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Clinical Utility of Genotyping the 677C>T Variant of Methylenetetrahydrofolate Reductase in Humans Is Decreased in the Post-Folic Acid Fortification Era[1,2]

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Abstract

Moderate hyperhomocysteinemia is associated with many diseases. Major factors affecting plasma total homocysteine (tHcy) concentrations include folate concentrations and polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene. Because U.S.-mandated fortification of grain products with folic acid has improved folate and tHcy status in Americans, we investigated the effect of the MTHFR 677C>T variant before and after fortification. We determined tHcy and folate concentrations in sera from 844 Caucasian and 587 African American participants in the Coronary Artery Risk Development in Young Adults study before and after fortification and we genotyped the MTHFR 677C>T variant. MTHFR 677TT homozygotes had higher ($P < 0.01$) tHcy concentrations both before and after fortification compared with MTHFR 677CC homozygotes. However, the difference between these 2 genotypes decreased from 2.5 μmol/L before fortification to <0.7 μmol/L postfortification ($P < 0.01$). In addition, the prevalence of moderate hyperhomocysteinemia (tHcy > 13 μmol/L) in 677TT homozygotes decreased from 33% before fortification to 12% postfortification ($P < 0.01$). Using a cutoff value of 13 μmol/L to define moderate hyperhomocysteinemia, the sensitivity of the MTHFR 677TT genotype to predict elevations in homocysteine was low (~30%) both before and after folic acid fortification. Increasing the cutoff from 13 to 19 μmol/L increased the sensitivity of the assay before fortification to 62% but decreased the sensitivity to 17% postfortification. We conclude that after folic acid fortification in the US, measurement of tHcy rather than genotyping of MTHFR 677TT should be used as the primary assay for the diagnosis and monitoring of moderate hyperhomocysteinemia. J. Nutr. 139: 33–37, 2009.

Introduction

Moderately elevated concentrations of plasma homocysteine have been linked to a variety of disease conditions, including cardiovascular disease (CVD)[8] (1–4), end stage renal disease, neurodegenerative diseases, osteoporotic fractures, and neural tube defects (5–7). It has been shown that there is an inverse relationship between plasma B vitamin concentrations that are involved in the metabolism of homocysteine, especially folate, and plasma total homocysteine (tHcy) concentrations (8–10). In 1996, the U.S. FDA required that all enriched grain products be fortified with folic acid by January, 1998, to increase folate intake among childbearing-aged women and thus reduce the risk of neural-tube defects in newborns. As a result of this fortification, folic acid intake increased by a mean of 190 μg/d (11) and a recent meta-analysis has shown that the incidence of stroke has been significantly reduced (12).

Studies of 2 cohorts, the Framingham Offspring Study and the NHANES, have demonstrated that folic acid fortification resulted in significantly improved folate status. Moreover, the prevalence of moderate hyperhomocysteinemia (tHcy > 13 μmol/L) was also significantly reduced (13,14). However, neither of these studies compared the genetic influence on tHcy concentrations before and after folic acid fortification.

Of the many genetic variants in the transsulfuration and remethylation pathways affecting the concentration of homocysteine, the most often studied is the 677C>T variant of

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[8] Abbreviations used: CARDIA, Coronary Artery Risk Development in Young Adults; CVD, cardiovascular disease; MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine.
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methylenetetrahydrofolate reductase (MTHFR). Kang et al. (15) first reported a variant of MTHFR that was thermolabile in vitro. Subsequent studies demonstrated that the thermolabile MTHFR variant is caused by the transition of cytosine to thymine at position 677, resulting in the substitution of alanine by valine (16). The 677C>T polymorphism is associated with reduced enzyme activity and moderately increased fasting tHcy concentrations (17), especially in the presence of low serum folate concentrations (18). Thus, individuals with this variant are thought to have a higher folate requirement for regulation of homocysteine. The uncovering of the molecular basis of the thermolabile variant of MTHFR led to a large number of studies demonstrating that homozygosity of the 677T allele predisposes individuals to hyperhomocysteinemia (5,19). This variant has been intensely studied in part due to the generally high prevalence in most races/ethnicities, with 677T homozygosity ranging from 20% or more among Italians and U.S. Hispanics, 10–12% among Caucasians, and 1% or less among Blacks from Africa and the US (20).

