNA was extracted from the peripheral blood leukocytes with a Puregen DNA Isolation Kit (Gentra systems, Inc., MN, USA). All the polymerase chain reactions (PCR) to detect the MTHFR mutation were carried out with 300ng of genomic DNA as a template, and primers as previously reported¹¹⁾. The temperature conditions for the PCR included an initial denaturation at 94°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, extension at 72°C for 3 min, and a final extension at 72°C for 7 min, after which the PCR- amplified fragment (198-bp) was digested with the restriction enzyme Hinf I, followed by 3% agarose gel electrophoresis and ethidium bromide staining.

Statistical analysis

All of the analyses were performed using StatView 5.0 (for Macintosh). Baseline differences between cases and controls were examined by means of the χ^2 test or the Fisher's exact test for categorical data and the Student t test for continuous data. Allele and genotype frequencies among the case and control subjects were compared by the χ^2 test with Hardy-Weinberg predictions. The association between MTHFR genotype (independent variable) and ischemic stroke (dependent variable) was examined by means of a logistic regression model, with adjustment for age, sex, and conventional vascular risk factors. Results were expressed as OR together with their 95% CI. Statistical significance was taken as $P < 0.05$.

RESULTS

The clinical characteristics of the study sample as a whole, and after stratification according to sex are shown in Table I. We found that the patients had a significantly higher prevalence of hypertension, hyperlipidemia, diabetes mellitus, and smoking habit in all the subjects, hypertension and diabetes mellitus in both sexes after a separate account, and hyperlipidemia and smoking habit only in men, which are known as major risk factors for ischemic stroke.

Table I. Baseline characteristics of patients and controls.

	All		Men		Women	
	Case (n=77)	Control (n=229)	Case (n=49)	Control (n=120)	Case (n=28)	Control (n=109)
Age	61.4±6.8	59.6±6.9	61.7±6.9	59.4±7.8	60.9±6.7	59.8±5.7
Hypertension, n (%)	46(59.7)*	50(21.8)	31(63.3)*	33(27.5)	15(53.6)*	17(15.6)
Hyperlipidemia, n (%)	46(59.7)*	80(34.9)	31(63.3)*	28(23.3)	15(53.6)	52(47.7)
Diabetes mellitus, n (%)	29(37.7)*	8(3.5)	19(38.8)*	7(5.8)	10(35.7)*	1(0.9)
Smokers, n (%)	41(53.2)*	56(24.5)	38(77.6)*	49(40.8)	3(10.7)	7(6.4)
Alcohol consumers, n (%)	43(55.8)	115(50.2)	36(73.5)	84(70.0)	7(25)	31(28.4)

Alcohol consumers were divided into current drinkers and subjects who never drank or past consumers; smokers were divided into subjects who currently smoke and who never did.

*P<0.0001

The calculated OR and 95% CI for the T/T genotype were 2.33 (1.05-5.20) compared with the C/C genotype in all, and this trend was significant (P=0.036) (Table II). The allele frequency of the T mutation was also significantly higher in the case group than in the control group (0.44/0.35, P=0.046). With a separate analysis for sex, the OR and 95% CI for the C/T genotype were 3.83(1.20-12.23) and 6.71(1.68-26.75) for the T/T genotype compared with the C/C genotype in women. Both of these effects were significant (P=0.017, P=0.003, respectively), and the T allele frequency was also significantly higher in case group than in the control group (0.55/0.34, P=0.004), but the significant trend was not identified in men.

Table II. Odds ratio of ischemic stroke associated with MTHFR genotypes.

	C/C n (%)	C/T n (%)	T/T n (%)	T Allele Frequency
All				
Case (n=77)	23 (29.9)	40 (51.9)	14 (18.2)	0.44
Control (n=229)	92 (40.2)	113 (49.3)	24 (10.5)	0.35
OR (95% CI)	1	1.42 (0.79-2.53)	2.33 (1.05-5.20)*	
Men				
Case (n=49)	19 (38.8)	23 (46.9)	7 (14.3)	0.38
Control (n=120)	46 (38.3)	62 (51.7)	12 (10)	0.36
OR (95% CI)	1	0.90 (0.44-1.84)	1.41 (0.48-4.14)	
Women				
Case (n=28)	4 (14.3)	17 (60.7)	7 (25)	0.55**
Control (n=109)	46 (42.2)	51 (46.8)	12 (11.0)	0.34
OR (95% CI)	1	3.83 (1.20-12.23)*	6.71 (1.68-26.75)*	

*P<0.05 compared with C/C genotype.

**P<0.05

Table III. Risk factors identified independently associated with ischemic stroke by multiple logistic regression analysis.

