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Original Article

The Methylentetrahydrofolate Reductase Gene Variant (C677T) as a Risk Factor for Essential Hypertension in Caucasians

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Essential hypertension (EH) is a common, multifactorial disorder likely to be influenced by multiple genes of modest effect. The methylenetetrahydrofolate reductase (MTHFR) gene C677T mutation is functionally important, being strongly associated with reduced enzyme activity and increased plasma levels of homocysteine. Mild hyperhomocysteinemia is a known risk factor for cardiovascular disease (CVD) and hypothesised also to be involved in hypertension pathophysiology. The present study was performed to determine the prevalence of the 677T mutation in Australian Caucasian patients diagnosed with EH and to test whether the C677T variant is associated with the disorder. A case-control cohort, consisting of 250 EH patients and 250 age, sex and racially matched normotensive controls, were used for the association study. Comparison of C677T allele frequencies revealed a higher proportion of the mutant allele (T) in the EH group (40%) compared to unaffected controls (34%) (p 0.07). Furthermore, genotypic results indicated that the prevalence of the homozygous mutant genotype (T/T) in the affected group was higher than that of controls (14%:10%) (p 0.17). Interestingly, conditional logistic regression showed that the MTHFR C677T mutation conferred a mild, yet significant increase in risk of essential hypertension after adjusting for body mass index (odds ratio 1.57, 95% confidence interval: 1.04–2.37, p 0.03). These findings require further investigation in large independent samples, but suggest that essential hypertension, like CVD, may be mildly influenced by the MTHFR C677T variant. (*Hypertens Res* 2004; 27: 663–667)

Key Words: **association, homocysteine, hypertension, methylenetetrahydrofolate reductase gene, body mass index**

Introduction

Essential hypertension (EH) is a condition that causes a greatly increased risk for stroke, heart disease and kidney failure. As such EH is a major public health problem with approximately 20% of the adult population affected in westernized societies. Many physiological abnormalities have been associated with persistent high blood pressure, but the underlying causes have remained difficult to identify. In many patients with EH, there is a strong inherited predisposition with epidemiological data derived from twin, adoption and family studies indicating up to 50% of the variability in blood pressure levels is attributable to genetic effects (*1*). This genetic component of EH is likely to be comprised of multiple susceptibility genes each conferring a modest effect on the disease (*2*). The case-control association study of candidate genes remain an efficient approach for detecting the "modest-effect" genetic variants underlying disease susceptibility, provided the study is adequately powered (*3*, *4*). A

large number of such studies have been conducted to date and many different genes have been implicated (*5−7*), although few have been confirmed through replication. Given that hypertension is a primary risk factor for cardiovascular disease (CVD), such as ischaemic heart disease and stroke, the genes implicated in CVD risk should also be considered targets for involvement in EH susceptibility (*8*).

Recently, Wald *et al.* (*9*) conducted a comprehensive meta-analysis exploring the role of homocysteine in relation to CVD. The conclusions of these researchers was that mild hyperhomocysteinemia is causally associated with risk of heart disease and stroke and that homocysteine lowering therapy, by simple dietary folate supplementation, may reduce risk of CVD. Folate is a cofactor in the remethylation of homocysteine, without which, homocysteine levels in the plasma increase. Folate metabolism is partly governed by the methylenetetrahydrofolate reductase (MTHFR) enzyme which catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (*10*). The MTHFR gene has been mapped to the chromosomal region 1p36.3 and is composed of 11 exons (*11*). A common mutation of the MTHFR gene is the C to T transition, located at nucleotide 677 (C677T). This results in the amino acid change: alanine to valine. This mutation is associated with increased thermolability and reduced specific activity of the enzyme (mean activity is 65% in the Ala/Val heterozygote and 30% in the Val/Val homozygous state, respectively compared to the mean activity in the Ala/Ala homozygote) (*10*). Patients with a deficiency in MTHFR have elevated blood and urinary levels of homocysteine. Thus, The MTHFR C677T, causing mild hyperhomocysteinemia, is an important genetic risk factor for CVD (*9*). A recent study has also indicated that the TT genotype is associated with an increased risk of hypertension specifically in males (*12*). Alternatively, a large study conducted by Inamoto (*13*) showed that the TT genotype was associated with hypertension and carotid stenosis in women. The objectives of our study were to employ a powerful case-control association approach to test for a relationship between the MTHFR (C667T) gene variant and EH susceptibility in Caucasians.