Today there are nearly 1500 genetic tests available from clinical and commercial laboratories on well-established genetic mutations as well as genetic variants that are thought to predispose individuals to a higher risk for certain diseases. It has been advocated that there should be a specific Clinical Laboratory Improvement Amendments regulation on issues such as the proficiency, ethics, and cost-effectiveness of these genetic tests (21). MTHFR 677C>T is among the most popular polymorphism tested for nutritional genomics purposes due to its involvement in hyperhomocysteiniemia and folate metabolism.

The Coronary Artery Risk Development in Young Adults (CARDIA) is a biracial, prospective cohort study of 5115 participants in which Caucasian and African American young adults are followed for the development and progression of CVD (22). In CARDIA, we studied the influence of the 677C>T variant of the MTHFR gene on tHcy levels before folic acid fortification (year 1985 and 1992) and after fortification (year 2000) in both Caucasians and African Americans in an attempt to discern the effects of nutrition and genetics on tHcy concentrations in different race/ethnicity groups.

Materials and Methods

Subjects and blood samples. The CARDIA study is a National Heart, Lung, and Blood Institute-funded, longitudinal epidemiological study of an initial cohort of 5115 participants from 4 clinical centers (Birmingham, AL; Chicago, IL; Minneapolis, MN; and Oakland, CA). A primary aim of CARDIA is to follow the development and progression of risk factors for CVD in both Caucasian and African American young adults initially aged 18–30 y. Background information concerning objectives and design of the CARDIA study are described elsewhere (22).

In this ancillary study of CARDIA, we studied a population of 844 Caucasians (462 females and 382 males, mean age 25.7 ± 3.3 y) and 587 African Americans (372 females and 215 males, mean age 24.7 ± 3.8 y). Serum samples that were collected from 3 different visits [y 0 (1985), y 7 (1992), and y 15 (2000)] and stored frozen at −70°C were used for tHcy and B vitamin analysis. DNA was extracted from peripheral leukocytes obtained from blood collected from each participant. Institutional Review Board approval was obtained at all CARDIA sites and all participants signed informed consent.

Biochemical assays. Serum tHcy was measured in serum by a fluorescence polarization immunoassay (IMx Homocysteine Assay, Axis Biochemicals) using the IMx Analyzer (Abbott Diagnostics).

We measured folate and vitamin B-12 in serum on the Hitachi 911 (Roche Diagnostics) using the CEDIA homogeneous enzyme immunoassay system (Boehringer Mannheim). Vitamin B-6 was measured in serum using a radioenzymatic assay (American Laboratory Products). B vitamin supplementation data were not available.

Mutation analysis. DNA was extracted from peripheral lymphocytes and MTHFR 677C>T genotype was determined using selective PCR amplification, followed by restriction enzyme digestion and agarose gel electrophoresis, as previously described (17). *P*-values for chi-square tests on Hardy Weinberg Equilibrium were 0.04 in Caucasians and 0.87 in African Americans. The deviation from the Hardy Weinberg Equilibrium in Caucasians was significant but small. Genotyping results were confirmed by real-time PCR analysis on the LightCycler (Roche Molecular Biochemicals) and by Taqman SNP Genotyping Assay (Applied Biosystems). Thus, the small deviation from the Hardy Weinberg Equilibrium was more likely due to chance rather than methodology. In addition, the prevalence of 677TT genotype in the CARDIA participants is comparable to previously published literature (20).

