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Genotyping 5,10-Methylenetetrahydrofolate Reductase for Patients with Coronary Artery Disease in Southern Taiwan

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ABSTRACT

Hyperhomocysteinaemia has been identified as a risk factor for cerebrovascular, peripheral vascular and coronary heart disease (CAD). Individuals with homozygous MTHFR (5,10-Methylenetetrahydrofolate reductase) mutation have significantly elevated plasma homocysteine levels. Two common mutations in MTHFR (MTHFR 677C→T and MTHFR 1298A→C) may represent an important genetic risk factor in vascular disease. The aim of this research was to investigate these two point mutations of MTHFR for CAD patients and non-CAD controls in Southern Taiwan by PCR-restriction fragment length polymorphism assay. We found that the distribution of the homozygous mutation for MTHFR677 was higher in 54 CAD patients than in 55 non-CAD subjects (11.1% versus 3.6%, $P = 0.275$). On the other hand, the frequency of MTHFR 1298CC in CAD patients was similar to that of control subjects ($P = 1.000$). This may suggest there is a trend toward an increased risk of coronary heart disease for individuals that have a 677TT genotype in the Taiwanese population. Finally, the frequency of homozygosity for the two MTHFR common mutations was still low in CAD cases. There may not be an appreciable association of MTHFR mutation to cardiovascular disease in Taiwan. Perhaps a further study including assessment of plasma concentrations of homocysteine, folate and vitamin B12 would be needed to better clarify the relationship between MTHFR polymorphisms and the occurrence of vascular disease.

Key words: MTHFR, homozygous mutation, coronary artery disease, RFLP

INTRODUCTION

Hyperhomocysteinaemia, defined as a mildly elevated plasma homocysteine (Hcy) level, is associated with cardiovascular diseases. An elevated level of Hcy has been implicated in the role of promoting growth of vascular smooth muscle cells along with inhibiting endothelial cell growth. Additional putative mechanisms of thrombosis in hyperhomocystinemia include increased platelet adhesiveness, enhanced LDL deposition in the arterial wall, and direct activation of the coagulation cascade [1]. Hyperhomocysteinaemia has been identified as a risk factor for cerebrovascular, peripheral vascular and coronary artery disease (CAD)[2-5].

Homocysteine is a sulphur amino acid formed by demethylation of the essential amino acid methionine, and it can be degraded by cystathionine-β-synthase (CBS). Alternatively, Hcy may be remethylated to methionine, a process requiring the sufficient function of several enzymes. Methionine synthase (MS), one of the key enzymes, catalyzes the remethylation of Hcy in the presence of methylcobalamin (Me-Cbl) and 5-methyl-tetrahydrofolate (Me-THF). Formation of Me-THF requires both an adequate supply of reduced folate and proper function of methylenetetrahydrofolate reductase (MTHFR)[6]. Dysfunctional MTHFR caused by genetic mutations or inadequate amount of reduced folate may therefore result in elevated concentrations of Hcy[7]. The plasma concentrations of Hcy varied among different populations, ages, sexes, diet habits and individual health conditions[8].

Two common point mutations have been found in MTHFR. The first identified polymorphism (677C→T), a missense mutation in exon 4 of the MTHFR gene found by Frosst, is a cytosine (C) to thymine (T) substitution at nucleotide 677, which converts an alanine (A) to a valine (V)[9]. Reduction in activity or thermolability of MTHFR has been associated with the heterozygous or homozygous 677T genotype. Homozygotes for the 677T allele are predisposed to hyperhomocysteinemia by the 50% decrease of MTHFR activity, particularly in the context of suboptimal folate status [7]. A high proportion of people with the 677T homozygous genotype show a satisfactory Hcy lowing response to modest daily folate supplements in the range 100-200 μg/d [10]. Recently, a second common polymorphism in the MTHFR gene has been reported that results in a glutamate-to-alanine conversion (1298A→C)[11]. This polymorphism also results in diminished enzyme activity, elevated Hcy concentration, and decreased folate concentration in plasma[12]. Therefore, these two common mutations (MTHFR677C→T and 1298A→C) may represent an important risk factor in vascular diseases[13].

