Prevalence of methylene tetrahydrofolate reductase polymorphism in South Indian population

A. Radha Rama Devi[†,*], V. Govindaiah[†], G. Ramakrishna[#] and S. M. Naushad[†]

[†]Diagnostic Division and [#]Cancer Biology Division, Center for DNA Fingerprinting and Diagnostics, Hyderabad 500 076, India

Prevalence of methylene tetrahydrofolate reductase (MTHFR) gene mutations in South Indian population was investigated from a total of 608 samples, 420 adults and 188 newborns. Detection of mutation was carried out focussing on the two most common mutations of the MTHFR gene (C677T and A1298C) using PCR-based RFLP method. T-allele frequency was almost similar between the newborns and adults (0.0904, 0.1012). However, a higher T-allele frequency was observed in females (0.1538 and 0.12 in adults and newborns respectively) than males (0.0556 and 0.05 in adults and newborns respectively). In the case of the other mutation, C-allele frequency was almost similar with no sex-bias (0.414 and 0.402 in males and females respectively). Biochemical correlation of fasting plasma total homocysteine to MTHFR genotype revealed mild to moderate hyperhomocysteinemia in mutants. Plasma total homocysteine in males was found to be higher than in females in both normal and mutant individuals. TT homozygous women had higher plasma homocysteine. The high T-allele frequency, elevated plasma homocysteine and low folate intake in women could well be a risk factor for birth defects. The gender bias observed in this autosomal recessive trait was an interesting finding and is discussed.

METHYLENE tetrahydrofolate reductase (MTHFR) plays a significant role in methionine metabolism. MTHFR deficiency is inherited as an autosomal recessive trait. In South India, with lower dietary intake of folate, MTHFR deficiency could be a significant risk factor for a number of defects, e.g. vascular events[1,2]. Specially so, in women this could be responsible for bad obstetric history[3], neural tube defects[1,2] and possibly Down's syndrome[4]. MTHFR enzyme catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. A non-covalently bound flavin adenine dinucleotide (FAD) cofactor accepts reducing equivalents from NADPH and transfers them to 5,10-methylenetetrahydrofolate. 5-methyltetrahydrofolate is the predominant circulating form of folate which participates in single carbon transfer that occurs as part of the synthesis of nucleotides and *S*-adenosyl methionine; the remethylation of homocysteine to methionine, and the methylation of DNA, proteins, neurotransmitters and phospholipids.

MTHFR gene comprising 11 exons has been localized on chromosome 1p36.3. Eighteen mutations have been reported so far in the MTHFR gene, the most common being C677T and A1298C mis-sense mutations which have been reported to induce milder form of MTHFR deficiency. C677T transition in exon 4 extensively studied in the West, makes the enzyme thermolabile with decrease in its enzymatic activity[5] due to dissociation of dimer into monomers and loss of FAD-binding capacity[6]. The other frequent MTHFR mutation in exon 7, i.e. A1298C transversion, is not associated with thermolability of the enzyme with no impact on plasma total homocysteine; it has not been studied in detail. Compound heterozygosity for (677CT/1298AC) will have similar clinical impact as C677T homozygosity. An individual with a 677TT genotype is always reported to have 1298AA genotype and vice versa[7].

MTHFR mutations were reported to have a heterozygote advantage. The incidence of acute lymphoblastic leukaemia and colorectal cancer is low due to increased availability of precursors required for DNA repair. Enhanced availability of methylenetetrahydrofolate in the DNA synthesis pathway reduces misincorporation of dUMP in place of dTMP into the DNA, which might otherwise result in double-stranded breaks during uracil excision repair process[8].

MTHFR genotype is also found to interact with certain drugs and increase the risk of diseases associated with homocysteinemia, especially if pregnant mothers are on antifolate drugs. Thus MTHFR is an important factor to be considered in pharmacogenomics[8].

MTHFR polymorphism among Asians was studied only in Japanese and Sri Lankans. Limited data are available for other Asian populations, especially Indians[9–11]. A low prevalence of MTHFR polymorphism was recently reported from case-control studies of coronary artery disease at Mumbai[10]. No data are available on the South Indian population, which prompted this study of SNPs in MTHFR and their impact on homocysteine.

