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Original Research

MTHFR C677T and A1298C Gene Polymorphisms and Ischemic Heart Disease (IHD) in a Sri Lankan Population - A Preliminary Study

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ABSTRACT

MTHFR A1298C and C677T SNPs are now recognised as important genetic mutations which would give rise to hyperhomocysteinemia. In this study, we analysed the prevalence of these two SNPs in 79 ischemic heart disease (IHD) patients awaiting coronary artery bypass grafting and 79 healthy subjects. MHFR polymorphisms were analysed using polymerase chain reaction followed by a restriction fragment analysis. Prevalence rates for MTHFR C677T polymorphism were 72.8%, 24.7%, and 2.5% for CC, CT, and TT genotypes, respectively, for the whole study population with 677CC genotype being the predominant genotype among both the IHD patients and the controls. The 677TT genotype was detected only among the IHD patients. There was no significant difference in MTHFR 677 genotype variations between IHD patients and the control group. Prevalence rates for the MTHFR A1298C polymorphism were 50%, 37.3%, and 12.7% for the AA, AC, and CC genotypes, respectively, for the whole study population with 1298AA genotype being the predominant genotype among controls and 1298AC the predominant genotype among IHD patients. There was a significant difference ($p < 0.01$) between IHD patients and controls when the MTHFR 1298 genotype variations were compared. Allele frequencies for the mutant T allele for C677T mutation at 0.149 are the highest reported from Sri Lanka. The frequency of the C for the A1298C mutation was 0.313. Results of this study indicate that MTHFR A1298C SNP is more prevalent in Sri Lankans when compared to C677T SNP and that the mutant forms of the MTHFR A1298C SNP are associated with ischemic heart disease.

KEYWORDS: Methylene tetrahydrofolate reductase, Single nucleotide polymorphisms, Ischemic heart disease (IHD) Hyperhomocysteinemia, C677T, A1298C, Allele Frequency

INTRODUCTION

Moderate hyperhomocysteinemia is an independent risk factor for arteriosclerosis, including ischaemic heart disease (IHD), stroke, as well as venous thrombosis [1–4]. One cause of hyperhomocysteinemia is genetic mutations in the Methylene tetrahydrofolate reductase (MTHFR) gene. MTHFR catalyses the reduction of 5,10, methyle netetrahydrofolate to 5, methyltetrahydrofolate which is the major form of folate found in the circulation. 5, methtyltetrahydrofolate also acts as a methyl donor in the remethylation pathway of homocysteine metabolism. This metabolic pathway plays an important role in regulating the fasting plasma levels of homocysteine [5]. Fourteen rare mutations of MTHFR have been found and some are associated

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with severe MTHFR deficiency and hyperhomocysteinemia [6,7]. Defects in the MTHFR gene could give rise to mild to moderate hyperhomocysteinemia and two common single nucleotide polymorphisms (SNP, namely C677T and A1298C that lead to altered amino acids are among them) [8]. The C to T transition at nucleotide 677 (C677T) of the MTHFR gene leads to substitution of Ala-222 by valine whereas the A to C transition at nucleotide 1298 (A1298C) results in substitution of Glu-429 by alanine [9–11].

The reported prevalence of MTHFR 1298C allele outside the Indian subcontinent varies among different populations with a prevalence of 35% in neonates in Canada [12], 33% among healthy individuals in the Netherlands [11], 33% among healthy individuals in Italy [13], 28% among an elderly population in the United Kingdom, and 31.7% among individuals with vascular disease from the United States [14]. All these studies indicated that the average prevalence of 1298C allele of MTHFR gene was approximately 30–35%. The highest prevalence of the MTHFR 1298C allele outside the Indian subcontinent was reported as 49% among healthy Lebanese subjects [15] whilst the lowest of 16% was reported in Japan [15].