Statistical methods. Fasting tHcy and B vitamins had a skewed distribution; therefore, these variables were natural log transformed and geometric means (95% CI) are presented in the text and tables. Moderate hyperhomocysteinemia was defined as >13 μmol/L, because this definition was also used by the NHANES and Framingham studies (13,14). To test the difference of tHcy and B vitamins across 3 visits, repeated-measures analysis was used by Proc Mixed with an unstructured covariance structure for within-subject correlation. The differences in tHcy and genotypes between different groups of individuals, both before and after folic acid exposure, were tested for significance. The Generalized Estimating Equation was employed to fit correlated binary outcome (high tHcy) and comparison between different time points was tested in the model adjusted with other covariates. Significance was defined as $P < 0.05$. *P*-values indicating higher significance were noted in tables for comparison between different genotypes and time points. Tests for an additive interaction between pre- and postfortification levels of folate and the 677C>T variant and tHcy levels were conducted and were nonsignificant at the $P < 0.05$ level. All statistical analyses were performed using SAS version 9.1.

Results

We compared the mean of serum B vitamins and tHcy concentrations and the prevalence of folate deficiency (serum folate ≤ 6.8 nmol/L) (18) and moderate hyperhomocysteinemia (tHcy > 13 μmol/L) (13,14) in 844 Caucasian and 587 African American participants before folic acid fortification (y 0 and y 7) and after fortification (y 15) (**Table 1**). Mandatory folic acid fortification improved the nutritional status of folate in both Caucasians and African Americans, with an approximate 3-fold increase in folate concentrations at y 15 compared with year 0. Vitamin B-6 levels slightly increased over the years, whereas vitamin B-12 levels at y 7 and 15 were lower compared with that in y 0. Concurrently, tHcy concentrations at y 15 decreased significantly compared with y 0 and 7 in both populations. The high prevalence of folate deficiency at y 0 (26.2% in Caucasians and 43.6% in African Americans) decreased to ~1% (0.4% in Caucasians and 1.4% in African Americans) at y 15 and the percentage of participants with moderate hyperhomocysteinemia decreased from 15.3% at y 0 to 4.5% at y 15 in Caucasians and from 16.9% to 6.5% in African Americans.

The geometric mean tHcy concentrations from y 0, 7, and 15 in Caucasian and African American participants grouped by MTHFR genotypes varied with presence of the MTHFR 677T allele (**Table 2**). Caucasian 677TT homozygotes had significantly higher tHcy levels compared with CC homozygotes at y 0, 7 and 15. However, the absolute difference between 677TT and 677CC decreased from 2.5 μmol/L at y 0 to 0.7 μmol/L at y 15. In African Americans, carriers of at least 1 MTHFR 677T allele had significantly elevated tHcy levels compared with CC

TABLE 1 Serum B vitamin and tHcy concentrations and prevalence of folate deficiency and moderate hyperhomocysteinemia in 844 Caucasian and 587 African American participants in the CARDIA study[1]

	y 0, 1985	y 7, 1992	y 15, 2000
Caucasian			
Folate, *nmo/L*	11.96 (11.17–12.80)	17.08* (16.31–17.87)	34.07*# (32.04–36.22)
Vitamin B-6, *nmol/L*	39.54 (35.21–44.40)	46.28§ (43.14–49.64)	50.76§ (44.63–57.74)
Vitamin B-12, *nmol/L*	470.73 (450.72–491.62)	439.97§ (428.44–451.81)	448.49 (427.24–470.81)
tHcy, *μmol/L*	10.35 (10.04–10.67)	9.4* (9.20–9.61)	8.22*# (7.96–8.48)
Folate <6.8 nmol/L, *%*	26.2 (23.2–29.2)	10.7* (8.6–12.8)	0.4*# (0–08)
tHcy >13 μmol/L, *%*	15.3 (12.9–17.7)	11.1 (9.0–13.3)	4.5*# (3.1–5.9)
African American			
Folate, *nmol/L*	7.93 (7.36–8.58)	10.71* (10.15–11.33)	24.64*# (23.19–26.16)
Vitamin B-6, *nmol/L*	22.08 (19.28–25.28)	27.90* (25.61–30.40)	31.95* (28.07–36.37)
Vitamin B-12, *nmol/L*	591.72 (562.49–622.47)	517.65* (501.11–534.74)	505.11* (481.32–530.08)
tHcy, *μmol/L*	10.67 (10.30–11.06)	10.17 (9.91–10.44)	8.66*# (8.40–8.93)
Folate <6.8 nmol/L, *%*	43.6 (39.6–47.6)	26.6* (23.0–30.2)	1.4*# (0.4–2.3)
tHcy >13 μmol/L, *%*	16.9 (13.8–19.9)	17.7 (14.6–20.8)	6.5*# (4.5–8.5)

