Mol Cell Biochem (2007) 302:125–131
DOI 10.1007/s11010-007-9434-5

MTHFR 677 CT/MTHFR 1298 CC genotypes are associated with increased risk of hypertension in Indians

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Received: 23 November 2006 / Accepted: 9 February 2007 / Published online: 1 March 2007

Abstract The goals of our present study were to measure plasma homocysteine levels and determine their association with methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms (C677T and A1298C) in essential hypertensive subjects. Plasma total homocysteine and folic acid levels were measured in essential hypertensive patients (n = 153) before and after oral supplementation with either 5 mg folic acid tablet/day or 5 mg placebo/day for 4 weeks and compared with age and sex matched normotensive controls (n = 133). MTHFR gene polymorphisms (C677T and A1298C) were studied by restriction fragment length polymorphism and correlated with plasma homocysteine levels. Homocysteine levels were significantly higher in hypertensive patients as compared to controls and showed a negative correlation with plasma folate levels. Folic acid supplementation (5 mg/day) for 4 weeks resulted in a significant decrease in plasma homocysteine concentrations in these patients. Patients carrying MTHFR 677T allele (OR = 1.90; 95%CI: 1.14–3.19) or MTHFR 1298C (OR = 2.6, 95%CI: 1.55–4.40) allele were at increased risk of hypertension. The frequency of co-occurrence of MTHFR 677 CT/1298 CC genotypes was significantly higher in the patients compared to controls ($P < 0.05$) and was associated with increased risk of hypertension (OR = 3.54, 95%CI: 0.37–4.30). Subjects with MTHFR 1298 CC genotype had significantly higher homocysteine levels compared to those with MTHFR 1298 AA genotype ($P < 0.05$). Our results indicate that MTHFR 677T and 1298C alleles and co-occurrence of MTHFR 677 CT/MTHFR 1298 CC genotypes are associated with increased risk of hypertension and MTHFR 1298 CC genotype is associated with higher homocysteine levels in our subjects.

Keywords Methylenetetrahydrofolate reductase (MTHFR) · Homocysteine · Folate · MTHFR C677T · MTHFR A1298C

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Introduction

A relatively higher prevalence of hypertension has been reported in people with plasma homocysteine levels in the highest quartile as compared to those in the lowest quartile [1–5]. A causal role for homocysteine in the pathogenesis of hypertension is further suggested by the observation that homocysteine lowering treatment is associated with a reduction in systolic and diastolic blood pressure [6, 7]. The vascular risk associated with hyperhomocysteinemia has been found to be stronger in hypertensive individuals [8] and it has been suggested that adverse risk associated with hyperhomocysteinemia might be in part mediated by the positive association of homocysteine with hypertension [9–12].

A common C to T transition at nucleotide 677 (C677T) and A to C transition at nucleotide 1298 (A1298C) of the methylene tetrahydrofolate reductase (MTHFR) gene coding sequence, have been shown to be the most frequent genetic causes for mild hyperhomocysteinemia [13, 14].

Homozygosity for MTHFR C677T (TT), homozygosity for MTHFR A1298C (CC), and compound heterozygosity for C677T and A1298C (677CT/1298AC) genotype are associated with a reduced MTHFR enzyme activity [13–15], which may result in increased homocysteine levels. A point mutation in MTHFR gene (C677T) in Caucasian population has been shown to be associated with mild hyperhomocysteinemia, particularly in subjects with low plasma folate levels [16]. MTHFR (TT) genotype has also been found to be associated with an increased risk of hypertension [16]. In a recent study, Koupepidou et al [17] have proposed that MTHFR 677TT and 677CT/1298AC genotypes may be predisposing the hypertensive patients to hypertensive nephrosclerosis and chronic renal failure. MTHFR 677C–1298C haplotype has been also shown to modulate response to short-term treatment with ACE inhibitor in Chinese essential hypertensive patients [18].