Materials and methods

Study population

Fasting blood samples of 420 randomly selected adults, with 225 males and 195 females between the age group of

*For correspondence. (e-mail: radha@www.cdfd.org.in)

20 and 45 years were collected in EDTA for plasma homocysteine estimation and mutation analysis.

A second group comprised of 188 newborns (mean age 4 +/– 2 days), with 108 females and 80 males, born in maternity hospitals. Dried blood spots collected for newborn screening were used as study material for mutation analysis.

Plasma homocysteine analysis

Plasma from EDTA samples was separated immediately within 1 h and stored at – 20°C until the time of analysis. Plasma homocysteine was estimated by reverse-phase HPLC with pre-column derivatization of sulphur-containing amino acids with thiol-specific fluoregenic reagent, ammonium 7-fluorobenzo-2-oxa-1, 3-diazole 4-sulphonate and tributyl *N*-phosphine. ODS 3v column was used as the stationary phase and 0.1 M KH2PO4 : acetonitrile (96 : 4) as the mobile phase with a flow rate of 2 ml/min. Isocratic separation was coupled with fluorescence detection.

MTHFR genotype analysis

Genomic DNA was isolated from the specimens using standard protocols.

C677T mutation

PCR amplification of exon 4 of MTHFR gene using primers 5′TTT GAG GCT GAC CTG AAG CAC TTG AAG GAG-3′ and 5′GAG TGG TAG CCC TGG ATG GGA AAG ATC CCG-3′ resulted in a 173-bp product. The PCR product was digested with *Hinf*I restriction enzyme. C677T mutation creates *Hinf*I restriction site causing cleavage of the 173 bp fragment into 125 bp and 48 bp.

A1298C mutation

PCR amplification of exon 7 of MTHFR gene using primers 5′CTT TGG GGA GCT GAA GGA CTA CTA C-3′ and 5′CAC TTT GTG ACC ATT CCG GTT TG-3′ resulted in a 163-bp product. The PCR product was digested with *Mbo*II restriction enzyme. A1298C mutation abolishes one restriction site of *Mbo*II, resulting in the merger of the 56 and 28 bp bands into 84 bp[5,7].

The PCR conditions for both MTHFR mutations were standardized (Figures 1 and 2).

Statistical analysis

Allele frequencies were calculated and Hardy–Weinberg equilibrium was employed. Statistical analysis was done based on c^2 distribution. The allele frequencies of MTHFR polymorphisms in adults and newborns are presented in Table 1.

Results

The 677T allele frequencies established by this study among newborns and adults are 0.09 (9.0%) and 0.1012 (10.12%) respectively. There is no significant difference in the T-allele frequency between adults and newborns (odds ratio 1.124) and the population was found to be in Hardy–Weinberg equilibrium. The 677T allele frequency distribution among different sexes showed that South Indian women have T-allele frequency of 0.153 which is

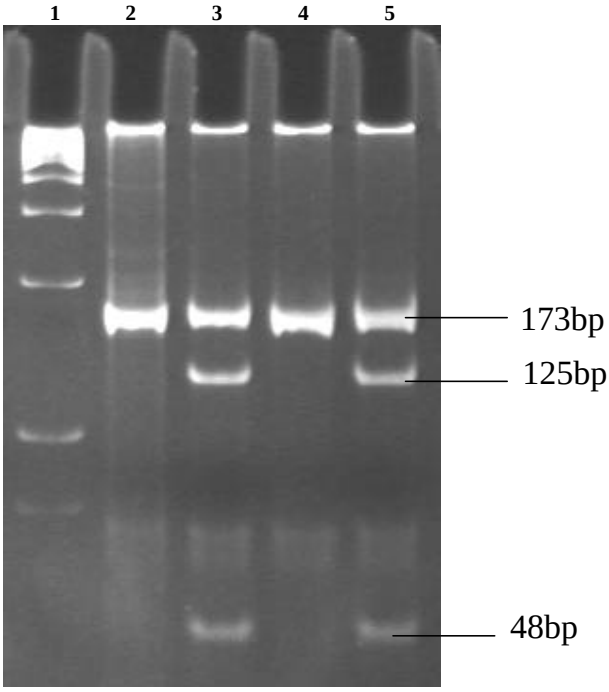

Figure 1. C677T mutation analysis. Lane 1, 100 bp DNA ladder; lane 2, CC (normal); lane 3, CT (heterozygote); lane 4, CC (normal), and lane 5, CT (heterozygote).