[1] Concentrations are expressed as geometric mean (95% CI). B vitamin concentrations were adjusted for age and gender and tHcy concentrations were adjusted for age, gender, and smoking status. *Different from y 0, $P < 0.001$; #Different from y 7, $P < 0.001$; §Different from y 0, $P < 0.05$.

homozygotes at y 0 and 7, but at y 15, the difference was no longer significant.

In Caucasians, the percentage of individuals who had hyperhomocysteinemia decreased from 34.2% (39/114) in y 0 and 25.4% (29/114) in y 7 to 10.5% (12/114) in y 15 (**Table 3**). However, deficient folate levels (<6.8 nmol/L) and 677TT genotype were not always associated with hyperhomocysteinemia. Thus, among Caucasians with deficient folate levels, 14 of 41 (34%) at y 0, 14 of 26 (54%) at y 7, and 1 of 3 at y 15 did not have hyperhomocysteinemia even though they had combined folate deficiency and homozygosity of the 677T allele. Conversely, 6 of 98 individuals in y 15 with serum folate > 15.4 nmol/L still had tHcy > 13 μmol/L. The range of serum folate in these 6 677TT homozygotes was 17.2–67.7 nmol/L, the highest 2 values being 43.9 and 67.7 nmol/L.

There were only 7 African Americans who were homozygous for the 677T allele and prior to folic acid fortification, 6 of these 7 had less than optimal folate concentrations (Table 3). Postfortification, 2 677TT homozygotes had moderate hyperhomocysteinemia, one with less than optimal folate concentration, the other with folate concentration of 17.4 nmol/L.

We used the sensitivity and specificity of MTHFR 677C>T genotyping to predict elevated tHcy concentrations using various tHcy cutoffs to define hyperhomocysteinemia (**Table 4**). Sensitivity of the MTHFR genotyping for predicting moderate hyperhomocysteinemia is low (30%) before and after folic acid fortification using the definition of >13 μmol/L as the tHcy cutoff. When higher tHcy values (up to 19 μmol/L) were used for defining hyperhomocysteinemia, the sensitivity of the MTHFR 677TT genotype test in detecting hyperhomocysteinemia increased from ~30% to >60% before folic acid fortification (y 0 and 7) and decreased from 32 to 17% after folic acid fortification (y 15). Specificity of MTHFR 677C>T genotyping in predicting hyperhomocysteinemia remained essentially unchanged (87–88%) after folic acid fortification.

Discussion

Mandatory folic acid fortification of enriched cereal grain products was initiated in the United States in 1998. The results from a subsample of the CARDIA study reported here agree well with the results from those of the Framingham and NHANES studies, which showed in cross-sectional analyses that folic acid fortification was associated with more favorable concentrations of serum folate and tHcy (13,23). Unlike these previous studies, we measured serum folate and tHcy at 2 time points before folic acid fortification and once postfortification in the CARDIA cohort. An interesting observation was that folate status improved moderately even before mandatory folate fortification, perhaps due to dietary changes in this young adult cohort.

