A Cross-Sectional Study to Find Out the Relationship of Methyleneheterofofale Reductase (MTHFR) C677T Genotype with Plasma Levels of Folate and Total Homocysteine by Daily Folate Intake in Japanese

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Summary In those with the methylenetetrahydrofofale reductase (MTHFR) 677TT genotype, enzyme activity is lowered. Therefore, these individuals might require an increased intake of folate to maintain or control blood levels of plasma folate or total homocysteine (tHcy). We examined associations of dietary folate intake with fasting plasma folate and total homocysteine (tHcy) according to genotype among 554 Japanese (207 men and 347 women aged 39–89 y) recruited in 2009. Intake of folate was estimated with a food frequency questionnaire. The MTHFR polymorphism was genotyped by a polymerase chain reaction with confronting two-pair primers. The log-transformed concentration of folate or tHcy was regressed on energy-adjusted folate intake in a linear regression analysis. Higher folate intake was associated with higher plasma folate among those with the CC (β=0.165, p=0.066) or CT (β=0.248, p<0.001) genotypes, and with lower tHcy levels only among those with the CC (β=−0.141, p=0.013) genotype. Plasma folate was significantly and inversely associated with tHcy, irrespective of MTHFR genotype. When the analysis was restricted to those with tHcy levels higher than the reference range (≥13.5 nmol/mL, n=20), these significant associations were not found. The interaction between folate intake or plasma folate and genotype was not significant in any analysis. In conclusion, dietary folate intake was positively associated with plasma folate among those with the CC or CT genotypes and inversely associated with tHcy among those with the CC genotype, but the associations were not clear among those with higher levels of tHcy.

Key Words folate intake, plasma folate, homocysteine, MTHFR C677T genotype, Japanese

L-5-Methyl-tetrahydrofofale (THF), derived from dietary folate, is involved in the conversion of homocysteine into methionine (Fig. 1; 1–3). Folate deficiency increases total homocysteine (tHcy) levels (4). Hyperhomocysteinemiam is one of the risk factors of cardiovascular disease (4, 5) and neural tube defects (NTDs) (6). Maintaining adequate blood folate levels and controlling tHcy levels are important in preventing these diseases.

In the folate metabolic pathway, 5,10-methylene-THF is converted into 5-methyl-THF by the enzyme 10-methylene-THF reductase (MTHFR). In the MTHFR C677T polymorphism (rs 1801133), the T allele reduces thermostability of the enzyme and lowers its activity, which were found to be decreased by 30% in heterozygotes and by 65% in homozygotes of minor alleles compared with the CC genotype in an in vitro study (7). However, folate intake could increase blood folate levels even among those with the TT genotype. In previous observational studies, decreased tHcy levels were related to increased blood folate levels (8–11), and increased folate intake was associated with increased blood folate levels (8, 12) and decreased tHcy levels (8, 13, 14) in each of the MTHFR genotype. These correlations were substantially stronger among those with TT (8, 9, 11–15) and those consuming relatively lower amounts of folate (8, 12, 13). In previous interventional studies using folate supplements or fortified foods, increased folate intake raised blood folate (16–21) and reduced tHcy levels (17–21).

However, one study in Japan found no association of serum folate with energy-adjusted folate intake among those with the TT genotype (22). This may be partly explained by the difference in the amount of folate intake and sources of folate. The mean folate intake among Japanese aged 20 y or more was 329 µg/d for men and 189 µg/d for women and the mean intake from supplements or fortified foods among the population was only 1 µg/d in 2008 (23). In contrast, the mean and standard deviation (SD) of folate intake was 772±11 µg dietary folate equivalents (DFE)/d, including only 199±2 µg DFE/d from natural sources among the American population from 2003 to 2006 (24). In several countries including the United States, fortified foods or folic acid supplements are important sources of folate (24–26), and supplemented folate is more ther-
mostable than folate from natural foods (27). The role of the MTHFR polymorphism in folate metabolism may be different in areas where folate intake is relatively low. Nevertheless, only a few observational studies have been conducted in such areas, including Japan (22). Thus, this study aimed to investigate associations of dietary folate intake with plasma folate or tHcy levels according to genotype among Japanese.