Recently, there have been several reports on the correlation between MTHFR polymorphism and vascular diseases in Taiwan, published by research teams from National Taiwan University Hospital, the Veterans Hospital and National Cheng-Kung University Hospital[14-16]. No report on the prevalence of MTHFR 1298A→C or the relationship between MTHFR 1298A→C and MTHFR677C→T in the

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Taiwanese population has been found so far. The aim of this study was to use PCR and restriction fragment length polymorphism (RFLP) to screen the prevalence of MTHFR 1298A→C and MTHFR677C→T polymorphisms in CAD patients and healthy subjects in the Tainan area and to find out the association between cardiovascular disease and the genotypes. The results will form the basis for future studies, including assessment of plasma concentrations of Hcy, folate and vitamin B12 in order to better clarify the relationship between MTHFR polymorphisms and occurrence of vascular disease.

MATERIALS AND METHODS

I. *Subjects*

Blood was collected from fifty-four patients with CAD from the internal cardiovascular department at Chi-Mei Hospital in Tainan. The average age of the group was 64.3, and consisted of forty-one males and thirteen females. Control samples were randomly collected from fifty-five patients with non-CAD or non-vascular diseases history at Chi-Mei Hospital. Their average age was 66.7, and consisted of thirty-one males and twenty-four females.

II. *Isolation of Genomic DNA*

Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) from blood drawn from CAD patients and control subjects. The purity, size and concentration of isolated DNA were measured by A260, A280 and agarose gel electrophoresis.

III. *PCR Amplification and MTHFR Polymorphisms Detection*

The MTHFR677C→T mutation alters an alanine into a valine residue, creating a *Hin*fI site. The presence of this mutation was analyzed according to established procedures[9]. The primers for PCR were 5'-TGAAGGAG-AAGGTGTCTGGGGGA-3' (exonic) and 5'-AGGACG-GTGCGGTGAGAGTG-3' (intronic). The PCR was carried out in a total volume of 50 μL, containing 10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100, 200 μM dNTP, 1.5 mM $MgCl_2$, 100 ng genomic DNA, 10 pmol of each primer, and 2 units Taq DNA polymerase (Promega, Wisconsin, USA). PCR parameters were as follows: an initial denaturation step of 4 min at 94˚C, followed by 30 cycles of 94˚C/60 s, 55˚C/60 s, and 72˚C/60 s, and a final cooling at 15˚C.

The PCR amplified products were then cleaned by a GFX^{TM} PCR DNA Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove excess primers. Five μL purified PCR fragments were digested with 1 μL (10 U/mL) HinfI at 37˚C for 2 hours, followed by adding 1 μL (10 U/mL) HinfI and reacted for another 2 hours in order to completely digest the DNA. The restriction digested fragments were then separated on 4% agarose gel electrophoresis.

The MTHFR1298A→C mutation alters a glutamate into an alanine residue, abolishing a *Mbo*II site. The MTHFR1298A→C mutation was analyzed according to established procedures[11]. The PCR was carried out in a total volume of 50 μL, containing 10 pmol of forward primer 5'-CTTTGGGGAGCTGAAGGACTACTAC-3' and 10 pmol of the reverse primer 5'-CACTTTGTGACCATTCCG-GTTTG'3', 10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100, 200 μM dNTP, 1.5mM $MgCl_2$, 100 ng genomic DNA, and 2 units Taq DNA polymerase (Promega, Wisconsin, USA). PCR parameters were a 2-min denaturation step at 92˚C followed by 35 cycles of 92˚C/60 s, 51˚C/60 s, and 72˚C/60 s, and a final cooling at 15˚C.

The PCR amplified products were then cleaned by a GFX^{TM} PCR DNA Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove excess primers. Five μL purified PCR fragments were digested with 1 μL (10 U/μL) *Mbo*II at 37˚C for 2 hours. The restriction digested fragments were then separated on 4% agarose gel electrophoresis.

IV. *Agarose Gel Electrophoresis*

The restriction digested products were separated on 4% agarose gel made of Nusive 3:1 agarose gel (FMC, Maine, USA) and ethidium bromide. The running condition was 100 volts for 1.5 hours at 4˚C. The gel was then visualized under UV and the imagine was captured and stored by an Uvi system.