TABLE 2 Serum tHcy concentrations in 844 Caucasian and 587 African American participants of the CARDIA study classified by MTHFR genotypes[1]

			tHcy					
Race/ethnicity	MTHFR genotype	*n* (%)	y 0, 1985		y 7,1992		y 15, 2000	
			μmol/L					
Caucasian	677CC	387 (45.9)	9.35	(9.05–9.67)	9.04	(8.75–9.34)	8.44	(8.24–8.65)
	677CT	343 (40.6)	9.62	(9.29–9.95)	9.10	(8.79–9.42)	8.45	(8.23–8.67)
	677TT	114 (13.5)	11.92*	(11.25–12.63)	10.93*	(10.32–11.57)	9.08#	(8.70–9.47)
African American	677CC	465 (79.2)	9.46	(9.15–9.78)	9.44	(9.13–9.77)	8.60	(8.38–8.82)
	677CT	115 (19.6)	10.27#	(9.69–10.89)	10.29§	(9.67–10.95)	9.02	(8.60–9.47
	677TT	7 (1.2)	12.50§	(10.08–15.49)	19.10*	(15.15–24.09)	9.54	(7.91–11.51)

[1] Concentrations are expressed as geometric mean (95% CI) adjusted for age, gender, and smoking status. *677TT vs. 677 CC, $P < 0.001$; #677CT vs. 677CC and 677TT vs. 677 CC, $P < 0.01$; §677CT vs. 677CC and 677TT vs. 677 CC, $P < 0.05$.

TABLE 3 Prevalence of moderate hyperhomocysteinemia in MTHFR 677T homozygotes based on serum folate concentrations in a population of 844 Caucasian and 587 African American participants in the CARDIA study

	y 0, 1985		y 7, 1992		y 15, 2000	
Range of folate, *nmol/L*	677 TT	tHcy >13 μmol/L	677 TT	tHCY >13 *μmol/L*	677 TT	tHcy >13 *μmol/L*
Caucasian	*n*	*n (%)*	*n*	*n (%)*	*n*	*n (%)*
<6.8	41	27 (65.9)	26	12 (46.2)	3	2 (66.7)
6.8–15.4	38	11 (28.9)	36	14 (38.9)	13	4 (30.8)
>15.4	35	1 (2.9)	52	3 (5.8)	98	6 (6.1)
Total	114	39 (34.2)	114	29 (25.4)	114	12 (10.5)
African Americans						
<6.8	4	1 (25)	4	3 (75)	0	0 (0)
6.8–15.4	2	0 (0)	2	1 (50)	1	1 (100)
>15.4	1	0 (0)	1	0 (0)	6	1 (16.7)
Total	7	1 (14.3)	7	4 (57.1)	7	2 (28.6)

Nevertheless, a more dramatic impact on folate level was seen after folic acid fortification such that mean serum folate levels more than doubled from y 7 to 15. These findings suggest that the improved folate and tHcy status in the postfortification era, while largely due to folic acid fortification, may also be in part due to dietary changes.

The prevalence of folate deficiency in CARDIA (0.7%) at y 15 agreed with NHANES (1%), demonstrating that although overall folate level significantly increased in response to folic acid fortification, the average level of folate in African Americans was lower than that in Caucasians (14). As expected, tHcy concentrations decreased as folate levels increased. The prevalence of moderate hyperhomocysteinemia in the CARDIA population (4.5% in Caucasians and 6.5% in African Americans at y 15) was similar to that of the NHANES, which reported that ~5% of the individuals had moderate hyperhomocysteinemia post-folic acid fortification (14). We show that, although the prevalence of moderate hyperhomocysteinemia, especially at y 7, was higher in African Americans than in Caucasians before folic acid exposure, the effect of folic acid fortification resulted in a larger percent reduction of moderate hyperhomocysteinemia in African Americans than in Caucasians. Nevertheless, the percentage of African Americans with moderate hyperhomocysteinemia is still higher compared with Caucasians.