Although hyperhomocysteinemia is commonly seen in hypertensive patients, the relationship between MTHFR genotypes, folate levels and risk of hypertension is poorly defined. Hence, the present study was carried out to examine association between the MTHFR gene variants, C677T and A1298C, their haplotypes and plasma homocysteine levels in essential hypertensive subjects. Further, to evaluate the role of nutrient–gene interaction in essential hypertensive subjects, the effect of folate supplementation on plasma homocysteine levels was correlated with MTHFR genotypes.

Materials and methods

Study population

Ethical approval for this study was obtained from the Institute Research Ethics Committee, PGIMER, Chandigarh, India. Written informed consent was obtained from patients or caregivers and controls before the collection of blood samples. Patients and controls were of North Indian origin and were ascertained to have at least parents and grandparents born in Northern India to ensure ethnicity. Subjects with secondary hypertension, previous history of stroke, coronary artery disease, myocardial infarction, peripheral vascular disease, renal hypertension, and diabetes mellitus were excluded from the study. About 153 consecutive essential hypertensive patients attending the hypertension clinic of our institute between September 2003 and August 2006 were enrolled in the study. About 133 unrelated, age and sex matched normotensive (SBP/DBP < 130/85 mmHg) (NT) individuals who had no family history of hypertension or any other disease served as controls. Blood pressure (BP) was measured during each visit with a mercury sphygmomanometer. A standardized protocol in which the appropriate cuff size was used depending on the arm circumference was followed. Three measurements were taken 5 min apart in the right arm in the sitting position after 5 min of rest. The average of the second and third measurements was taken as the participant's BP. Essential hypertension was defined as systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg. The demographic profile of hypertensive patients and normotensive controls is given in Table 1.

Folic acid/placebo supplementation: Patients were given either 5 mg folic acid tablet/day or a placebo tablet/day for 4 weeks.

Blood sampling: A total of 5 ml of venous blood sample was drawn from overnight fasting subjects before and after 4 weeks of folic acid or placebo supplementation.

Biochemical investigations: Plasma total homocysteine was measured in overnight fasting plasma samples by ELISA-based kit (Axis Shield Diagnostics, UK). Blood glucose and lipids were measured using their respective analytical kits in a semi-auto analyzer. Folic acid was measured using AIA-Pack-Folate Assay Kit.

Genotyping of the MTHFR gene variant

DNA was isolated from the whole blood by the method of Lahiri and Nurnberger [19]. Genetic polymorphism in two MTHFR variants, C677T and A1298C were studied by restriction fragment length polymorphism (RFLP) analysis.

C677T polymorphism was analyzed by polymerase chain reaction (PCR) followed by digestion with *Hin*fI, using the primers described by Frosst et al. [13].

The reaction mixture containing 1.5 mmol/l of $MgCl_2$, 1× standard polymerase chain reaction (PCR) buffer, 0.2 mmol/l of deoxynucleotide triphosphates (dNTPs), 25 pmol/l each of forward and reverse primers, 5%

Table 1 Demographic profile

Variable	Patients (n = 153)	Controls (n = 133)
Age (Years)	47.7 ± 12.4	46.2 ± 10.8
Males	83	69
Females	70	64
SBP (mmHg)	139.9 ± 13.8*	116.9 ± 10.9
DBP (mmHg)	88.9 ± 8.0*	75.3 ± 5.8
BMI (Kg/m^2)	23.4 ± 4.9**	22.9 ± 4.3
Total cholesterol (mg/dl)	187.8 ± 40.6*	161.3 ± 36.5
Triglycerides (mg/dl)	164.6 ± 90.2*	125.7 ± 52.9
HDL-C (mg/dl)	47.8 ± 19.1	44.5 ± 11.8

Values are mean ± SD, * $P < 0.001$, ** $P < 0.05$. SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, Body mass index; HDL-C, High density lipoprotein cholesterol

Dimethyl Sulphoxide (DMSO), 1 unit of *Taq* polymerase (Perkin-Elmer), 40 ng of genomic DNA was made to a final volume of 25 µl with sterile distilled water. The cycle parameters were as follows: 1 cycle at 95°C for 5 min for an initial denaturation, followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 58°C, primer extension for 1 min at 72°C and a final extension for 7 min at 72°C. The amplification resulted in the synthesis of a 198-bp fragment. The MTHFR gene contains a C to T substitution at nucleotide 677 [13]; the alteration created a *Hin*fI site that was used to screen the 198-bp fragment. For the restriction digestion, 2 units of *Hin*fI (Bangalore Genei), 1× assay buffer, and sterile water were added to each reaction mix in final volume of 30 µl and samples were digested overnight at 37°C. *Hin*fI did not digest the fragment derived from the C allele, whereas *Hin*fI digested the fragment of the same length from the T allele into 175- and 23-bp fragments. These fragments were then electrophoresis by using a 12% polyacrylamide gel followed by silver staining.

