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Polygenic Association with Total Homocysteine in the Post Folic Acid Fortification Era: the CARDIA Study

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Abstract

Elevated plasma concentration of total homocysteine (tHcy) has been linked with many diseases. tHcy is associated with a variety of factors, including polymorphisms in genes involved in homocysteine metabolism. It is not clear whether US-mandated fortification of grain products with folic acid has affected the association of genetic variants with tHcy levels. We determined tHcy concentrations in sera from 997 Caucasians and 692 African Americans participants in the Coronary Artery Risk Development in Young Adults (CARDIA) study before and after folic acid fortification. DNA was genotyped for variants present in four genes involved in homocysteine metabolism: cystathionine β -synthase (CBS) 844ins68, methionine synthase (MS) 2756A>G; methionine synthase reductase (MTRR) 66A>G, and methylenetetrahydrofolate reductase (MTHFR) 677C>T and 1298A>C. A greater number of African Americans were homozygous for the MS 2756GG, MTRR 66GG and CBS 844ins68 genotypes compared to Caucasians, while prevalence of MTHFR 677TT and 1298CC genotypes was substantially lower in African Americans compared to Caucasians. The overall variance in tHcy levels at y 0, 7, and 15 that can be explained by the combined presence of all five variants increased slightly over time in Caucasians (17%, y 0; 21%, y 7 and 26%, y 15) and in African Americans (13%, y 0; 17% y 7; 18% y 15) largely due to decrease in tHcy variance.

Keywords

Cystathionine B-synthase; folic acid; homocysteine; methylenetetrahydrofolate reductase; methionine synthase; methionine synthase reductase

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INTRODUCTION

Moderately elevated concentrations (>13 $\mu\text{mol/L}$) of total homocysteine (tHcy) have been associated with many diseases including coronary artery disease (CAD) [1–5], age-related disease states such as cognitive impairment, dementia and depression [6], osteoporotic fractures [6,7], end stage renal disease [8] and cancer [9]. Homocysteine has also been known to be elevated in mothers of children with neural tube defects [10].

Genetic defects in a number of enzymes involved in either the transsulfuration or the remethylation pathways of homocysteine metabolism may influence tHcy levels [2]. Cystathionine β -synthase (CBS) is the key enzyme in the transsulfuration of homocysteine to form cystathionine and cysteine. A large number of mutations of CBS have been identified in patients with homocystinuria [11]; however, these mutations are rare. A relatively prevalent 68-bp insertion (844ins68), present in the heterozygous state in approximately 12–15% of the Caucasian population [2, 12, 13], has been associated with decreased levels of homocysteine, especially in individuals with low levels of vitamin B6 [14].

Three enzymes involved in the remethylation of homocysteine to methionine are methionine synthase (MS), methionine synthase reductase (MTRR) and methylenetetrahydrofolate reductase (MTHFR). Only one polymorphism, the 2756A>G (D919G) transition, is relatively prevalent in the MS gene [15]. In a study of 1025 individuals, 32.1% of the population was heterozygous and 3.8% was homozygous for the G2756 allele, and individuals homozygous for the 2756A>G variant had a mild but significantly lower concentration of plasma tHcy [2]. A common polymorphism of MTRR, 66A>G (I22M), has been reported to be present as the AA variant in 19 – 29% and as the GG variant in 17 – 31% of the Caucasian population; however, reports on the association of the MTRR 66A>G polymorphism with plasma tHcy have been inconsistent [16–18]. The MTRR genotype is independent of serum folate, vitamin B6 and vitamin B12 concentrations [16–18]. The MTHFR 677C>T (A222V) variant is the most widely studied polymorphism associated with homocysteine metabolism. Frequency of this recessive trait varies from 1% or less among African Americans from the United States and Africa to 10 – 12% in Caucasians and 20% or more among Italians and US Hispanics [19–23]. Numerous studies have demonstrated that individuals homozygous for the 677T allele are predisposed to higher concentrations of tHcy, especially in the presence of low folate concentration [20, 24, 25]. A second MTHFR polymorphism, the 1298A>C variant, is present in the homozygous state in about 10% of the Caucasian population. This polymorphism alone does not significantly affect plasma tHcy but has been shown to do so when combined with the 677T variant [23, 26, 27].