Studies conducted in Sri Lankan populations have shown an increased risk of cardiovascular disease in persons having hyperhomocysteinemia [16,17]. While nutritional deficiencies may contribute, genetic alterations in the MTHFR gene are now recognised as an important cause of hyperhomocysteinemia. Only few studies have been conducted in Sri Lanka to investigate the allelic frequency of MTHFR C677T single nucleotide polymorphism [18–20]. Thus, the knowledge regarding the prevalence of the MTHFR C677T is sparse whilst there are no studies conducted to assess the prevalence of A1298C SNP. This study is the first to report the allelic frequency of A1298C and the combination of both C677T and A1298C single nucleotide polymorphisms in IHD patients in Sri Lanka.

Thus, in the present study, we have analysed the MTHFR C677T and A1298C genotypes in diagnosed IHD patients as well as a similar number of normal individuals and compared the frequency of the genotypes between the two study populations.

MATERIALS AND METHODS

Study Participants

A total of 158 subjects including 79 IHD patients awaiting coronary artery bypass grafting and 79 healthy subjects (without any history of IHD) were recruited to the study. Informed written consent was obtained from all participants prior to enrolment to the study. The study protocol was approved by the institutional review board of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka, and the experiments were conducted in conformity with guidelines of the Helsinki declaration.

Collection of Blood Samples and DNA Isolation

Whole blood (2 ml) was collected from each of the study subjects into vacutainer tubes containing K_3-EDTA anticoagulant. 150 µl of whole blood was used in DNA extraction. DNA extraction was performed using Promega Wizard DNA purification kits (Promega Cooperation., Madison, WI) according to the manufacturers' protocol and isolated DNA stored at –20 °C pending analysis.

Amplification of DNA by Polymerase Chain Reaction (PCR)

PCR for the C677T single nucleotide polymorphism analysis was conducted in a reaction mix containing 2.0 µl of sample template genomic DNA, 2.5 µl of 2mM deoxynucleotides, 2.5 µl of 10X PCR reaction buffer (containing $MgCl_2$), 1.0 µl of 5 µM primer mix (forward TGA AGG AGA AGG TGT CTG CGG GA; reverse AGG ACG GTG CGG TGA GAG TG), 0.25 µl of *Taq* DNA polymerase, and 16.75 µl of distilled water to make a total reaction volume of 25 µl [9].

PCR for the A1298C single nucleotide polymorphism analysis was conducted in a reaction mix containing 2.0 µl of sample template genomic DNA, 2.5 µl of 2

mM deoxynucleotides, 2.5 µl of 10X PCR reaction buffer (containing $MgCl_2$), 1.0 µl of 5 µM (forward CTT TGG GGA GCT GAA GGA CTA CTA C; reverse CAC TTT GTG ACC ATT CCG GTT TG), 0.25 µl of *Taq* DNA polymerase, and 16.75 µl of distilled water to make a total reaction volume of 25 µl [11]. The PCR amplification was done using the same PCR conditions for both A1298C and C677T polymorphisms and involved an initial heat denaturing step at 94 °C for 5 min followed by 35 repeated cycles of heat denaturation of the genomic DNA at 94 °C for 30 s, annealing of the primers to their complementary sequences at an annealing temperature of 65 °C for 30 s, and extension of the annealed primers at 72 °C for 30 s. The temperature cycles were concluded with 4 min at 72 °C.

A restriction digestion internal control to use in the C677T polymorphism analysis was developed using *Enterobacteria phage lambda* genomic DNA with a *HinfI* restriction site which gives a fragment of 132bp in size on *HinfI* digestion. A 154 base pair fragment (named 150 bacteriophage lambda fragment) was amplified using PCR in a reaction mix containing 2.0 µl of sample bacteriophage λ template DNA, 2.5 µl of 2mM deoxynucleotides, 2.5 µl of 10X PCR reaction buffer (containing $MgCl_2$), 2.5 µl of 5 µM forward primer (TGC AAT GCC ACA AAG AAG AG), 2.5 µl of 5 µM reverse primer (GCG AAT TAA CCC ATC GTT GA), 0.25 µl of *Taq* DNA polymerase, and 12.75 µl of distilled water to make a total reaction volume of 25 µl. The PCR amplification was done using the same PCR conditions as described above.

