Original Article

Serum folate, total homocysteine levels and methylenetetrahydrofolate reductase 677C>T polymorphism in young healthy female Japanese

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Environmental and genetic factors influence serum total homocysteine (tHcy), a risk factor for vascular diseases. The gene polymorphism of methylenetetrahydrofolate reductase (*MTHFR*) is reported to be a genetic factor for influencing tHcy. However, it is not clear whether *MTHFR* polymorphism influences tHcy in the younger generation. To investigate the influence of *MTHFR* polymorphism on vascular disease risks in young Japanese females, we determined dietary intakes, serum folate and tHcy, and examined the influence of *MTHFR* 677C>T polymorphism in healthy junior and high school students (n=192, 12-18y). The relationships between *MTHFR* polymorphism and folate intake, serum folate or tHcy were investigated by dividing participants into CC, CT and TT types. Among individuals with the TT genotype, folate and tHcy levels were significantly lower ($p<0.05$) or higher ($p<0.0001$), respectively, than in those with the other genotypes; although there were no significant differences in the intake of folate among genotypes. In addition, a significant inverse correlation between folate and tHcy ($p<0.05$) was noted in all genotypes, even in young females, so far not examined in Asian populations. Therefore, *MTHFR* genotypes were proven to be a significant determinant for folate and tHcy concentrations. However, the association of increased folate intake with lower tHcy concentration, even in cases of the mutation TT type, indicates the importance of folate intake in young Japanese females for early detection of risk, as well as the prevention of vascular diseases.

Key Words: *MTHFR*, polymorphism, folate, homocysteine, young female

INTRODUCTION

Homocysteine is a sulfur-containing amino acid absent in natural dietary sources. Many studies demonstrated the association of hyperhomocysteinemia with vascular diseases[1,2] such as extracranial carotid-artery stenosis[3] and with cardiovascular disease mortality rates.[4] Both folate and vitamin B influence serum total homocysteine (tHcy) levels. Higher tHcy levels have been associated with low folate and vitamin status in a cohort study.[5] tHcy levels are controlled by remethylation or transsulfuration pathways related to folate and vitamin B-6, 12. In addition, these pathways are influenced by, not only environmental factors, but also genetic factors. Several enzymes are responsible for modulating intracellular levels of homocysteine. One of these enzymes, methylenetetrahydrofolate reductase (*MTHFR*) promotes remethylation of homocysteine to methionine by catalyzing from 5,10-methylenetetra- hydrofolate to 5-methyltetrahydrofolate. Therefore, the decreased activity of the enzyme *MTHFR* elevates tHcy level. A missense mutation of *MTHFR* that converts alanine to valine (C to T substitution at nucleotide 677) encodes a thermolabile enzyme with lower specific activity. The frequency of this mutation may vary among different ethnic groups[7-9] and appear to be higher in the Japanese compared to Europeans. The mutant genotype of *MTHFR* has been reported to increase the risk of several common disorders, including cardiovascular diseases,[10,11] neural tube defects,[9] and neuropsychiatric disorders[12]. The homozygous mutant (TT) genotype elevates tHcy levels.[6,13]. However, individuals with high folic acid levels are not hyperhomocysteinemic, even in the case of the individuals who have the homozygous mutation[7]. Another risk that may influence tHcy level is age, since a genetic modifier is likely to have a greater role in the young. The metabolic functions in the processes of growth and development are higher in the young than the elderly. Therefore, mutations in enzymes involved in the metabolism of nutrients might be expected to be of greater consequence in the young. Mager *et al* reported that the *MTHFR* genotype was associated with risk in the persons with an earlier age onset of diseases.[14]

In this study, we conducted a health survey on192 healthy school girls aged 12-18 years at a junior and senior high school in Japan, to evaluate the potential rela-

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tionship between 677 C>T *MTHFR* polymorphism and the risk of vascular disease in young Japanese females.

MATERIALS AND METHODS

Subjects

We carried out a health survey in a junior and senior high school located in Hyogo Prefecture, Japan, during June 2009. The study samples were 192 healthy school students, aged 12 to 18 years, who were informed of the purpose and procedures of the study and signed informed consent forms .The study design was approved by the research ethics committee of the Mukogawa Women's University.