In 1996 the U.S. Food and Drug Administration required that all enriched grain products be fortified with folic acid to reduce the risk of neural-tube defects in newborns [28]. As a result, studies have shown that folate status has significantly improved with a concurrent decrease in prevalence of moderate hyperhomocysteinemia [29, 30]. Our group, in a previous study of a subset of 844 Caucasian and 587 African American participants of the Coronary Artery Risk Development in Young Adults (CARDIA) study showed that homozygosity of the MTHFR 677T allele plays a less significant role in predisposing the United States population to moderate hyperhomocysteinemia in the post folic acid fortification era [31]. Although polygenic regulation of tHcy has been examined in populations with premature CAD or at high risk for CAD [2,32], there are no studies investigating the effect of multiple homocysteine metabolizing genes on the level of tHcy after folic acid fortification in the U.S. In the current study of a larger subset of CARDIA, we investigated the role of genes involved in homocysteine metabolism in addition to MTHFR 677C>T in order to determine the polygenic influence on tHcy concentrations in

the era of folic acid fortification in the United States. In contrast to most studies on association of tHcy with genetic defects, which have been performed in Caucasians of northern European descent, this study includes both Caucasian and African American participants of the CARDIA study.

MATERIALS AND METHODS

Subjects and blood samples

The CARDIA study is a NHLBI-funded longitudinal epidemiological study of a biracial cohort of 5115 participants from four clinical centers (Birmingham, AL; Chicago, IL; Minneapolis, MN; and Oakland, CA). The primary aim of CARDIA is to follow the development and progression of cardiovascular risk factors in both Caucasian and African American young adults initially aged 18–30 years. Background information concerning objectives and design of the CARDIA study are outlined elsewhere [33].

In this ancillary study of CARDIA, a population of 997 Caucasians (503 females and 494 males, mean age 25.7 ± 3.3) and 692 African Americans (417 females and 275 males, mean age 24.7 ± 3.8) was studied. Fasting serum samples that were collected from three different visits (y 0 [1985], y 7 [1992], and y 15 [2000]) and stored frozen at -70°C were used for tHcy analysis. DNA was extracted 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 folate was measured on the Hitachi 911 (Roche Diagnostics, Indianapolis, IN) using the CEDIA homogeneous enzyme immunoassay system (Boehringer Mannheim). tHcy was measured in serum by a fluorescence polarization immunoassay (IMx Homocysteine Assay, Axis Biochemicals ASA, Oslo, Norway) using the IMx Analyzer (Abbott Diagnostics, Abbott Park, IL).

Mutation Analysis

DNA was extracted from peripheral leukocytes isolated from acid-citrate-dextrose-anticoagulated blood using a commercially available DNA isolation kit (Puregene, Gentra Systems, Minneapolis, MN). The 844ins68 variant of the CBS gene was detected by selective PCR amplification of a 184-bp DNA fragment containing exon 8 of the CBS gene, as described by Tsai et al. [12]. To detect the 2756A>G polymorphism of the MS gene, a 189-bp fragment was amplified and digested with *Hae*III, as described by Leclerc et al. [15]. Primers that introduce an artificial *Nde*I restriction site were used to detect the MSRR 66A>G polymorphism [34]. The 677C>T polymorphism in exon 4 of the MTHFR gene was detected by selective PCR amplification of a 198-bp fragment followed by digestion with the restriction enzyme *Hinf*I, as described by Frosst et al. [20]. In order to distinguish the 1298A>C polymorphism from the 1317T>C silent polymorphism, primers that introduce artificial *Fnu*4HI restriction sites were used to amplify the MTHFR 1298A>C polymorphism, as described by Weisberg et al. [26]. All cleaved products were applied to a 2% agarose gel containing ethidium bromide, electrophoresed, and visualized on an ultraviolet transilluminator (Fotodyne, Hartland, WI). All genotyping results follow Hardy Weinberg Equilibrium with the exception of MTHFR 677C>T for Caucasians (*P* value for Chi-square test = 0.04). This deviation is small, but significant. Genotyping results for MTHFR 677C>T were confirmed by real-time PCR analysis on the LightCycler™ (Roche Molecular Biochemicals, Indianapolis, IN) and by Taqman® SNP Genotyping Assay (Applied Biosystems, Foster City, CA). Thus, the small deviation from the Hardy Weinberg Equilibrium is more likely to be due to chance rather than methodology. In addition, the

prevalence of 677TT genotyping in the CARDIA participants is comparable to previously published literature [22].