A1298C missense mutation in MTHFR gene was analyzed by polymerase chain reaction (PCR) followed by digestion with *Mbo II*, using the primers described by Weisberg et al. [15].

The PCR reaction mixture consisted of 4 mmol/l of $MgCl_2$, 1× standard polymerase chain reaction (PCR) buffer, 0.2 mmol/l of deoxynucleotide triphosphates (dNTPs), 25 pmol/l each of forward and reverse primers, 5% DMSO, 1 unit of *Taq* polymerase (Perkin-Elmer), 40 ng of genomic DNA which was made to a final volume of 25 µl with sterile distilled water. The cycle parameters were as follows: 1 cycle at 95°C for 5 min for an initial denaturation, followed by 35 cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 60°C, primer extension for 1 min at 72°C and a final extension for 7 min at 72°C. This amplification reaction resulted in the synthesis of a 163-bp fragment. The 1298(A–C) mutation abolishes an *Mbo II* restriction site. Digestion of the 163-bp fragment of the 1298 AA genotype gives five fragments, of 56,31,30,28, and 18 bp, whereas the 1298 CC genotype results in four fragments, of, namely, 84, 31, 30, and 18 bp. The restriction pattern was analyzed after 12% polyacrylamide gel electrophoresis and silver staining.

Statistical analysis

Paired *t*-test was performed to compare the homocysteine and folic acid levels before and after folic acid intervention. Unpaired students *t*-test and ANOVA were carried out to analyze the data between control and patient groups. The genotypes and allele frequencies for each mutation were stratified for wild-type, heterozygosity and homozygosity of the respective allelic variant. We also calculated the allelic frequencies for the two mutations in the study population and tested for deviations from the Hardy–Weinberg equilibrium. We performed pair-wise cross-tabulations of both the *MTHFR* allelic variants. In these tables, we contrasted the observed frequency with the expected cell count for each cell. We also conducted analysis of all allelic combinations of the two genetic variants. Pearson's chi-square test was used for statistical comparisons. The Conditional logistic regression analysis was performed to estimate the main effects of the MTHFR C677T variants on essential hypertension susceptibility after adjustment for body mass index (BMI), folate, cholesterol, HDL, and TG. Risk magnitudes were estimated by calculating odds ratios (OR) with 95% confidence intervals (CI). The conventional *P*-level of 0.05 was specified as the significance threshold. All the statistical analysis was performed using the SPSS (V10).

Results

Plasma total homocysteine and folate levels before and after folic acid supplementation are shown in Table 2. Homocysteine levels were significantly higher ($P = 0.02$) and serum folate levels were significantly lower ($P = 0.001$) in patients as compared to controls. Hyperhomocysteinemia was found to be associated with increased risk of hypertension (OR = 17.12; 95%CI: 7.94–36.8). Homocysteine levels showed a significant negative correlation with folate ($r = –0.093$), ($P < 0.05$). A significant decrease in the plasma total homocysteine levels ($P < 0.001$) was observed in patients after 4 weeks of folic acid supplementation as compared to the patients on placebo. The mean change in homocysteine levels after folic acid supplementation was found to be positively correlated to folate levels ($r = 0.324$, $P = 0.001$). Homocysteine levels were not found to be associated with age, sex, BMI, and lipids ($P > 0.05$) in patients and controls. No significant correlation between folate levels and SBP or DBP was observed in patients and controls.