All				
	β	SE	OR (95% CI)	P
Age	0.024	0.027	1.02 (0.97-1.08)	0.3682
Hypertension	1.541	0.347	4.67 (2.37-9.23)	<0.0001*
Hyperlipidemia	1.004	0.349	2.73 (1.38-5.42)	0.0040*
Diabetes mellitus	2.846	0.494	17.21 (6.53-45.35)	<0.0001*
Smoking habit	1.548	0.427	4.70 (2.04-10.85)	0.0003*
Alcohol-consumption	-0.609	0.401	0.54 (0.25-1.19)	0.1291
MTHFR (C/T)	0.334	0.383	1.40 (0.66-2.96)	0.3829
(T/T)	0.996	0.514	2.71 (0.99-7.42)	0.0528
Men				
	B	SE	OR (95% CI)	P
Age	0.034	0.034	1.03 (0.97-1.11)	0.3270
Hypertension	1.417	0.462	4.12 (1.67-10.20)	0.0022*
Hyperlipidemia	1.842	0.490	6.31 (2.41-16.49)	0.0002*
Diabetes mellitus	2.372	0.625	10.72 (3.15-36.48)	0.0001*
Smoking habit	1.615	0.497	5.03 (1.90-13.31)	0.0012*
Alcohol-consumption	-0.917	0.534	0.40 (0.14-1.14)	0.0857
MTHFR (C/T)	-0.377	0.507	0.69 (0.25-1.85)	0.4572
(T/T)	0.053	0.730	1.05 (0.25-4.41)	0.9425
Women				
	β	SE	OR (95% CI)	P
Age	-0.007	0.049	0.99 (0.90-1.09))	0.8878
Hypertension	2.712	0.619	8.78 (2.61-29.52)	0.0004*
Hyperlipidemia	0.081	0.580	0.92 (0.30-2.88)	0.8888
Diabetes mellitus	4.171	1.190	64.76 (6.28-667.38)	0.0005*
Smoking habit	0.585	1.079	1.80 (0.22-14.88)	0.5875
Alcohol-consumption	-0.331	0.762	0.72 (0.16-3.20))	0.6638
MTHFR (C/T)	1.080	0.695	2.95 (0.75-11.50)	0.1201
(T/T)	2.250	0.863	9.49 (1.75-51.47)	0.0091*

The multivariate logistic regression model contained age, hypertension, hyperlipidemia, diabetes mellitus, smoking habit, alcohol consumption and MTHFR genotype variables.

β , indicates estimated coefficient; SE, standard error; OR, adjusted odds ratio.

*Statistically significant.

The findings from the univariate analysis were further investigated in a multiple logistic model with adjustment for age (in years), sex, alcohol and smoking habit, and conventional vascular risk factors (Table III). Under these circumstances, the conventional vascular risk factors, such as diabetes mellitus (adjusted OR, 17.21, 95% CI, 6.53-45.35), hypertension (adjusted OR, 4.67; 95% CI, 2.37-9.23), smoking habit (adjusted OR, 4.70; 95% CI,

2.04-10.85) and hyperlipidemia (adjusted OR, 2.73; 95% CI, 1.38-5.42) were identified independently related to ischemic stroke, but the MTHFR T/T mutation was not. A separate analysis for sex was carried out. In men, significant associations were found in hypertension (adjusted OR, 4.12; 95% CI, 1.67-10.20), hyperlipidemia (adjusted OR, 6.31; 95% CI, 2.41-16.49), diabetes mellitus (adjusted OR, 10.72; 95% CI, 3.15-36.48) and smoking habit (adjusted OR, 5.03; 95% CI, 1.90-13.31). In women, the statistical relevance of hypertension (adjusted OR, 8.78; 95% CI, 2.61-29.52), diabetes mellitus (adjusted OR, 64.76; 95% CI, 6.28-667.38) and MTHFR T/T genotype (adjusted OR, 9.49; 95% CI, 1.75-51.47) with ischemic stroke was found.

DISCUSSION

With the separate analysis for sex, in women a statistical significance in the association between the T/T polymorphism of the MTHFR gene and ischemic stroke was identified. On the other hand, multivariate analysis demonstrated that this association was independent on conventional vascular risk factors related to ischemic stroke.

After stratification for sex, we observed that the MTHFR T/T mutation was independently associated with an increased risk of ischemic stroke in women. Elevating plasma homocysteine concentration being associated with the occurrence of ischemic stroke was reported by many studies ^{4,7,34}. Recently, sex as a non-biochemical variable independently associated with homocysteine has been reported in an Italian study ²⁸, and homocysteine levels were significantly higher in healthy men than in women (most women were in the premenopausal state) in all 3 MTHFR genotypes. There was another consistent report that sex differences exist in levels of homocysteine, which were lower in premenopausal women than in men or postmenopausal women ³³. In our study, most of women subjects were in the postmenopausal state and the relevance of MTHFR T/T mutation was restricted to women. These facts suggest that estrogens may play a role of increasing the resistance to elevated homocysteine levels due to MTHFR T/T mutation. In the postmenopausal state after losing the protective function by these hormones, the potential risk of elevated homocysteine levels for ischemic stroke would manifest although in the present study the small number of women patients and absence of data of homocysteine levels did not allow us to evaluate this hypothesis.

The MTHFR T/T homozygosity has been related to elevated plasma homocysteine levels ^{8,14,21}, however, recently several studies indicated this association is dependent, at least in part on the serum folate levels ^{16,23}. At the time of collecting samples it was impossible to measure the levels of homocysteine and folate, therefore we could not examine the above possibility.

Investigators have failed to find any association between the T/T mutation and ischemic stroke ^{15,20}, however, the association which was statistically significant was identified from another studies in differential ethnic group studies ^{24,35}. These inconsistencies may reflect the difference separating sex analysis or adjustment of confounding factors for ischemic stroke. In this study we also identified that diabetes mellitus, hypertension, hyperlipidemia and smoking habit were independently related to ischemic stroke by a multiple logistic regression model, and it is consistent with other previous studies. Certainly, the sample of case was so small that it will be addressed in further study.

We conclude that the relevance of the MTHFR T/T mutation appears to be restricted to women, suggesting a role of female hormones in the resistance to elevated homocysteine levels due to the MTHFR T/T mutation. The quantity of case samples and the measurement of homocysteine and red cell folate levels should be addressed in further studies.

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