Statistical analysis

Fasting serum tHcy and folate levels had a skewed distribution, therefore, results were natural log transformed and geometric means were used. Chi-square tests were employed to test genotype frequency between ethnic groups and Hardy-Weinberg equilibrium for all genetic polymorphism stratified by both ethnic groups. Polygenic effects were assessed by starting with the assumption of a codominant model for the SNPs to determine if the dominant assumption is appropriate. ANOVA models were used to test for interaction between each genotype and to identify which of the genotypes are significantly associated with tHcy levels after adjusting for the other genes. Significance was defined as $P < 0.05$. P values indicating higher significance were noted in tables for comparison between different genotypes and time points. All statistical analyses were performed using SAS version 9.1 (Cary, NC).

RESULTS

Prevalence of polymorphisms

Table 1 shows the prevalence of the 844ins68 of the CBS gene, the 2756A>G polymorphism of the MS gene, the 66A>G polymorphism of the MTRR gene, and the 677C>T and 1298A>C polymorphisms of the MTHFR gene in 997 Caucasians compared to 692 African Americans in the CARDIA cohort. A larger number of African Americans (7.7%) were found to be homozygous for the 844ins68 of the CBS gene compared to 0.8% of the Caucasian population. Occurrence of the MS 2756GG genotype is twofold greater in African Americans compared to Caucasians. For the MTRR 66A>G polymorphism, African Americans have a greater number of GG homozygotes (51.5%) and fewer AA homozygotes (7.8%) compared to Caucasians, who have similar distributions between the GG and AA genotypes. In contrast, there were fewer African American MTHFR 677TT homozygotes (1.3%) compared to Caucasians (12.8%), and also less prevalence of MTHFR 1298CC homozygotes in African Americans (3.3% compared to 11.6% in Caucasians).

Variation in tHcy levels with genetic variants

Table 2 shows the overall geometric folate and tHcy concentrations and the influence of CBS, MS, MTRR and MTHFR genotypes on tHcy concentrations in sera at y 0, 7 (pre-fortification) and y 15 (post-fortification) in Caucasian and African American participants of the CARDIA study. Mandatory folic acid fortification improved the nutritional status of folate in both Caucasians and African Americans, with approximately a threefold increase in folate concentrations at y 15 compared to y 0. Regardless of genotype, tHcy values were significantly lower in both ethnic groups after folic acid fortification compared to that in the pre fortification era. When individual genotypes were considered, tHcy levels were significantly lower in African Americans who are homozygous for the MS 2756G allele compared to AA homozygotes at y 0, 7 and 15. Caucasian 677TT homozygotes showed significantly higher tHcy concentrations compared to CC homozygotes at y 0, 7 and 15. In African Americans, carriers of at least one MTHFR 677T allele had significantly elevated tHcy levels compared to CC homozygotes at y 0 and 7, but at y 15, the difference was no longer significant.

Multivariable association of genetic variants with tHcy

Table 3 shows the multivariable association of CBS, MS, MTRR, and MTHFR variants with tHcy concentrations before (y 0 and 7) and after (y 15) folic acid fortification adjusted for

age and gender. The polygenic association is significant ($P < 0.001$) for y 0, 7 and 15 with tHcy concentrations and with the difference in tHcy concentrations between y 0–15 and y 7–15. The overall variance in tHcy levels at y 0, 7, and 15 that can be explained by the combined presence of all five variants increased slightly over time in Caucasians (17%, y 0; 21%, y 7 and 26%, y 15) and in African Americans (13%, y 0; 17% y 7; 18% y 15). The overall variance in tHcy values measured as the difference between y 0 and 15 and between y 7 and 15 that can be explained by the combined presence of the five variants is 6 and 5%, respectively, in Caucasians and 4 and 17% in African Americans. In Caucasians, only the 677C>T polymorphism of the MTHFR gene was significantly ($P < 0.001$) associated with tHcy concentrations before folic acid fortification after controlling for other genes. This significance decreased ($P = 0.004$) in the post folic acid fortification era. In African Americans, the MTHFR 677C>T variant was significantly associated with tHcy concentrations at y 0 ($P = 0.003$) and y 7 ($P < 0.001$) after controlling for other genes; however, no significance was observed after folic acid fortification (y 15). The MS 2756A>G genotype was significantly associated with tHcy concentrations in African Americans after controlling for other genes both before ($P = 0.03$ and 0.01 in y 0 and 7, respectively) and after ($P = 0.007$ in y 15) folic acid fortification; however, the MS A2756G genotype was not significantly associated with tHcy levels in Caucasians.