V. *Statistics*

Statistical analyses were performed using SPSS window-10.0. Comparisons were analyzed by the Chi-square test and the Fisher exact probability test.

RESULTS AND DISCUSSION

Genomic DNA was isolated from the blood of CAD patients and control subjects by a QIAamp DNA Blood Mini Kit. The isolated DNA was then analyzed on 0.5% agarose gel electrophoresis. Most of the molecular weights of the DNA were more than 15 kb, although some appeared to contain tailing. Overall, the effect of purification was good and the tailing might be due to pipeting shearing or gel overloading.

The purified genomic DNA was then amplified by PCR as described in Materials and Methods. The amplified products were then analyzed by a 2% agarose gel electrophoresis and the molecular weights were 198 bp and 163 bp for MTHFR677C→T and MTHFR1298A→C, respectively.

The MTHFR677C→T alters an alanine into a valine residue, creating a *Hin*fI site. After restriction analysis of the 198-bp PCR fragment with *Hin*fI, we expected and observed the following: the $MTHFR^{677}$+/+ (+ indicates wild type) gives a 198-bp band, whereas $MTHFR^{677}$-/- (- indicates mutant) results in a 175-bp band, and $MTHFR^{677}$+/- generates 198-bp and 175-bp bands (as shown in Figure 1). The

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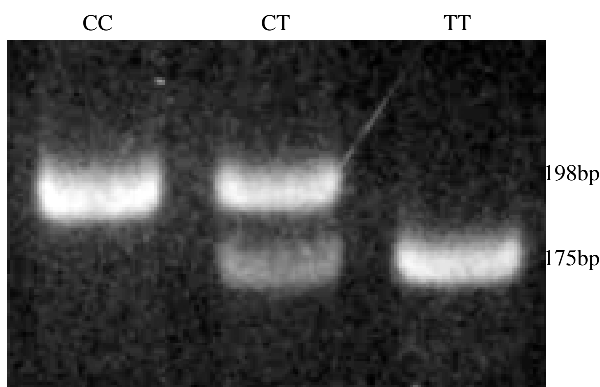

Figure 1. Restriction enzyme analysis for the alanine to valine substitution. The substitution creates a *Hinf*I recognition sequence which digest the 198 bp fragment into 175 and 23 bp fragments; the latter fragment has been run off the gel. All three possible genotypes are shown.

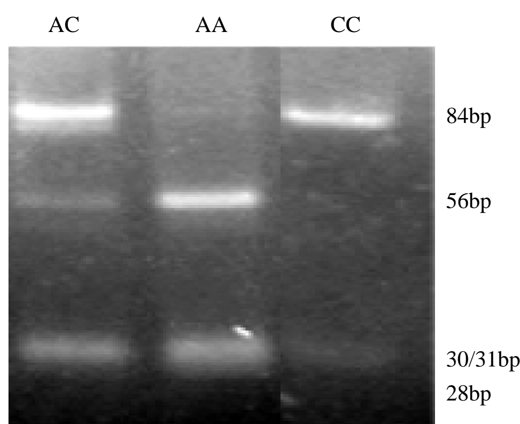

Figure 2. Restriction enzyme analysis for the glutamine to alanine substitution. The substitution abolishes an *Mbo*II recognition site. Digestion of the 163 bp fragment of the 1298 CC gives four fragments, of 84, 31,30 and 18 bp, whereas the 1298AA genotype results in five fragments, of, namely, 56, 31, 30, 28, and 18 bp. The 18 bp fragment has been run off the gel. All three possible genotypes are shown.

MTHFR1298A→C mutation alters a glutamate into an alanine residue, abolishing an *Mbo*II site. After restriction analysis of the 163-bp PCR fragment with *Mbo*II, we expected and observed the following: the $MTHFR^{1298}$+/+ gives 5 fragments, namely 56, 31, 30, 28 and 18-bp fragment, whereas $MTHFR^{1298}$-/- results in 4 fragments, namely 84, 31, 30 and 18-bp fragment, and $MTHFR^{1298}$+/- generates 6 fragments, namely 84, 56, 31, 30, 28 and 18-bp fragment (as shown in Figure 2).