MATERIALS AND METHODS

Study population. Subjects were recruited during a health checkup at Yakumo, Hokkaido, in 2009. Among 593 examinees, 573 (96.6%) participated in this study. One subject was not included because of no available DNA sample. We excluded one participant with plasma folate levels less than 2.0 mg/mL \((n=1)\), or those with plasma vitamin B\(_{12}\) levels greater than 3,000 pg/mL \((n=4)\) because they were outliers and vitamin B\(_{12}\) is involved in folate metabolism (4). We also excluded those with estimated glomerular filtration rates (eGFR) less than 30 mL/min/1.73 m\(^2\) (chronic kidney disease [CKD] stage 4 or 5 \([n=3]\)) because CKD patients tended to have higher tHcy levels (28). An outlier (eGFR≥200 mL/min/1.73 m\(^2\)) also was omitted \((n=1)\). Additionally, we excluded those who had implausibly low estimated energy intakes (less than 1,000 kcal; \(n=8\)) or high energy intakes (greater than 4,000 kcal; \(n=1\)). The remaining 554 subjects (207 men and 347 women aged from 39 to 89 y) were included in the present analysis. This study complied with the code of ethics of the World Medical Association (Declaration of Helsinki), and was approved by the Ethics Committee of the Nagoya University School of Medicine (approval number 643).
Data and sample collection. The participants completed a self-administered questionnaire on their health and lifestyle at the time of the health checkup. Data obtained from the questionnaire included consumption of ten kinds of alcoholic beverages (frequency [rarely, 1–3 times/mo, 1–2 times/wk, 3–4 times/wk, 5–6 times/wk, or every day] and the amount per time) for calculation of alcohol consumption, and smoking habits (current, former, or never). Dietary habits over the previous year were reported with a validated food-frequency questionnaire (FFQ). It included intake frequency of 43 food items (rarely, 1–3 times/mo, 1–2 times/wk, 3–4 times/wk, 5–6 times/wk, once/d, twice/d, or 3 times/d or more), and consumption of coffee (rarely, 2 cups/wk or less, 3–4 cups/wk, 5–6 cups/wk, 1–2 cups/d, 3–4 cups/d, or 5 cups/d or more). Intakes of folate and total energy were estimated based on the responses to the FFQ. De-attenuated Pearson’s rank correlation coefficients between folate intakes measured with 3-d weighed diet records, and log-transformed and energy-adjusted folate intakes estimated from the FFQ were 0.36 for men and 0.38 for women.

Peripheral blood was drawn from the participants in the morning after overnight fasting or in the afternoon after skipping lunch. Plasma and buffy coat samples were stored at −80 °C until analysis. Plasma folate, vitamin B_{12}, tHcy, and serum creatinine concentrations were determined with an auto-analyzer (JCA-RX20, Nihon Denshi Co Ltd, Tokyo, Japan) in a single laboratory (SRL Inc, Hachioji, Japan). Reference ranges in the laboratory were ≤4.0 ng/mL for serum folate, 180–914 pg/mL for serum vitamin B_{12}, and 3.7–13.5 nmol/mL for plasma tHcy. eGFR (mL/min/1.73 m^2) was calculated by the following Japanese eGFR equation:

\[ \text{eGFR} = 194 \times \frac{\text{serum creatinine (mg/dL)}}{\text{age}^{0.287}} \times 0.739 \] (for females) (30). Body height and weight were measured during the health checkup, and body mass index (BMI) was calculated from body weight (kg) divided by height (m) squared.

Genotyping procedure. DNA was extracted from the sampled blood using a BioRobot M48 Workstation (Qiagen Group, Tokyo, Japan). MTHFR C677T polymorphism was genotyped by a polymerase chain reaction with confronting two-pair primers (PCR-CTPP) (31). Details of the procedure have been described elsewhere (22).