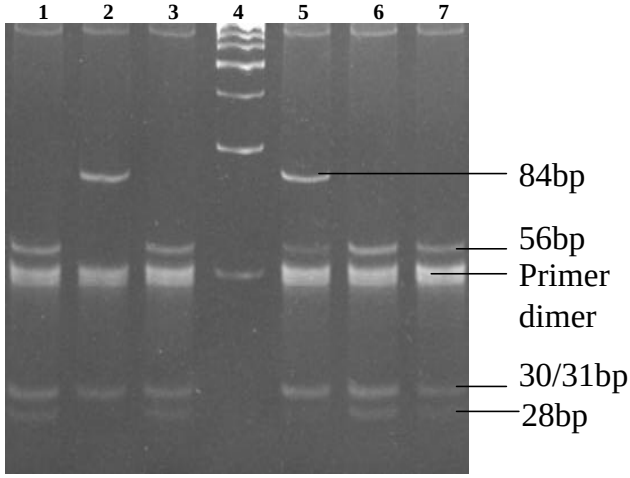

Figure 2. A1298C mutation analysis. Lane 1, AA (normal); lane 2, CC (homozygous); lane 3, AA (normal); lane 4, 100 bp DNA ladder; lane 5, AC (heterozygote); lane 6, AA (normal), and lane 7, AA (normal).

significantly higher than the T-allele frequency in men, i.e. 0.055 with an odds ratio of 2.676, $P > 0.05$. Similar results were seen in case of newborns also, with the allele frequencies of male to female being 0.05 and 0.12 respectively (odds ratio: 2.4, $P > 0.05$). A1298C polymorphism shows C-allele frequencies of 0.414 and 0.402 in males and females respectively. There is no appreciable difference in the allele frequencies in this mutation (Table 1) among both the sexes.

Out of the 225 adult males tested, 25 were heterozygous for C677T mutation and no homozygous mutant was found. Out of the total 195 women in this study, 50 were heterozygous and five were homozygous for this mutation. Four of the TT homozygous women had babies with neural tube defects and one had a bad obstetric history. Out of 80 newborn males tested, eight were heterozygous and no homozygous mutant was found. In case of 108 female newborns, 22 were heterozygous and two were homozygous.

Correlating the MTHFR genotype with total homocysteine in plasma, the mean plasma homocysteine in males and females with wild genotype (677CC) was 13.52 and 8.95 μmol/l respectively. In heterozygotes the mean values were 29.68 and 12.72 μmol/l in males and females respectively. In homozygous mutant females the mean plasma homocysteine was 50.09 μmol/l (Table 2).

Discussion

T-allele frequency for C677T mutation of MTHFR gene in the South Indian population (0.10) established by this study is lower compared to UK (0.186) and USA (0.322) and much higher when compared to Sri Lanka (0.049)[12]. Similar findings were observed among case-control studies conducted in Mumbai[10] and Pune[11]. The allele frequencies were almost similar between newborns and adults. However, a surprising sex bias of this mutation was observed, both in newborns and adults.

Females were found to have higher T-allele frequency than males. TT homozygosity was found to be very low. Out of 195 females five were homozygous for TT, whereas out of 225 males no homozygous TT genotype was observed. Four TT homozygous females had babies with neural tube defects and one had repeated foetal loss. This probably indicates a strong association of maternal TT genotype with higher risk for neural tube defects and bad obstetric history. Newborn population data also showed the presence of two homozygous TT females who are twins, but no homozygous TT males. This sex-bias is an autosomal recessive trait which cannot be explained solely on the basis of genetic factors. We have to consider the gene–environment interaction which may be playing a vital role in this sex-bias. Possibly, there could be a greater mortality associated with TT homozygous or with double-heterozygous male foetuses. To support the hypothesis whether the TT genotype is deleterious or not, a study is under way to establish the impact of MTHFR mutation and gender on foetal viability. Preliminary data indicate a higher mortality among heterozygous male foetuses (unpublished data).