Because each individual has both a MTHFR677 and a MTHFR1298 genotype, the associations between disease and allelic variants of one polymorphism are first reported so that their effects are described independently of allelic variants of the other polymorphism. Among the 54 cases diagnosed with CAD and 55 matched controls, we found the $MTHFR^{677}$-/- genotype present among 5 (11.1%) cases and 2 (3.6%) controls, the $MTHFR^{677}$+/- genotype among 19 (35.2%) cases and 18 (32.7%) controls, and the $MTHFR^{677}$+/+ allele among 29 (53.7%) cases and 35 (63.6%) controls. It seemed that the occurrence of $MTHFR^{677}$-/- allele was a little bit higher in CAD patients than in control, indicating that subjects with homozygous mutations may be prone to cardiovascular diseases. However, the association between disease and the allelic variants analyzed by the Chi-square test and the Fisher exact probability test showed an insignificant correlation (P=0.275). There have been several reports on the correlation between MTHFR polymorphisms and CAD in Taiwan [14-16]. A report published by National Taiwan University Hospital showed the frequency of the $MTHFR^{677}$-/- genotype for CAD patients and control subjects was similar, both were 10%[14]; while a slightly higher frequency can be found in CAD (6.7%) than in the control (4.6%) according to a National Chen-Kung University Hospital report[16]. The frequency of the $MTHFR^{677}$-/- genotype in CAD patient in this study (11.1%) was a little bit higher than previous reports. Generally speaking, distribution of $MTHFR^{677}$-/- genotype varied significantly among different populations. The worldwide survey of 881 unrelated individuals published by Schneider *et al*. showed that the $MTHFR^{677}$-/- genotype frequency in Africans was the lowest at 0%, Europeans at 7.4%, and Asia populations (including Chinese, Mongolia, Vanuatu and Indonesia) varying from 1.5% to 12.8%[19]. Another report on 114 unselected French Canadians showed the $MTHFR^{677}$-/- genotype frequency was 12%[9]. Another report showed that the $MTHFR^{677}$-/- genotype frequency for European, Middle East and Japanese populations was from 12 to 15 %[17]. Our report on the frequency of $MTHFR^{677}$-/- genotype for Southern Taiwanese is higher than that of Africans but lower than European, Middle East or Japanese populations.

We further analyzed the MTHFR1298 A→C polymorphism among the same samples. We found that the MTHFR1298-/- allele present among 2 (3.7%) cases and 3 (5.45%) controls, the $MTHFR^{1298}$+/- genotype among 16 (29.6%) cases and 16 (29.1%) controls, and the $MTHFR^{1298}$+/+ allele among 36 (66.7%) cases and 36 (65.5%) controls. The frequency of $MTHFR^{1298}$-/- genotype reported here was much lower than that of Dutch[11] or Canadians[12] (10%). From the above results, we concluded that the association between CAD and $MTHFR^{677}$-/- genotype (P=0.275) is insignificant and no association between CAD and $MTHFR^{1298}$-/- can be found (P=1).

We next investigated the distribution of the joint effects of the two polymorphisms as shown in Table 1. Here we found the genotype frequency for normal allele ($MTHFR^{677}$+/+, $MTHFR^{1298}$+/+) is the highest, about 35%, among both the CAD and the control; followed by heterozygous MTHFR677 and normal MTHFR1298 ($MTHFR^{677}$+/-, $MTHFR^{1298}$+/+), about 24%; and followed by normal MTHFR677 and heterozygous MTHFR1298 ($MTHFR^{677}$+/+, $MTHFR^{1298}$+/-), about 19%. The trends were compatible with the report of Skibola[18]. The frequency of double heterozygous mutations ($MTHFR^{677}$+/-, $MTHFR^{1298}$+/-) is higher in CAD patients than in the control (13% vs. 7.3%). It has been shown that the enzyme activity of double heterozygous mutations is similar to that of homozygous mutation ($MTHFR^{677}$-/-) and is only 50-60% of normal

Table 1. Frequency of the MTHFR 677and MTHFR 1298 genotypes in coronary artery disease patients and controls