DISCUSSION

The purpose of the current study was to compare the association of multiple genetic variants with four homocysteine metabolizing genes on tHcy concentrations before and after folic acid fortification. The study was done on a subset of CARDIA participants of both African American and Caucasian ethnicity attending the y 0, 7 and 15 exams allowing us to determine the polygenic association of these variants with moderate hyperhomocysteinemia in two different races. Additionally, the study allowed us to compare these two populations with respect to the prevalence of these five variants in African Americans and Caucasians.

We show that the distribution of genotypes in the CBS, MS, MTRR and MTHFR genes is different in African Americans compared to the more frequently studied Caucasian population. While we confirmed findings that homozygosity for the 68 bp insertion is rare ($<1\%$) in Caucasians [12,13], our findings show that the 68bp insertion is relatively prevalent (7.7%) in African Americans. Our results also show that the frequency of MS 2756GG and MTRR 66GG alleles are approximately 2 fold higher in African Americans compared to Caucasians. Our finding that the frequency of MTHFR 677TT homozygotes is lower in African Americans compared to Caucasians (1.3% vs 12.8%, respectively) agrees with the HuGE Review where the frequency of 677TT homozygosity was reported to be 1 to 2% in African Americans and 10 to 14% in Caucasians living in the United States [22]. Frequency of the 1298A>C allele of the MTHFR gene was not documented in the HuGE Review; however, we found that frequency of the 1298A>C allele is also lower in African Americans compared to Caucasians. This is in agreement with the reported linkage disequilibrium for these two variants [35].

There is wide agreement that both genetic and nutritional factors are important in the regulation of tHcy concentrations. In the current study we are able to compare the association of polymorphisms in the CBS, MS, MTRR and MTHFR genes with tHcy concentrations before and after folic acid fortification. We have previously shown in a smaller subset of CARDIA that folic acid fortification was associated with increased concentrations of folate and decreased prevalence of moderate hyperhomocysteinemia [31], similar to results from the Framingham and the National Health and Nutrition Examination Survey (NHANES) studies [29, 30]. In this study, we also examined how folic acid fortification influenced the association of the MTHFR 677C>T polymorphism with tHcy

concentrations. We showed that both Caucasians and African Americans homozygous for the 677T allele had higher tHcy concentrations compared to those with 677CC/677CT genotype before and after folic acid fortification, although the magnitude of the differences significantly decreased in the post-fortification visit. Additionally we showed that the prevalence of moderate hyperhomocystenemia in MTHFR 677TT homozygotes decreased from 37% before fortification to 12% post fortification ($P<0.01$), thus suggesting that genotyping for the MTHFR 677C>T variant has decreased clinical utility in the post fortification era [31]. In the current study, we examined the combined association of MTHFR 677C>T and 1298A>C, MS 2756A>G, MTRR 66A>C, and CBS 844ins68 with tHcy concentrations before and after folic acid fortification in an increased number of CARDIA participants.

We confirmed that after folic acid fortification, the significant association between MTHFR 677C>T and tHcy decreased in Caucasians and disappeared in African Americans. However, the small number of African Americans with the MTHFR 677TT genotype makes meaningful assessment in this group difficult.

The MS 2756GG allele has previously been shown to be associated with lower tHcy concentrations [36], and we show that this association is significant in African Americans both before and after folic acid fortification. Our work agrees with others that MTHFR 1298C>A, MTRR 66G>A and CBS 844ins68 variants do not significantly affect tHcy values in Caucasians [17, 23, 26, 27, 36, 37], and we show for the first time that this is also true in African Americans. Moreover this finding is independent of folic acid fortification.

In the era of post-folic acid fortification, the variance in tHcy that can be explained by the polygenic effect is 26% in Caucasians and 18% in African Americans. The slight increase in the percentage of genetic variance compared to the pre fortification era is primarily due to decreased variance in tHcy values post folic acid fortification, thus the slightly increased percentage is associated with a net decreased effect on the level of tHcy in serum. The current study involves a relatively young and healthy population, thus renal insufficiency as measured by elevated serum creatinine was rare (< 1%) either at y 0 or at y 15. On the other hand, the study of a relatively young and healthy population also constitutes a limitation of the study, as these results may not be extrapolated to either an older cohort or cohorts with specific diseases.