Health examination

Anthropometric characteristics, including height and weight, were measured. Body mass index (BMI) was calculated as body weight (kg)/height (m^2). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice with an automated blood pressure measurement system (HEM-970; OMRON, Kyoto, Japan) in sitting position after over 10 minute rest and the average was used in the analysis. According to the criteria of risks established by the Ministry of Health, Labour and Welfare in Japan, overweight and underweight were defined as follows: overweight, BMI ≥25; underweight, BMI <18.5.

Blood chemical analysis

Blood samples were obtained after more than 3 hrs fasting. Serum levels of tHcy, folate, triglycerides, total cholesterol and HDL, and plasma levels of insulin and glucose were determined by SRL (Special Reference Laboratory), Inc (Tokyo, Japan). Insulin resistance (homeostasis model assessment of insulin resistance: HOMA-IR) was calculated using the following formula: HOMA-IR = fasting plasma insulin (μIU/ml)×fasting plasma glucose (mg/dL)/405.[15]

Assessment of dietary intake

All subjects were interviewed by experienced dietitians using a FFQ, for energy and nutrient intake estimation. The FFQ was validated by a comparison with weighed dietary records for 7 continuous days.[16] From the FFQ, the mean daily nutrient intake was calculated according to the fifth revised and enlarged edition of the Standard Tables of Food Composition in Japan.[17] For the data analysis, we excluded 17 participants whose energy intakes were calculated either under 600 kcal or over 3,500 kcal.

Genetic analysis

The *MTHFR* polymorphism was identified by polymerase chain reaction (PCR) followed by restriction enzyme digestion and separation on a 3% agarose gel.[18] The following sense and antisense primers within exon 4 were used: 5'GCCCAGCCACTCACTGTTTTA3' and 5'AGGACG GTGCGGTGAGAGTG3'. The PCR product of 418 bp was digested with HinfI, resulting in 77 and 341 bp bands for wild-type homozygote, 77, 165, 176, and 341 bands for heterozygote, and 77, 165, and 176 bands for mutant homozygote.

Statistical analysis

We used SPSS software, version 15.0. One-way ANOVA was used to study the effect of the *MTHFR* genotype. Pearson's correlation test was used to correlate tHcy concentrations with folate and age. A *p*-value less than 0.05 is regarded as statistical significant.

RESULTS

As shown in Table 1, subjects with *MTHFR* wild-type allele (CC), heterozygous allele (CT) and homozygous for the mutant allele (TT) were 37.6, 46.4 and 16.1% respectively. Anthropometric characterization and blood chemical analysis are shown in Table 1. Among the three groups, no significant differences were observed in all parameters (age, BMI, SBP, DBP, heart rate, cholesterol, triglycerides, insulin, glucose, HbA1c and HOMA-IR) (Table 1). The rate of overweight was 2.6%, whereas that of underweight was 26.0% (data not shown). To understand dietary habits in young Japanese females, we calculated nutritional intakes such as folate, vitamin B-6 and B-12. There were no significant differences among groups (Table 2). However, it was ascertained that higher intake of folate showed a significant reduction in serum