Methods

Subject Groups

For this case-control study we utilised an affected group of 250 hypertensive (HT) patients and a carefully age and sex matched control group of 250 normotensive (NT) individuals. All participants of the study were unrelated and of Caucasian origin. Qualified physicians diagnosed hypertension if blood pressure was above 140 mmHg systolic and 95 mmHg diastolic as recorded on at least 3 occasions over a 2-month period prior to the administration of anti-hypertensive medication. Importantly, 72% of the HT patients included in the case group reported that at least 1 parent was hypertensive,

Table 1.   Demographic and Clinical Parameters

Variable	Cases	Controls
n (total)	250	250
Male	90 (36%)	90 (36%)
Female	160 (64%)	160 (64%)
Age (years)	55 (11.0)	55 (11.0)
SBP (mmHg)	143.4 (18.6)	122.8 (11)
DBP (mmHg)	86.3 (11.3)	77.9 (7.8)
BMI (kg/m^2)	28.8 (5.7)	26.5 (5.3)

Data are mean (SD). SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.

41% of cases had two hypertensive parents. This provides some genetic enrichment for this affected group. NT individuals had no past or current history of hypertension and all had a blood pressure of less than 140/90 mmHg. The demographic variables are listed in Table 1. The study protocol complied with the Australian Ethics Standards and was approved by the Griffith University Ethics Committee for experimentation on humans. All participants signed informed consent agreements prior to collection of DNA and clinical information.

Genotyping of the MTHFR Gene Variant

DNA was isolated from frozen whole blood, containing ethylene diamine tetra acetic acid (EDTA) or lithium heparin as anticoagulant, by a standard salting out procedure and resuspended in TE (1.0 mol/l Tris-HCl pH 8.0, 0.5 mol/l CaCl$_2$) buffer as previously described (*14*). Genomic DNA (40 ng) was amplified by polymerase chain reaction, in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, USA) using the MTHFR primers designed by Frosst *et al.* (*10*) (5′-TGAAGGAGAAG GTGTCTGCGGGA-3′ for the sense oligonucleotide primer and 5′-AGGACGGTGCGGTGAGAGTG-3′ for the antisense primer; synthesized by GeneWorks, Adelaide, Australia) in the following singleplex reaction: 1.75 mmol/l of MgCl$_2$, IX standard polymerase chain reaction (PCR) buffer, 0.2 mmol/l of deoxynucleotide triphosphates (dNTPs), 0.2 μmol/l each of forward and reverse primers, 0.16 g/l of bovin serum albumin (BSA), 1 unit of *Taq* polymerase (Perkin-Elmer), 40 ng of genomics DNA, made to a final volume of 25 μl with sterile distilled water. The cycle parameters were as follows: 1 cycle at 95 °C for 3 min for an initial denaturation, followed by 35 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 65 °C, primer extension for 2 min at 72 °C and a final extension for 10 min at 72 °C (*10*). This amplification reaction resulted in the synthesis of a 198-bp fragment.