While the fraction of the variance in tHcy explained by the 5 genetic variants increased after the introduction of fortification, measurement of tHcy remains as the logical first line of assay for screening of moderate hyperhomocystenemia. Genetic assays are not cost effective for screening purposes but may be of importance particularly in patients with hyperhomocystenemia not explained by nutritional, lifestyle factors or those resulting as co-morbidity from diseases such as renal failure.

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Abbreviations

CAD	coronary artery disease
CARDIA	Coronary Artery Risk Development in Young Adults
CBS	Cystathionine β -synthase

MS	methionine synthase
MTRR	methionine synthase reductase
MTHFR	methylenetetrahydrofolate
tHcy	total homocysteine

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Table 1

Prevalence of five polymorphisms in homocysteine metabolizing genes in 997 Caucasian and 692 African American participants of the CARDIA study.

Genotype	Prevalence n (%)	
	Caucasian	African American
CBS 844ins68^a		
−/− ^b	854 (85.7)	409 (59.1)
−/+ ^c	135 (13.5)	230 (33.2)
+/+ ^d	8 (0.8)	53 (7.7)
MS 2756A>G		
AA	622 (62.4)	378 (54.6)
AG	327 (32.8)	251 (36.3)
GG	48 (4.8)	63 (9.1)
MTRR 66A>G^a		
AA	278 (27.9)	54 (7.8)
AG	502 (50.4)	282 (40.8)
GG	217 (21.8)	356 (51.5)
MTHFR 677C>T^a		
CC	452 (45.3)	547 (79.1)
CT	417 (41.8)	136 (19.7)
TT	128 (12.8)	9 (1.3)
MTHFR 1298A>C^a		
AA	478 (47.9)	472 (68.2)
AC	403 (40.4)	197 (28.5)
CC	116 (11.6)	23 (3.3)

^a $P < 0.05$ for Caucasians vs. African Americans

^b no 844ins68 present

^c heterozygous for 844ins68

^d homozygous for 844ins6

Table 2

Overall geometric mean concentrations of folate and tHcy by genotype in 997 Caucasians and 692 African American participants of the CARDIA study.