Table 1. Clinical characteristics of participants in three *MTHFR* genotypes

	CC (n=72)	CT (n=89)	TT (n=31)	Total (n=192)
%	37.6	46.4	16.1	100
Age	15.1 ± 0.2	15.7 ± 0.2	15.4 ± 0.3	15.4 ± 0.1
BMI (kg/m^2)	19.9 ± 0.2	20.0 ± 0.2	19.9 ± 0.4	19.9 ± 0.2
SBP (mmHg)	105 ± 1.3	102 ± 1.2	103 ± 2.2	103 ± 0.8
DBP (mmHg)	62.1 ± 1.0	60.1 ± 0.8	59.0 ± 1.0	60.7 ± 0.6
Heart rate (beat/min)	80.1 ± 1.4	76.2 ± 1.3	75.1 ± 2.1	77.5 ± 0.9
Total cholesterol (mg/dL)	173 ± 3.6	172 ± 2.9	177 ± 6.0	173 ± 2.1
HDL cholesterol (mg/dL)	65.0 ± 1.6	63.8 ± 1.2	62.7 ± 2.0	64.1 ± 0.9
LDL cholesterol (mg/dL)	92.4 ± 2.8	93.7 ± 2.5	99.9 ± 5.0	94.2 ± 1.8
Triglyceride (mg/dL)	84.5 ± 6.2	73.0 ± 3.8	70.0 ± 5.9	76.8 ± 3.1
Insulin (μIU/mL)	12.9 ± 1.2	15.4 ± 1.1	13.0 ± 1.8	14.1 ± 0.8
Glucose (mg/dL)	91.4 ± 0.9	93.5 ± 1.0	92.2 ± 1.3	92.5 ± 0.6
HbA1c (%)	4.96 ± 0.03	4.97 ± 0.02	4.96 ± 0.03	4.96 ± 0.02
HOMA-IR	2.94 ± 0.28	3.70 ± 0.31	2.96 ± 0.41	3.29 ± 0.19

CC, homozygous for the wild-type allele: CT, heterozygous; TT, homozygous for the mutant allele
It checked that the frequency of gene polymorphism followed Hardy-Weinberg principle by Pearson's chi-square test.
Values are expressed as means ± SD. There were no significant differences by the one-way ANOVA.

Table 2. Dietary intake of vitamins B-6, B-12, folate in three *MTHFR* genotypes

	CC (n=66)	CT (n=82)	TT (n=27)	Total (n=175)
Vitamin B-6 (mg)	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.0
Vitamin B-12 (μg)	6.1 ± 0.4	6.0 ± 0.3	6.2 ± 0.7	6.1 ± 0.2
Folate (μg)	243 ± 9.8	242 ± 12.1	219 ± 12.4	239 ± 7.1

To derive data, we first excluded the participants who reported an intake of less than 600 kcal or 3,500 or more kcal. Values are expressed as means ± SD. There were no significant differences by the one-way ANOVA.

Table 3. Correlation between serum tHcy and intakes of vitamins B-6, B-12 and folate

	CC (n=66)		CT (n=82)		TT (n=27)		Total (n=175)	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Vitamin B-6	-0.156	0.210	-0.199	0.074	-0.048	0.813	-0.147	0.053
Vitamin B-12	-0.171	0.169	-0.137	0.218	-0.022	0.913	-0.094	0.215
Folate	-0.194	0.118	-0.190	0.088	-0.056	0.782	-0.170	0.025

The Pearson correlation test was used to establish the relations between serum tHcy and the intakes of vitamins B-6, B-12 and folate

Table 4. Correlation between serum tHcy, folate and age

	CC (n=72)		CT (n=89)		TT (n=31)		Total (n=192)	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Folate	-0.353	0.002	-0.425	<0.001	-0.400	0.026	-0.424	<0.001
Age	0.215	0.069	0.197	0.064	0.182	0.328	0.285	<0.001

The Pearson correlation test was used to establish the relations between serum tHcy, folate and age.

Table 5. Distribution of tHcy and folate concentrations by *MTHFR* genotype

		CC (n=72)	CT (n=89)	TT (n=31)	Total (n=192)
Homocysteine (nmol/mL)	mean	6.9 ± 0.2	7.2 ± 0.2	8.9 ± 0.5**	7.4 ± 0.1
	range	4.5 - 10.5	4.4 - 11.9	4.9 - 19.4	4.4 - 19.4
Folate (ng/mL)	mean	6.5 ± 0.2	6.2 ± 0.3	4.8 ± 0.5*	6.1 ± 0.2
	range	3.1 - 11.6	2.6 - 20.6	2.4 - 16.9	2.4 - 20.2

Values are expressed as means ± SD. There were significant differences between genotype groups by one-way ANOVA.
* p = 0.05, ** p < 0.001

tHcy (r = -0.170, *p*=0.025) in all subjects (Table 3). Moreover, the tHcy levels were inversely related with serum folate; the correlations in *MTHFR* genotypes CC, CT, TT and in total were all significant ($p<0.01$, 0.001, 0.05 0.001), respectively (Table 4). tHcy and folate (means±SD) in *MTHFR* genotypes CC, CT, TT and in total were shown in Table 5. When folate and tHcy were analyzed as the function of *MTHFR* genotypes, the TT genotype showed significantly higher tHcy and lower folate than CC or CT genotypes (Table 5).