Genotyping

MTHFR C677T gene polymorphism

The genotype and allele frequencies for MTHFR C677T polymorphism are shown in Table 3. A significant difference in C677T genotype prevalence was seen between patients and controls ($P = 0.013$) and homozygous mutant genotype (TT) was found only in patients. The MTHFR CT genotype was found to be associated with increased risk of hypertension (OR = 1.32; 95%CI: 0.74–2.39). T allele

Table 2 Biochemical parameters at baseline and following 4 weeks of folic acid administration

Variable	Folate supplemented Patients (n = 103)		Placebo supplemented Patients (n = 50)		Controls (n = 103)
	Pre-folate	Post-folate	Pre-placebo	Post-placebo	
Plasma homocysteine (μmol/l)	16.5 ± 5.8*	11.2 ± 5.3	16.4 ± 4.0	15.5 ± 3.0	8.0 ± 3.7
Serum folate (ng/ml)	6.8 ± 4.5	13.4 ± 7.0**	7.7 ± 4.8	7.2 ± 4.7	8.3 ± 3.5

** $P < 0.01$, * $P < 0.05$

Table 3 Distribution of MTHFR C677T and MTHFR A1298C frequencies in hypertensives patients and controls

MTHFR C677T	Genotypes			Alleles	
Group	CC	CT	TT	C	T
Patients (n = 153)	105(68.6%)	40(26.2%)	8(5.2%)	0.82	0.18
Control (n = 133)	105(78.9%)	28(21.1%)	0(0%)	0.89	0.11
Genotypic comparison: $\chi^2 = 8.76$, $P = 0.013$					
MTHFR A1298C	AA	AC	CC	A	C
Patients (n = 153)	99(64.7%)	43(28.1%)	11(7.2%)	0.78	0.22
Control (n = 133)	112(84.2%)	17(12.8%)	4(3%)	0.90	0.10
Genotypic comparison: $\chi^2 = 14.0$, $P = 0.001$					

frequency was significantly higher in the patients ($P = 0.008$) and was found to be associated with the increased risk of hypertension (OR = 1.90; 95%CI: 1.14–3.19). No significant difference for either genotype or allele frequencies of the C677T variants were observed between males and females ($P > 0.05$).

Logistic regression adjusted for BMI, homocysteine, folate levels, cholesterol, HDL, and TG did not confirm any of the genotypes as a risk factor for essential hypertension ($P = 0.211$) (Data not shown).

MTHFR A1298C gene polymorphism

Genotype frequencies of MTHFR A1298C polymorphism are shown in Table 3. There was a significant difference in the prevalence of MTHFR A1298C genotypes between patients and control subjects ($P = 0.001$). The frequency of C allele was found to be significantly higher in patients as compared to the controls ($P = 0.0001$) and it was also found to be associated with the increased risk of hypertension (OR = 2.6, 95%CI: 1.55–4.40). MTHFR 1298 CC genotype was found to be associated with increased risk of hypertension (OR = 2.49; 95%CI: 0.71–9.56).

MTHFR C677T and A1298C combined genotype analysis and homocysteine levels

Cross tabulation of patients and normotensive controls by genotype combination of MTHFR C677T and A1298C SNPs is shown in Table 4. There was no evident discrepancy between the observed and the expected genotype frequencies ($P = 0.706$). One patient was found to be homozygous for both the mutations, i.e., (MTHFR677TT, MTHFR1298CC). When the combined effects of these two SNPs were analyzed (Table 4), the genotype frequency for the combined genotype MTHFR677CC/MTHFR 1298 AC was significantly higher among patients than the controls ($P = 0.014$). This combined genotype was found to be associated with increased risk of hypertension (OR = 2.35, 95%CI: 1.12–4.99). The co-occurrence of MTHFR 677 CT/MTHFR 1298 CC genotype was also significantly higher in patients as compared to the controls ($P = 0.04$)

Table 4 Frequencies of combined MTHFR C677T and MTHFR A1298C allelic variants in patient and control groups