Statistical analysis. The Hardy-Weinberg equilibrium was tested by the chi-square test. Characteristics of participants were compared among MTHFR C677T genotypes by the analysis of variance, Kruskal-Wallis
Fig. 2. Plots and regression lines estimated from simple regression analysis for each methylenetetrahydrofolate reductase C677T genotype (CC, n=199; CT, n=274; and TT, n=81). (A) Log-transformed plasma folate levels against energy-adjusted folate intake; (B) log-transformed plasma total homocysteine (tHcy) levels against energy-adjusted folate intake; and (C) log-transformed tHcy levels against plasma folate levels.
Effect of Folate Intake by MTHFR Genotype in Japanese

Interaction between folate intake and genotype: \( p = 0.892 \)

Interaction between folate intake and genotype: \( p = 0.056 \)

Interaction between plasma folate and genotype: \( p = 0.418 \)

MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine; 95% CI, 95% confidence interval.

1 Adjusted for sex, age, smoking habit, and alcohol intake for plasma folate levels, and adjusted for sex, age, body mass index, plasma vitamin B12 levels, estimated glomerular filtration rate (eGFR), smoking habit, alcohol intake, and coffee consumption for tHcy levels.

2 Adjusted for sex, age, smoking habit, and alcohol intake for plasma folate levels, and adjusted for sex, age, body mass index, plasma vitamin B12 levels, estimated glomerular filtration rate (eGFR), smoking habit, alcohol intake, and coffee consumption for tHcy levels.

test, or chi-square test where appropriate. Natural logarithmic transformation was applied for plasma folate and tHcy, and folate and energy intakes because these variables had a skewed distribution and the transformation improved the normality of the distribution. Folate intake was adjusted for energy intake by using the residual method (32). We conducted multiple linear regression analyses to examine the associations between folate intake and plasma folate or tHcy, and between plasma folate and tHcy by MTHFR C677T genotype. For the analysis considering plasma tHcy as a dependent variable, we included age, BMI, plasma vitamin B12, eGFR, and plasma folate or folate intake as continuous variables, and sex, smoking habits (never, former or current), alcohol intake (nondrinker, \(<23\) g/d, or \(\geq23\) g/d: one Japanese cup (180 mL) of rice wine (sake) usually includes 23 g alcohol), and coffee consumption (nondrinker, \(<1.0\) cup/d, 1.0–2.9 cups/d, or \(\geq3.0\) cups/d) as categorical variables in the multivariate models as covariates (28, 33). When the plasma folate levels were considered a dependent variable, we included sex, age, smoking habits, and alcohol intake as confounding factors (34, 35) as in the model for plasma tHcy. Additionally, we examined the interaction between folate intake or plasma folate and the MTHFR genotype by incorporating the number of \(T\) alleles and the interaction term \([\text{folate intake or plasma folate}] \times [\text{number of } T\text{ allele}]\) into the regression model and examined the significance of coefficients of the interaction term. To examine the associations among those with high risk levels of tHcy, we also performed similar analyses restricting subjects to those with tHcy levels higher than the reference range (\(\geq13.5 \text{ nmol/mL}, n=20\)). The analysis by genotype was not conducted because of the small number of participants with high levels of tHcy. We also calculated regression coefficients for high tHcy (\(\geq13.5 \text{ nmol/mL}\)) compared with lower tHcy by multiple linear regression analysis, setting plasma folate or folate intake as the dependent variable. When the dependent variable was folate intake, we used the same confounding factors in the model including plasma folate as a dependent variable. Additionally, we examined the interaction between higher tHcy and the number of \(T\) alleles of the MTHFR genotype using the same method mentioned above. All statistical analyses were conducted with STATA software version 13.0 (STATA, College Station, TX). Two-sided \(p\)-values under 0.05 were considered to be statistically significant.