Table 1. Allele frequencies of the two common polymorphisms of MTHFR gene in adults, newborns and products of conception in South India

	Allele frequency (95% C.I.)			
	Adult		Newborn	
Polymorphism	M	F	M	F
C677T	0.055 (0.058–0.12)[a] *N* = 450	0.153 (0.138–0.168)[a] *N* = 390	0.05 (0.039–0.06)[a] *N* = 160	0.12 (0.10–0.139)[a] *N* = 216
A1298C	0.414 (0.409–0.419)[a] *N* = 140	0.402 (0.397–0.407)[b] *N* = 236	–	–

N, Number of alleles examined. [a], P value ≥ 0.05; [b], P value = 0.975.

Table 2. Relationship between thermolabile variant of MTHFR and plasma homocysteine in adults

	Plasma homocysteine in μmol/l	
Genotype	Male	Female
677CC	13.52 ± 6.98 (*N* = 100)	8.95 ± 4.48 (*N* = 100)
677CT	29.68 ± 9.77 (*N* = 30)	12.72 ± 8.11 (*N* = 38)
677TT	–	50.09 ± 16.73 (*N* = 5)

Our study on the frequency of the second common mutation showed the 1298C allele frequency in adults as 0.4069, which is again relatively lower than the Whites, Japanese and Africans who have 0.64, 0.79 and 0.91 frequencies respectively[12,13]. However, there was no significant difference in the C-allele frequencies of males (0.4143) and females (0.4025). This mutation being in the regulatory domain will not have much impact on the thermolability, unless it is combined with heterozygosity for C677T mutation[7]. No comparable data are available for this mutation from India.

From previous studies[7] it has been indicated that an individual with a 677TT genotype always has a 1298AA genotype and vice versa, thus concluding that these two alleles are always in trans-configuration. Most of our data are in accordance with this except in two cases with 677CT/1298CC genotype, which has also been reported by Hanson *et al.*[13,14]. The exceptional genotype of 677CT/1298CC could be rare and presumably due to crossing over.

C677T mutation in MTHFR gene was found to cause mild to moderate homocysteinemia. This is due to dissociation of the active dimer into monomers and loss of FAD-binding capacity of the enzyme, thus leading to hypomethylation[6]. Gender difference was observed in homocysteine in normal and heterozygous states. Females were found to have low homocysteine than males. This difference is possibly due to the protective effect of oestrogen and other physiological factors[15] in women. Elevation is more pronounced in homozygous TT genotype. Due to lack of TT homozygous men, gender difference in plasma homocysteine in homozygous mutants cannot be commented upon.

For adequate functioning of MTHFR enzyme in mutants both in heterozygous and homozygous conditions, folate supplementation is required. Low folate intake and defective folate absorption will result in hyperhomocysteinemia which will be more pronounced in mutants for C677T. TT homozygous or compound heterozygous women with low circulating folate have increased risk of birth defects resulting in increased mortality and morbidity. Multi-disease association, including cancer has been reported in individuals with negative folate status and a mutation in the MTHFR gene. MTHFR polymorphism is now a recognizable genetic risk factor in individuals with recurrent abortions, in treatment with methotrexate causing clinically significant elevation in liver enzymes and toxicity. Anti-epileptics such as methotrexate, sulfasalazine, phenytoin, carbamazepine and valproic acid given to individuals with MTHFR mutation, especially in pregnancy, can cause birth defects. The possible toxicity is probably mediated through homocysteine.

Hyperhomocysteinemia is multifactorial, involving genetic and environmental factors. Apart from mutations in the genes involved in the metabolic pathway, nutritional deficiencies, viz. folic acid, B6 and B12 also are the causative agents for hyperhomocysteinemia. Hence by correcting these deficiencies by supplementation one can reduce homocysteine levels to some extent and decrease the risk for associated disorders.

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