	CAD n(%) total: 54 (100%)			Control n(%) total: 55 (100%)		
	$MTHFR^{677}$+/+	$MTHFR^{677}$+/-	$MTHFR^{677}$-/-	$MTHFR^{677}$+/+	$MTHFR^{677}$+/-	$MTHFR^{677}$-/-
$MTHFR^{1298}$+/+	18(33.3%)	12(22.2%)	6(11.1%)	20(36.3%)	14(25.5%)	2(3.6%)
$MTHFR^{1298}$+/-	9(16.7%)	7(13.0%)	0(0%)	12(21.8%)	4(7.3%)	0(0%)
$MTHFR^{1298}$-/-	2(3.7%)	0(0%)	0(0%)	3(5.5%)	0(0%)	0(0%)

genotype(12). Furthermore, we were unable to identify any subject with double homozygous mutations ($MTHFR^{677}$-/-, $MTHFR^{1298}$-/-) in CAD patients or normal control. Similar results have been reported in Dutch(11) , Canadian(12), and British(18) populations.

The above results may indicate: 1) The frequency of genotype of $MTHFR^{677}$-/- or $MTHFR^{1298}$-/- is lower in the Taiwanese population than in most other populations. Therefore, although the distribution of $MTHFR^{677}$-/- was higher in CAD patients than in the control, the association between CAD and $MTHFR^{677}$-/- is insignificant. The distribution of $MTHFR^{1298}$-/- genotype is similar between CAD patients and the control, suggesting that Southern Taiwanese with the $MTHFR^{677}$-/-, not the $MTHFR^{1298}$-/-, genotype may be at an increased risk for cardiovascular disease. 2) The patients we screened may carry other genetic mutations such as cystathionine-β-synthase (CBS) and methionine synthase (MS), which may also cause hyperhomocysteinaemia. 3) MTHFR mutation exerts its influence on CAD through the action of Hcy. However, the plasma Hcy level is not only influenced by the genetic variations, but also by non-genetic factors such as diet. The nutritional compensation may make a substantial difference in Hcy levels among those with $MTHFR^{677}$-/- genotype. It has been known that in addition to hyperhomocysteinaemia, high blood pressure, hypercholesterolaemia, diabetes or smoking are risk factors cardiovascular disease. As the MTHFR mutations account only for a fraction of the risk factors, a much larger sample size might be necessary to demonstrate the relationship between MTHFR polymorphisms and occurrence of vascular disease. Perhaps a long-term study of plasma concentrations of Hcy and the intake of folate and vitamin B12 would be needed to clarify the association between MTHFR polymorphisms and CAD.

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台南地區冠狀動脈患者5，10－次甲基四氫葉酸酶基因型之檢測

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摘　要

Hyperhomocysteinaemia（高半胱胺酸血症）被證實為腦血管、心血管、冠狀動脈等疾病之危險因子之一。最近有研究指出Homozygous變異的5，10－次甲基四氫葉酸還原酶（5,10-Methylenetetrahydrofolate reductase, MTHFR），會使血液中homocysteine濃度提高，所以MTHFR的突變可能是心血管疾病的重要危險因子。本研究之目的即為利用PCR和限制內切酶片段長度多型性（RFLP）來篩檢大台南地區心血管疾病患者及正常者之MTHFR基因型，我們發現在54名冠狀動脈病患中MTHFR 677C→T的homozygous變異（MTHFR^{677}-/-）佔11.1%，而在55名正常受檢者為3.6%（P = 0.275）。至於MTHFR1298基因型之檢測方面，結果顯示冠狀動脈病患在MTHFR^{1298}-/-僅有2位（3.7%），與正常者之比率5.5%差異不大（P = 1.000）。以上結果顯示MTHFR^{677}-/-可能為國人冠狀動脈病變的遺傳因素之一，但MTHFR 1298A→C的變異則與國人冠狀動脈病變並無關聯。此外由於在冠狀動脈病患中MTHFR^{677}-/-僅佔11.1% ，而MTHFR^{1298}-/-僅佔3.7% ，顯示南台灣地區的冠狀動脈病變並非單由MTHFR677及MTHFR1298基因變異所造成；此與大部分文獻所敘，相去不遠。將來我們將進一步檢測血清中homocysteine、葉酸及維生素B12之含量，以期了解台灣地區MTHFR變異分佈及與血管疾病之關聯性。

關鍵詞：5，10－次甲基四氫葉酸還原酶，同型變異，冠狀動脈疾病，限制內切酶片段長度多型性