The MTHFR gene contains a C to T substitutions at nucleotide 677 (*10*); the alteration created a *Hinf*I site that was used to screen the 198 patients. For the restriction digestion, 7 units of *Hinf*I, 2 μl of New England Biolabs buffer II and

Table 2.   Distribution of MTHFR C677T Frequencies in Hypertensives (HT) and Controls

Group	Genotypes			N (genotypes)	Alleles	
	C/C	C/T	T/T		C	T
HT	87 (35%)	125 (51%)	35 (14%)	247	299 (60%)	195 (40%)
Control	105 (42%)	119 (48%)	25 (10%)	249	329 (66%)	169 (34%)

Genotypic comparison: χ^2 3.49, p 0.17; allelic comparison: χ^2 3.27, p 0.07. MTHFR, methylenetetrahydrofolate reductase.

Table 3.   Conditional Multivariate Logistic Regression Analysis of MTHFR, BMI and EH

Variable	OR	p-value	95% CI for OR	
			Lower	Upper
MTHFR (CT/TT)	1.57	0.03	1.04	2.38
BMI	1.08	0.00	1.04	1.13

MTHFR, methylenetetrahydrofolate reductase; BMI, body mass index; EH, essential hypertension; CI, confidence interval; OR, odds ratio.

2.3 μl of sterile water were added to each extension mix at a final volume of 20 μl and samples digested overnight at 37 °C. *Hinf*I did not digest the fragment derived from the C allele, whereas *Hinf*I digested the fragment of the same length from the T allele into 175- and 23-bp fragments. These fragments were then electrophoresed by using a 5% ultra-high-resolution agarose gel, stained with ethidium bromide and visualised under ultraviolet (UV) light.

To confirm results, samples were also subjected to a DNA fragment analysis by using a Perkin-Elmer Applied Biosystems 310 Genetic Analyser (Perkin-Elmer). One of the PCR primers (the reverse primer) was labeled with FAM fluorescent dye. Samples were prepared using the same protocols as the restriction fragment length polymorphism (RFLP) analysis and 1 μl of each digest products were added with 8 μl of a mix contained 1,500 μl of formaldehyde and 63 μl of Genescan-350 TAMRA as size standard (Perkin-Elmer). This mixture was denatured for 2 min at 95 °C and 2 min at 4 °C before running in the Genescan. The size analysis was done with the GeneScan Analysis Software (Applied Biosystems, Foster City, USA).

An independent laboratory technician, blinded to the initial genotype data, performed quality control analysis for both gene variants by repeat PCR and genotyping of a random selection of 50 cases and controls. All genotype discrepancies were rectified or excluded from subsequent data analysis.

Statistical Analysis

The MTHFR C677T genotype and allele frequencies were calculated from observed genotype counts. As a statistical control for systematic genotyping error and/or population stratification, the expected genotype proportions according to the Hardy-Weinberg law were calculated and compared to observed genotypes. Genotype and allele frequencies were initially assessed for association with EH using standard contingency table analysis incorporating the χ^2 test of independence. This analysis produces a χ^2 statistic with 1 or 2 degrees of freedom and corresponding p-values for allele and genotype distributions, respectively. Conditional logistic regression analysis was performed to estimate the main effects of the MTHFR C677T variant on EH susceptibility after adjustment for body mass index (BMI). Risk magnitudes were estimated by calculating odds ratios (OR) with 95% confidence intervals (CI). The conventional α-level of 0.05 was specified as the significance threshold. All the statistical analyses were performed using the SPSS (v10).

Results

The genotype and derived allele frequencies for the MTHFR C677T variant and the results of the χ^2 analyses are shown in Table 2. The frequency of genotypes in the case and control groups conformed to the expectations under the Hardy-Weinberg equilibrium law. The prevalence of the T allele and the homozygous TT genotype in the control group was found to be 34% and 10%, respectively. These frequencies are consistent with control groups in other CVD studies of this variant (*9*). Comparison of the HT group against the control group showed that the frequency of the T allele was over-represented in the HT group by 6% (40% in the HT group *vs.* 34% in the control). The distribution of genotypes in the case group was also different to controls, with the TT homozygotes and CT heterozygotes showing frequencies of 14% and 51%, respectively compared to 10% and 48% in the control population. However, these observed allele and genotype differences were not significant at the α-level of 0.05 (p 0.07 and p 0.174, respectively). Moreover, there were no gender differences observed for the C677T variant ($p > 0.05$).