MTHFR Polymorphism Detection by RFLP Analysis

The MTHFR C677T SNP creates a *HinfI* restriction enzyme recognition sequence which is detected by digestion of the PCR amplified products with *HinfI* enzyme [9]. To distinguish the undigested products from the wild type homozygous state (677CC), the developed 150 bacteriophage lambda fragment was added to the digestion mix.

The *HinfI* restriction enzyme digestion was conducted in a reaction mix containing 10µl of sample PCR product, 5.0 µl of 150bp bacteriophage λ product as internal control, 0.5µl of 10units/µl *HinfI* restriction endonuclease (Promega Corporation, Madison, WI) 2.0 µl of 10X restriction enzyme buffer, 0.15 µl of 0.1 mg/ml bovine serum albumin (BSA), and 2.35 µl of distilled water to make a reaction volume of 20 µl. The samples were incubated at 37 °C overnight in a dry bath. The digested PCR fragments were visualised and fragment size analysis performed after separation by gel electrophoresis in a 2% agarose gel and staining with ethidium bromide (Figure 1).

The MTHFR A1298C SNP abolishes a *Mbo* II restriction enzyme recognition sequence which is detected by digestion of the PCR amplified products with *Mbo* II enzyme [11].

The *Mbo* II restriction enzyme digestion was conducted in a reaction mix containing 10 µl of PCR product, 0.02 µl of 10 units/µl *Mbo* II restriction endonuclease (Promega corporation, Madison, WI) 1.5 µl of 10X restriction enzyme buffer B, 0.15 µl of 0.1 mg/ml BSA, and 3.33 µl of distilled water to make a reaction volume of 15 µl. The samples were incubated at 37 °C overnight in a dry bath. The digested PCR fragments were visualised and fragment size analysis performed after separation by gel electrophoresis in a 2% agarose gel and staining with ethidium bromide.

RESULTS

The control group consisted of 17 females and 52 males in the age group of 29—70 years whilst the IHD patients consisted of 18 females and 61 females in the age group 34—77 years.

Amongst the whole study population MTHFR 677CC and the MTHFR 1298AA genotypes showed the highest frequencies (Table 1).

The MTHFR 677CC genotype was the commonest among both the control subjects as well as the IHD patients whereas MTHFR 1298AC genotype was the