	Patients, n (%) total: 153			Controls, n (%) total: 133		
	MTHFR 677++	MTHFR 677+-	MTHFR 677–	MTHFR 677++	MTHFR 677+-	MTHFR 677–
MTHFR 1298++	68(44.5)	27(17.8)	4(2.8)	89(66)	23(17.6)	0(0)
MTHFR 1298+-	31(20.5)	9(5.9)	3(1.0)	3(9.8)	4(3)	0(0)
MTHFR 1298–	6(3.9)	4(2.8)	1(0.8)	3(2.7)	1(0.9)	0(0)

and was also found to be associated with increased risk of hypertension (OR = 3.54, 95%CI: 0.52–23.80). The prevalence of combined genotypes (MTHFR677CC/MTHFR1298AA) was significantly lower in patients compared to the controls ($P = 0.0001$). The co-occurrence of MTHFR genotypes with both variant alleles, i.e., MTHFR 677TT and MTHFR 1298CC was seen in one patient only.

Homocysteine concentration in different genotypes of MTHFR C677T and MTHFR A1298C SNPs are shown in Table 5. Higher homocysteine levels were seen in subjects (controls and hypertensives) with MTHFR 1298 CC ($P = 0.000$), and AC genotypes ($P = 0.006$) compared to MTHFR 1298 AA genotype. No significant difference in the homocysteine levels was observed in patients and controls belonging to different MTHFR C677T genotypes (Table 5)

Discussion

Over the past decade, several studies have implicated hyperhomocysteinemia and MTHFR C677T mutation as risk factors for cardiovascular diseases [1, 2, 8, 9], but few studies have examined the association between genetic polymorphisms in MTHFR gene and plasma homocysteine levels in essential hypertension [16, 18]. The goal of our present study was to investigate the homocysteine levels and their association with MTHFR gene polymorphisms (C677T and A1298C) and their haplotypes in essential hypertensive subjects. We observed significantly higher plasma homocysteine levels in hypertensive patients, which showed positive association with diastolic blood pressure. Several cross-sectional studies have reported a positive association of homocysteine with SBP and DBP and with hypertension [4, 20, 21]. A marked regional heterogeneity in plasma homocysteine levels has been observed, which is influenced by factors such as age, gender, folate, vitamin B-12 status, smoking, and nutritional habits [22, 23]. Hyperhomocysteinemia in Asian Indians has been reported to be associated with low folate levels, which were also observed in our study. We also found a negative correlation between homocysteine and folate levels suggesting that hyperhomocysteinemia in our patients may be related to lower folate levels. However, we did not observe any significant difference in homocysteine levels based on age, gender, or smoking habits in our study population.

Folic acid supplementation has been proposed as a therapeutic intervention for the treatment of elevated homocysteine levels [21–23]. In our study we too observed a significant reduction in the plasma homocysteine levels in hypertensive patients on oral supplementation with folic acid, however, this reduction in homocysteine levels was not accompanied by any significant lowering of either SBP or DBP. This lack of association between post-folate homocysteine levels and blood pressure, suggests that increased homocysteine levels in hypertensive subjects may be concomitant rather than a precursor of hypertension as also suggested earlier [20].

Genetic polymorphisms, MTHFR C677T, and A1298C have been observed to influence the plasma homocysteine concentrations and the risk of cardiovascular diseases [24–35]. However, there are a few studies which have examined the association of these two variants and their haplotypes with essential hypertension [16, 17, 28, 36]. The TT genotype is present at a frequency of about 9% in Caucasian and 15–16% in Chinese and Japanese population. However, in our population this polymorphism was present at a much lower frequency (5.2%). Although the frequency of MTHFR677 TT genotype was low in our population compared to Caucasians, it was found to be significantly associated with increased risk of hypertension. TT genotype has been shown to be associated with

Table 5 Relationship between MTHFR genotype and the homocysteine levels in patients and controls