DISCUSSION

Data derived from observational studies suggest that the *MTHFR* C>T polymorphism negatively influences folate and tHcy status.[6,19] Subjects who were TT homozygous for the C677T mutation had a significantly lower folate and higher tHcy than those with the other genotypes. In the present study, it was shown that *MTHFR* polymorphism influences folate and tHcy status in the young female, as well as the adult, even if there is no difference in dietary intakes of vitamins B-6, B-12 and folate. Some researchers reported that high folate diets significantly increased blood folate concentrations or significantly decreased tHcy.[20,21] We found a significant inverse correlation between folate and tHcy in each genotype.

The mean serum concentrations of tHcy in our study population (7.4 nmol/mL) were lower than those of healthy adults in Japan[22] and Korea.[23] As for many biological variables in humans, folate and tHcy concentrations reflect the balance between environmental and genetic factors. In our study, serum tHcy concentrations increased significantly with TT genotype and age. However tHcy decreased significantly with folate intake and serum folate concentrations. Therefore, other metabolic pathways in addition to folate levels might relate to the serum tHcy levels in the young female.

In the present study, the mean concentrations of folate from our study population (6.1 ng/mL) were lower than those of Japanese adults or Greek children.[24,25] Recent studies have indicated that folate status is very important for women in pregnancy because folate supplementation reduces the risk of neural tube defects.[26] Folate is also important to achieve normal birth weight because the low intake of folate in pregnant women is associated with low birth weight.[27] Therefore, the Japanese Government recommends for women expecting pregnancy to take 400 micrograms of supplementary folate per day. However, the folate status of Japanese young women had not been fully investigated because there had been little awareness in Japan. Because detailed reports of folate intake by the

young Japanese are limited, the data on serum folate that reflects intake is important.

These underscore the importance of adequate intake of folate for young people, particularly for Japanese girls who yearn to be slim. Therefore, we should emphasize through food education to current young Japanese females that higher folate intakes should be required in general and particularly in persons with the homozygous mutant genotype to prevent the possible deleterious effects of increased tHcy in the future.

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AUTHOR DISCLOSURE

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Original Article

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日本年輕健康女性的血清葉酸與總同半胱胺酸濃度及甲基四氫葉酸還原酶 677C>T 基因多型性

環境及基因會影響血清中總同半胱胺酸(tHcy)濃度，此為血管疾病的危險因子之一。甲基四氫葉酸還原酶(MTHFR)的基因多型性被認為是影響 tHcy 的基因因子。然而，在年輕世代的 MTHFR 基因多型性是否影響 tHcy 並不清楚。為研究年輕日本女性的 MTHFR 基因多型性對血管疾病的影響，我們測量膳食攝取、血清葉酸及 tHcy，並評估健康的中學生(n=192，12-18 歲)MTHFR 677C>T 基因多型性對它們的影響。依據參與者的 MTHFR 基因多型性分為 CC、CT 及 TT 型三組，並分析基因型態與葉酸攝取、血清葉酸或 tHcy 的相關性。雖然葉酸攝取量在各基因型組沒有顯著差異，但具 TT 基因型的參與者比起其他基因型者，有顯著較低的血清葉酸($p<0.05$)與較高的血清 tHcy 量($p<0.0001$)。此外，所有基因型的年輕女性均顯示葉酸與 tHcy 有顯著負相關，這到目前為止在亞洲族群尚未被探討過。因此，MTHFR 基因型被證明是血清葉酸與 tHcy 濃度的重要決定因子。再者即使在 TT 突變型，也呈現葉酸攝取的增加與較低的 tHcy 濃度有相關，這指出日本年輕女性的葉酸攝取在早期危險性監測及預防血管疾病的重要性。

關鍵字：甲基四氫葉酸還原酶、基因多型性、葉酸、同半胱胺酸、年輕女性