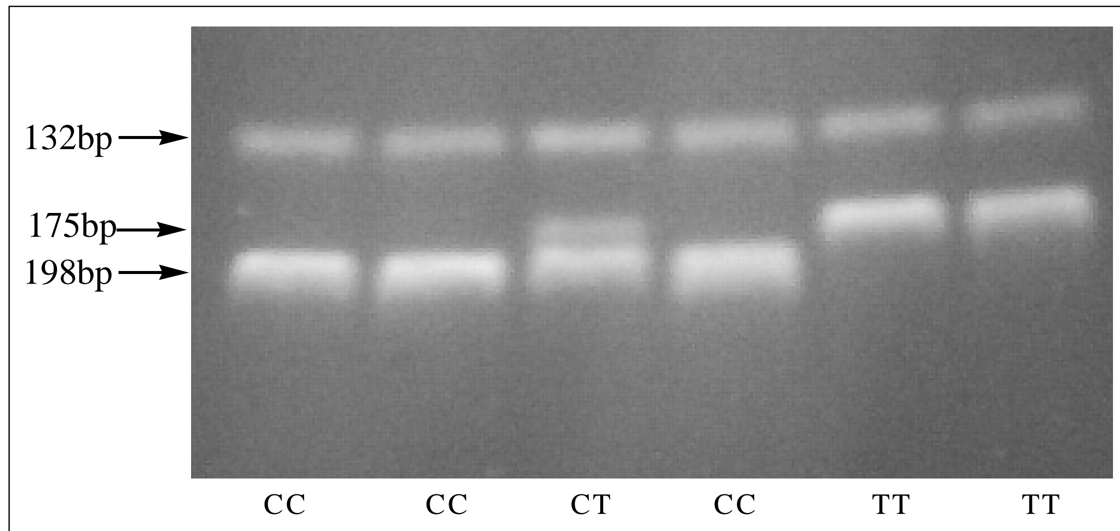

Figure 1: Visualisation of the MTHFR C677T polymorphisms. CC, CT, and TT refers to the MTHFR 677 genotypes. The 132bp fragment is the *HinfI* digested fragment from the developed 150 bacteriophage lambda internal control. The 175 and 198bp fragments are the fragments generated by *HinfI* digestion of the amplified DNA segment of genomic DNA of the subjects

commonest among the IHD patients compared to the MTHFR 1298 AA genotype in the control group. MTHFR 677TT genotype was not observed amongst the control subjects whereas it was present in four IHD patients (5.1%). There was no significant difference in the occurrence of the heterozygous MTHFR 677CT genotype between IHD patients (25.3%) and the control subjects (24.1%). There was no significant difference when the genotype variations in the IHD and the control group were compared. The allelic frequency for the MTHFR 677 C and T alleles was 0.851 and 0.149 with a higher frequency of the T allele being observed in IHD patients (17.7%) compared to controls (12%) in the study (Table 2).

When the MTHFR 1298 genotype frequencies between the IHD patients and the control groups were analysed, the 1298AA genotype showed the highest frequency

Table 1: Genotype frequencies for MTHFR A1298C and C677T polymorphisms in patients with IHD and controls

Polymorphism (genotype)	All, *n* (%) *n* = 158	Patients, *n* (%) *n* = 79	Controls, *n* (%) *n* = 79
MTHFR C677T*			
CC	115 (72.8%)	55(69.6%)	60 (75.9%)
CT	39 (24.7%)	20(25.3%)	19 (24.1%)
TT	4 (2.5%)	4 (5.1%)	–
MTHFR A1298C**			
AA	79 (50%)	29 (36.7%)	50 (63.3%)
AC	59 (37.3%)	32 (40.5%)	27 (34.2%)
CC	20 (12.7%)	18 (22.8%)	2 (2.5%)

*p >0.05 when prevalence of MTHFR 677 mutations in patient and control groups compared.
**p < 0.01 when prevalence of MTHFR 1298 mutations in patient and control groups compared.

Table 2: Allele frequencies for MTHFR A1298C and C677T polymorphisms in patients with IHD and controls

Polymorphism (allele)	All, *n* (%) *n*=158	Patients, *n* (%) *n* =79	Controls, *n* (%) *n* = 79
MTHFR C677T			
C	0.851	0.823	0.880
T	0.149	0.177	0.120
MTHFR A1298C			
A	0.687	0.570	0.804
C	0.313	0.430	0.196

amongst the control group compared to the 1298AC genotype which showed the highest frequency amongst the IHD patients. There was a significant difference ($p < 0.01$) when the genotype variations in the IHD and the control group were compared. The allelic frequency for the MTHFR 1298 A and C alleles was 0.687 and 0.313 with a higher frequency of the C allele being observed in IHD patients (43%) compared to controls (19.6%) in the study.

When the combined MTFR A1298C and C677T genotype frequencies in the whole study population were analysed, the normal allele type for the two SNP's (1298AA and 677CC) showed the highest frequency (Table 3). This combination was the commonest in the control group as well whereas the MTFR 1298AC/677CC combination was the commonest amongst IHD patients. Double homozygosity for the mutant alleles ((1298CC/677TT) were not observed in IHD patients as well as in the controls.