	Caucasian			African American		
	y 0 (1985)	y 7 (1992)	y 15 (2000)	y 0 (1985)	y 7 (1992)	y 15 (2000)
Folate^a, nmol/L						
	11.96(11.17–12.80)	17.08 ^b (16.31–17.87)	34.07 ^{b,c} (32.04–36.22)	7.93(7.36–7.58)	10.71 ^b (10.15–11.33)	24.64 ^{b,c} (23.19–26.16)
			tHcy^a, umol/L			
	10.35(10.04–10.67)	9.4 ^b (9.20–9.61)	8.22 ^{b,c} (7.96–8.48)	10.67(10.30–11.06)	10.17(9.91–10.44)	8.66 ^{b,c} (8.40–8.93)
CBS 844ins68						
–/–	9.83(9.60–10.06)	9.30(9.09–9.51)	8.51, 8.37–8.65)	9.93(9.63–10.23)	9.95(9.62–10.28)	8.79(8.56–9.03)
–/+	9.46(8.92–10.03)	8.82(8.33–9.34)	8.15(7.82–8.50)	9.75(9.37–10.15)	9.51(9.11–9.94)	8.57(8.28–8.87)
+/+	9.86(7.77–12.50)	8.66(6.87–10.93)	8.09(6.83–9.58)	9.44(8.69–10.26)	9.48(8.66–10.39)	8.56(7.97–9.19)
MS 2756A>G						
AA	9.74(9.48–10.00)	9.25(9.01–9.50)	8.42(8.26–8.58)	10.07(9.76–10.39)	10.02(9.68–10.36)	8.86(8.62–9.10)
AG	9.94(9.57–10.32)	9.17(8.84–9.51)	8.59(8.36–8.82)	9.67(9.30–10.05)	9.65(9.25–10.07)	8.68(8.40–8.98)
GG	9.29(8.43–10.24)	9.33(8.49–10.26)	8.18(7.63–8.76)	9.06 ^d (8.38–9.30)	8.73 ^d (8.01–9.51)	7.84 ^d (7.33–8.38)
MTRR 66A>G						
AA	9.97(9.58–10.38)	9.30(8.95–9.67)	8.48(8.24–8.72)	9.54(8.72–10.44)	9.83(8.93–10.82)	8.40(7.79–9.06)
AG	9.74(9.44–10.04)	9.29(9.01–9.57)	8.47(8.29–8.66)	9.97(9.61–10.34)	9.68(9.30–10.08)	8.69(8.42–8.97)
GG	9.62(9.20–10.07)	9.00(8.61–9.41)	8.41(8.15–8.69)	9.76(9.45–10.08)	9.82(9.48–10.17)	8.75(8.51–8.99)
MTHFR 677C>T						
CC	9.35(9.06–9.64)	8.97(8.70–9.24)	8.37(8.18–8.56)	9.65(9.40–9.90)	9.55(9.29–9.82)	8.62(8.43–8.81)
CT	9.65(9.35–9.97)	9.04(8.76–9.33)	8.39(8.19–8.59)	10.42 ^e (9.90–10.98)	10.26 ^d (9.70–10.85)	8.98(8.59–9.40)
TT	11.90 ^f (11.24–12.59)	10.85 ^f (10.26–11.48)	9.02 ^e (8.65–9.40)	12.64 ^d (10.19–15.68)	18.82 ^f (14.93–23.74)	9.64(8.00–11.62)
MTHFR 1298A>C						
AA	9.95(9.65–10.26)	9.33(9.06–9.62)	8.70(8.29–8.66)	9.91(9.64–10.20)	9.75(9.46–10.06)	8.48(8.49–8.91)
AC	9.55(9.23–9.87)	9.04(8.75–9.34)	8.66(8.20–8.60)	9.56(9.16–9.98)	9.68(9.24–10.15)	8.40(8.35–8.99)
CC	9.89(9.28–10.54)	9.44(8.88–10.04)	9.12(8.23–9.01)	10.56(9.28–12.00)	10.65(9.24–12.26)	8.61(8.16–10.18)

^aConcentrations are expressed as geometric mean (95% CI) adjusted for age, gender and smoking status

^b*P*<0.001 for differences compared to y 0

- ^c $P < 0.001$ for differences compared to y 7
- ^d $P < 0.05$ for 2756GG vs. AA, 677CT vs. CC and 677TT vs. CC
- ^e $P < 0.01$ for 2756GG vs. AA, 677CT vs. CC and 677TT vs. CC
- ^f $P < 0.001$ for 677TT vs. CC

Polygenic influence of variants in the CBS, MS, MTRR and MTHFR genes on tHcy concentrations before (y 0, y 7) and after (y 15) folic acid fortification in 997 Caucasians and 692 African Americans in the CARDIA study.

Table 3

Variable		y 0, 1985	y 7, 1992	y 15, 2000	y 0 – 15	y 7 – 15
Caucasian	<i>P</i> -value for model ^a	<.001	<.001	<.001	<.001	<.001
	R square for model	0.17	0.21	0.26	0.06	0.05
	<i>P</i> -value for F-test ^b					
	CBS 844ins68	0.61	0.37	0.19	0.91	0.98
	MS 2756A>G	0.44	0.84	0.33	0.71	0.43
	MTRR 66A>G	0.36	0.52	0.93	0.91	0.64
	MTHFR 677C>T	<.001	<.001	0.004	<.001	<.001
	MTHFR 1298A>C	0.06	0.1	0.29	0.38	0.64
	<i>P</i> -value for model ^a	<.001	<.001	<.001	0.015	<.001
	R square for model	0.13	0.17	0.18	0.04	0.17
African American	<i>P</i> -value for F-test ^b					
	CBS 844ins68	0.48	0.22	0.39	0.95	0.35
	MS 2756A>G	0.03	0.01	0.007	0.73	0.88
	MTRR 66A>G	0.5	0.93	0.61	0.52	0.58
	MTHFR 677C>T	0.003	<.001	0.17	0.001	<.001
	MTHFR 1298A>C	0.34	0.3	0.69	0.82	0.75

^aCodominant model was constructed for the genetic variants

^bANOVA was used to identify significant association between genetic variants and tHcy after adjusting for other genes.