RESULTS

The genotype frequency of MTHFR C677T was 36.2% for CC, 49.1% for CT, and 14.7% for TT genotypes. The frequency was in Hardy-Weinberg equilibrium \((p=0.394)\). Characteristics of participants by MTHFR C677T genotype are described in Table 1. Although median plasma folate levels among those with the TT
interaction between energy-adjusted folate intake and plasma folate levels was modified by the genotype of \textit{MTHFR} C677T polymorphism among healthy Japanese. Our study suggested that dietary folate intake may increase plasma folate levels with the CC or CT genotypes and decrease tHcy levels among those with the CC genotype. However, the effect was not significantly different among the genotypes and those associations were not clearly detected in the analysis restricted to those with tHcy exceeding the reference range (≥13.5 nmol/mL). We also found that plasma folate levels and folate intake were somewhat lower among those with higher levels of tHcy than those with lower tHcy levels.

As for the association between energy-adjusted folate intake and plasma folate levels, a previous study in Japan also reported no association among those with the TT genotype (22). In another study in Italy, mean serum folate levels were similar between the highest tertile and the middle tertile groups of folate intake among those with TT when adjusted for sex, age, energy intake, and other factors (12). One study in a sample from the Netherlands reported that a difference of mean serum folate between the middle and the highest tertile groups of food folate intake was smaller than the difference between the lowest and the middle tertile groups among those with the TT genotype (8). Findings in the two studies (12, 22) may be consistent with ours, but need further confirmation because the interaction between folate intake and the \textit{MTHFR} genotype was not significant in our study or a study reporting inconsistent findings (8). Therefore, differences in trends of plasma folate elevation by folate intake might be within the measurement error range.

There are several potential reasons why tHcy levels were slightly elevated among participants with the TT genotype with increasing habitual folate intake in our study. The subjects in our study had a wide range of folate intake (median and 5th–95th percentiles: 306 (187–536) mg/d), so effects of higher dietary folate intake could be elucidated. The recommended dietary allowance of folate intake in Japanese aged ≥12 y was 240 μg/d in 2010 (36). Because estimated folate intake using a FFQ is usually underestimated because of a limited number of food items on questionnaires (37), most subjects might have had sufficient dietary folate. The number of subjects with the TT genotype (n=81) was relatively small, so that the small elevation of tHcy might have been detected by chance.

Our study had some limitations to be addressed. First, several factors affecting folate and tHcy metabolism (Fig. 1) could not be measured for multivariate adjustment. Blood vitamin B₆, cystathionine β-synthase, and betaine, affect tHcy levels independently of blood folate (27), although folate is a stronger determinant for tHcy (38, 39). Second, plasma folate levels are influenced by folate intake during a relatively short term, so plasma folate levels of the subjects may indicate folate intake only
a few days before the health checkup. By contrast, we inquired about habitual dietary intake during the previous year with the FFQ. Erythrocyte folate levels may be more appropriate to detect folate deficiency because of long-term dietary habits (6). However, it has been reported that plasma folate levels were correlated with plasma tHcy more strongly than erythrocyte folate (38). Third, Japanese dietary folate intake may be more difficult to estimate than intake in other countries (40). This is probably because folate is derived mainly from fortified foods or supplements in other countries (24, 26) and the variety of folate sources in such countries may be relatively limited compared to the variety in Japan. Fourth, the number of subjects with the TT genotype and higher plasma tHcy levels in our study was relatively small to examine the associations in such groups. Finally, we did not inquire about intakes of folate supplements or fortified foods although a few Japanese take them (23). Although we excluded the outliers, plasma folate and vitamin B₁₂ levels might have been increased as a result of the use of supplements or fortified foods.

In conclusion, plasma folate increase with increasing dietary folate intake was clearer among those with the MTHFR 677CC or CT genotype, and a significant decrease in tHcy levels was associated with folate intake only among those with the CC genotype in Japanese. These associations, however, were not clearly detected among those with high-risk tHcy levels.

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