BMI is a well-known and significant risk factor for EH.

Indeed, the average BMI in our HT case group (28.8) was significantly higher than in NT controls (26.5) (*p*<0.001). Therefore, to adjust for BMI, conditional logistic regression (LR) analysis was performed incorporating the MTHFR C677T mutation and BMI as independent variables. Given the allelic trend observed for the T allele in our initial contingency table analysis, the MTHFR risk variable in the LR model was set as the dominant (CT/TT) genotype. Table 3 shows the results of the multivariate analysis. Interestingly, a modest, yet significant, independent effect of the dominant MTHFR genotype (CT/TT) on EH was revealed after adjusting for the effect of BMI (OR   1.57, 95% CI: 1.04−2.37, *p*   0.03).

Discussion

Over the past decade, a substantial body of evidence has accumulated implicating the MTHFR C677T mutation as a modest genetic risk factor for cardiovascular disease (OR   1.5, *p*<0.05) (*9*). Although hypertension is a primary risk factor for CVD, few studies have examined the MTHFR C677T variant specifically in relation to EH risk. A recent large scale study conducted by Inamoto *et al*. (*13*) examined hypertension and carotid atherosclerosis in in over 3,000 Japanese patients. These researchers reported that the TT genotype of the MTHFR gene was associated with an increased risk of hypertension, conferring a small, but significant, risk of   1.5 (*13*). A Spanish case-control study of similar size to our present study also detected an association between MTHFR TT genotype but in males with a greater risk (2.3) (*12*). In contrast, a recent study of CVD in an unrelated group from the Czech Republic examined the MTHFR C677T in 193 patients diagnosed with EH only (*15*). The frequency of the T allele was shown to be over-represented in the Czech group affected with EH compared to the healthy control group in this Czech study (38% *vs*. 33%). These data also indicated an increased prevalence of the dominant genotype group (CT/TT) compared to controls. Neither of these observed differences was significant, although these researchers did not report the BMI-adjusted effect of MTHFR C677T on EH in this study (*15*).

It is important to note that the frequency of the MTHFR 677T allele, and indeed EH prevalence, is known to vary substantially among different ethnic populations (*16*). It is these differences in gene and disease frequencies that may partly explain the conflicting association results obtained across independent studies. Our study tested the MTHFR C677T variant for association with EH in a Caucasian case-control panel from Australia. The results of this work were consistent with the trends reported by Benes *et al*. (*15*), showing that the prevalence of the T allele and its composite genotypes (CT/TT and TT) were increased in the HT group compared to NT controls. Interestingly, the MTHFR C677T considered under a dominant state (CT/TT) appeared to be significantly associated with EH after adjusting for the confounding effect of BMI. Thus, the effect of the T allele on the total EH group was partially masked by the effect of BMI on EH, but removal of the BMI effect (*i.e*. analysis of less obese subjects) revealed evidence for association between the gene and EH.

As with other studies of MTHFR and CVD phenotypes, our results indicated that the presence of the 677T allele confers only a modest increased risk for EH. It is possible that a more substantial risk may arise from combinations of MTHFR C677T variant and other genetic and/or environmental factors. Nevertheless, our findings provide evidence that the MTHFR C677T variant, causing mild hyperhomocysteine, may be an independent risk factor for EH. Further studies designed specifically to determine the adjusted risk that the MTHFR gene imposes on EH are now warranted to confirm the MTHFR gene as a risk factor for EH. Such studies should also consider the possibility that variation elsewhere in the MTHFR gene may also contribute to an association with EH. Finally, studies designed to measure plasma levels of homocysteine in lean patients with EH are required to determine the extent to which the MTHFR C677T mutation transfers to a biochemical change in patients affected with this important vascular disorder.

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