	Genotype		
	677 CC	677 CT	677 TT
Homocysteine (μmol/l)			
Patients	16.0 ± 5.0 ($n = 105$)	17.9 ± 5.6 ($n = 40$)	18.2 ± 6.3 ($n = 8$)
Controls	8.6 ± 3.4 ($n = 105$)	10.0 ± 4.7 ($n = 28$)	N.A. ($n = 0$)
Combined	12.4 ± 5.8 ($n = 222$)	12.4 ± 5.8($n = 52$)	16.9 ± 5.5 ($n = 12$)
	1298 AA	1298 AC	1298 CC
Patients	15.7 ± 4.7 ($n = 99$)*	18.0 ± 5.7 ($n = 43$)	19.1 ± 6.7 ($n = 11$)
Controls	8.7 ± 3.4 ($n = 112$)*	11.2 ± 4.1 ($n = 17$)**	14.7 ± 2.6 ($n = 4$)
Combined	12.3 ± 5.4($n = 204$)*	14.5 ± 6.4 ($n = 69$)**	18.3 ± 6.4 ($n = 13$)

* MTHFR 1298AA versus CC ($P < 0.05$)

** MTHFR 1298AA versus AC ($P < 0.05$)

increased risk of hypertension in Japanese and other ethnic groups too [16, 18, 27].

Although the MTHFR A1298C polymorphism has been associated with hyperhomocysteinemia, its prevalence in essential hypertensive Indian subjects has not been studied till date. Heterozygotes for the A1298C polymorphism show only a 10% reduction in the activity of MTHFR, while the homozygotes for this polymorphism show a reduction of 35–45% in the activity of the enzyme and as a consequence can potentially elevate the levels of homocysteine. We observed that MTHFR 1298 CC genotype and MTHFR 1298C allele were significantly associated with increased risk of hypertension in our subjects. We also observed significantly higher plasma homocysteine levels in subjects with MTHFR 1298CC genotype indicating a possible association between MTHFR 1298CC genotype and hyperhomocysteinemia in our subjects. A similar association between MTHFR 1298CC genotype and hyperhomocysteinemia has been recently reported in Indian subjects [37].

A co-occurrence of MTHFR C677T and A1298C polymorphisms have been suggested to be associated with hyperhomocysteinemia and increased risk of vascular occlusive diseases [38, 39]. We too observed that co-occurrence of MTHFR 677CT/MTHFR 1298CC genotypes conferred higher relative risk of hypertension compared to either C677T or A1298C mutant genotypes. We found seven patients with three mutations for MTHFR C677T and MTHFR A1298C, i.e., MTHFR 677TT/1298AC or 677 CT/1298CC indicating that genotypes with mutant alleles may be more prevalent in hypertensive patients. A higher risk of CAD has been also found to be associated with MTHFR CC/AC, CC/CC, and TT/AA genotypes (95%CI: 1.2–4.4) [38]. It has been proposed that MTHFR 1298C interacts with the MTHFR 677T allele to cause reduced MTHFR enzyme activity [39]. We observed one patient with homozygosity for mutant alleles in both the SNPs (MTHFR 677TT and MTHFR 1298 CC). Thus far, only a single compound homozygous individual with the MTHFR 677TT/1298CC genotype has been reported and a possible selection disadvantage for the individuals showing quadruple MTHFR C677T and A1298C mutations has been suggested [40].

Modest differences in homocysteine concentrations in different MTHFR genotypes have been observed in Caucasians and Asian Indians [16, 23, 26, 28]. Higher plasma homocysteine concentrations have been observed in persons with MTHFR 677 TT and MTHFR 1298 CC genotype [33, 35]. We observed higher plasma total homocysteine levels in subjects with 1298CC genotype. However, we did not observe any significant association between plasma total homocysteine and C677T genotypes in either patients or controls. The lack of association between MTHFR 677TT and homocysteine levels in our study may be due to low prevalence of TT genotype in our population. Similar association between MTHFR A1298C and C677T genotypes and homocysteine concentrations in Asian Indians was recently reported by Kumar et al. [37].

In conclusion, our study confirms a mild hyperhomocysteinemia in hypertensive patients that can be reduced effectively with folic acid supplementation. Our study also suggests that MTHFR 1298 CC genotype may be associated with hyperhomocysteinemia and the presence of mutant alleles (677T allele and 1298C allele) confers a modest risk while the co-occurrence of these mutations results in a higher risk for essential hypertension.

Acknowledgment Supported by research funding from Indian Council of Medical Research, New Delhi, India.

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