DISCUSSION

The prevalence of T allele frequency of MTHFR C677T gene in this study was found to be higher than that previously reported for Sri Lankan populations [18–20]. Two of the previous studies failed to find MTHFR 677TT homozygotes in Sri Lankans [18,19] while in the other only five (1.4%) 677TT homozygotes were found [20]. In the present study, four 677TT homozygotes were identified comprising of three males and one female. The MTHFR 677T allele frequencies in different communities vary according to research reports from around the world. For example, the allele frequency in Europeans is 24–40% [21], 26–37% in Japanese populations [22,23], and 11% in African Americans. In neighbouring India, the allele frequency for 677 T allele has been reported to be between 10% and 11% [24,25]. Thus, findings of this study give a higher 677 T allele frequency than that observed previously in Sri Lanka and India. The reason for the higher frequency observed merits further investigation as 677 T allele is the allele that is associated most with hyperhomocysteinemia.

In India, the allelic frequencies for the MTHFR 1298C allele have varied from a high prevalence rate of 43%

Table 3: The characteristics of combined genotype frequencies in study subjects

MTHFR genotype (1298/677)	All *n* (%)	Patients with IHD *n* (%)	Controls *n* (%)
AA/CC	55 (34.81)	17 (21.52)	38 (48.10)
AA/CT	22 (13.92)	10 (12.66)	12 (15.19)
AA/TT	2 (1.27)	2 (2.53)	–
AC/CC	43 (27.21)	23 (29.11)	20 (25.32)
AC/CT	14 (8.86)	7 (8.86)	7 (8.86)
AC/TT	2 (1.27)	2 (2.53)	–
CC/CC	17 (10.76)	15 (18.99)	2 (2.53)
CC/CT	3 (1.90)	3 (3.80)	–
CC/TT	–	–	–

among a population in New Delhi [26] to a lowest prevalence rate of 10% in Chandigarh [27]. A study in Tamil Nadu, India reported that the 1298C allele frequency among patients with MI and healthy controls was 40.4% and 35%. Thus, the prevalence of the MTHFR 1298C allele frequency in this study appears to be very much similar to the prevalence rates reported in India.

The missense mutation at position 677 of the MTHFR cDNA has been found to produce a thermolabile form of the enzyme resulting in increased plasma homocysteine [9]. Folate is an important cofactor in the conversion of homocysteine to methionine. It is known that 677TT homozygosity results in the need for a higher folate intake to obtain normal homocysteine levels. It has also been reported that homozygosity for 677T when associated with a decreased intake of or an increased requirement for folate can lead to vascular disorders especially as the individual ages [19].

The effect of 677T allele on homocysteine concentration has been found to be more pronounced in homozygous TT subjects with low folate concentrations [28], and folic acid supplementation has been reported to cause a marked decrease in plasma homocysteine in TT subjects who initially had the same homocysteine concentrations as subjects with the CC genotype [29]. Yet some newer studies have found that the association between elevated homocysteine and coronary heart disease is confined to carriers of the MTHFR 677 C-allele, which could have implications for the efficiency of homocysteine lowering treatment [30].

A significant decrease in the MTHFR enzyme activity has been observed in the homozygous 1298CC as well as in the heterozygous 1298AC state of the MTHFR gene [11], with the highest reduction of enzyme activity being observed in subjects with 1298CC genotype. Thus, the observation that the occurrence of the heterozygous 1298CC genotype was higher in IHD patients than the control group in the present study indicates a possible role for this genotype in ischemic heart disease. With this background our finding that IHD patients have a higher prevalence of the 1298AC and CC genotypes deserves further study to ascertain the effect of these two on serum homocysteine concentrations and subsequent effects of this on IHD.

CONCLUSION

The results of this study suggest that the mutant forms of the MTHFR 1298 AC and CC genotypes may play an important role in IHD patients and the MTHFR 677 mutations are not as important as the 1298 polymorphisms as a risk factor for IHD. Our study is the first to report on the association between IHD and the MTHFR 1298 SNP among Sri Lankans. The homocysteine, vitamins B_{12}, and folate concentrations have to be analysed in the future along with the MTHFR polymorphisms in Sri Lankan subjects to see their associations with IHD.

In conclusion, our preliminary study shows that the MTHFR A1298C SNP is more prevalent in Sri Lankans and its mutant forms are associated with ischemic heart disease.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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