A major focus of the present study was to examine how folic acid fortification influenced the effect of the MTHFR 677C>T polymorphism on tHcy concentrations and also to evaluate the clinical utility of genotyping of the MTHFR 677C>T polymorphism. The MTHFR 677C>T is a prevalent polymorphism and numerous studies have demonstrated that individuals homozygous for the 677T allele are predisposed to higher concentrations of tHcy (17,18). However, the predisposition to elevated plasma tHcy concentration was thought to be modulated by folate status (18). We show that both Caucasians and African Americans homozygous for the 677T allele have higher tHcy concentrations compared with those with the 677CC/677CT genotype before and after folic acid fortification, although the magnitude of the differences significantly decreased in the postfortification visit. Because the prevalence of the minor allele (677T) is low in African Americans, meaningful assessment can be best carried out in the Caucasian cohort. Although there is a significant difference in the mean values of tHcy between individuals who are homozygous for the 677T allele and those either homozygous or heterozygous for the 677C allele, the absolute difference is small even before folate fortification (2.5 μmol/L). After folate fortification, the mean difference was <0.7 μmol/L. Using the value of >13 μmol/L as the cutoff for moderate hyperhomocysteinemia, even at y 0 prior to folic acid fortification, only 34.2% (39/114) of the Caucasians homozygous for the 677T allele had moderate hyperhomocysteinemia (Table 3). Post-folic acid fortification (y 15), only 10.5% (12/114) of the Caucasian individuals with the 677TT genotype had moderate hyperhomocysteinemia. Moreover, whereas it is well known that high serum folate concentration modulates the predisposition of individuals homozygous with respect to the 677T allele to hyperhomocysteinemia, we found that post-folate fortification, among the 12 individuals with the 677TT genotype and moderate hyperhomocysteinemia, 6 had folate levels >15.4 nmol/L (range, 17.2–67.4 nmol/L). None of the individuals were deficient in either vitamin B-6 or B-12 and none had a creatinine level >0.11 mmol/L. In the present study, we did not measure riboflavin, which is associated with tHcy levels, particularly in 677TT individuals. However, the influence of riboflavin on tHcy is strongest at low folate levels and diminishes with increasing plasma folate concentration (24). Thus, it is not known whether the moderate hyperhomocysteinemia in those 6 individuals was due in part to riboflavin deficiency or, given the high folate level, to factors other than MTHFR 677TT genotype.

TABLE 4 Sensitivity and specificity of 677C>T MTHFR genotype for prediction of tHcy levels in 844 Caucasian participants in the CARDIA study

	y 0, 1985			y 7, 1992			y 15, 2000		
tHcy	*n*	Sensitivity	Specificity	*n*	Sensitivity	Specificity	*n*	Sensitivity	Specificity
μmol/L		%			%			%	
>13	129	30.2	89.5	94	29.9	88.6	38	31.6	87.3
>15	63	38.1	88.5	45	42.2	88.1	19	21.1	86.7
>17	38	52.6	88.3	30	43.3	87.6	10	20.0	86.6
>19	26	61.5	88.0	19	63.2	87.6	6	16.7	86.5

To further assess the utility of MTHFR 677C>T genotyping, we determined the sensitivities and specificities of this test before and after folic acid fortification using various tHcy cutoffs to define hyperhomocysteinemia. When the cutoff was increased from 13 to 19 μmol/L, sensitivity of the MTHFR 677TT genotype assay increased before folic acid fortification but decreased after folic acid fortification.

As genetic tests become increasingly available in both the clinical laboratory setting and in laboratories that directly market genetic testing to the public, it is increasingly important to determine the utility of each individual assay. The current study shows that, although the MTHFR 677C>T is one of the most studied polymorphisms and the test has been available in clinical laboratories as well as nutritional genetic counseling companies, the utility of this test is limited, particularly in the post-folic acid fortification era. Whereas others have argued the benefits of screening for MTHFR 677C>T and targeting 677TT homozygote individuals for folate supplementation as a means to lower tHcy levels and prevent CVD (25), our data do not support the efficacy of using MTHFR 677C>T as a screening test. In contrast, we show that the MTHFR 677TT genotype is neither necessary nor sufficient to cause hyperhomocysteinemia. Although the test might have had some utility in the prefolate fortification era in individuals with relatively high tHcy values, screening for MTHFR 677C>T in the US post-folic acid fortification offers little value either in the clinical or the nutritional genetic counseling setting so long as the interest in genotyping stems from its association with moderate hyperhomocysteinemia. For most individuals, the measurement of tHcy should be the primary assay used for the diagnosis and monitoring of moderate hyperhomocysteinemia.

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