MTHFR C677T Polymorphism as a Risk Factor of Neural Tube Defects in Malay: A Case Control Study

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SUMMARY

Major congenital malformations occur in about 3% of newborn. Several studies have suggested that homozygosity for the C677T methylenetetrahydrofolate reductase (MTHFR) variant is a potential risk factor for neural tube defects (NTDs). It has been hypothesized that the maternal folic acid supplementation prevents NTDs by partially correcting reduced MTHFR activity associated with the variant form of the enzyme. This association has not been found in some ethnic groups. In this study, we attempted to assess the association between NTDs and MTHFR C677T in Malaysian Malay population. Results show that MTHFR 677TT genotype was absent in both patient and control groups.

KEY WORDS:
Genetics, Malay ethnic, MTHFR C677T, Neural tube defects, Polymorphism

INTRODUCTION

Major congenital malformations occur in about 3% of newborn[1]. Central nervous system malformation comprised about a third of these cases. The defects usually lead to death or life-long handicap in the surviving children. These children may also need expensive medical maintenance throughout their lives[2,3,4]. In a population based study in Kinta district (Perak, Malaysia) between 2002 and 2003, there were 253 infants born with congenital abnormalities and of these, 14 were due to neural tube defects (NTDs). This gives a prevalence of 0.79 per 1000 birth[1]. In 1988, a study of 19,769 deliveries in Hospital Alor Setar, Malaysia reported a prevalence of 1.53% for all congenital malformation present at birth or diagnosed in the first week of life, while the prevalence of anencephaly was 1.29 per 1000 birth[1].

Methylenetetrahydrofolate reductase (MTHFR) is a flavoprotein and key enzyme in folate metabolism. It catalyzes the reduction of 5,10 methylenetetrahydrofolate (5,10-CH_2-H_4 folate) to 5 methyltetrahydrofolate (5, CH_3-H_4 folate) which is the major circulatory form of folate and methyl donor for homocysteine remethylation to methionine. The common C677T missense mutation in the MTHFR gene is known to be a risk factor for NTDs[5,6,7]. Other forms of MTHFR polymorphism include A1298C, T1317C and T1068C but are not proven to be clinically important[8,9]. The frequency of the allele in certain ethnic groups roughly correlates with the incidence of NTDs[4].

Study has shown that mutation of the folate dependent enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) is responsible for the reduction of its activity[6]. The homozygous (TT) and heterozygous (CT) genotypes are associated with decreased MTHFR activity. The MTHFR activity among homozygous (TT) is 50-60% lower at 37°C. Heterozygous are in the intermediate range[5]. Individual with a homozygous (TT) genotype demonstrates elevated plasma homocysteine at low folate levels, whereas those with (CT) and (CC) have normal plasma homocysteine levels, indicating that the TT homozygosity can cause a defective homocysteine methylation to methionine[5,6]. Individuals who are homozygous (TT) tend to have mildly elevated blood homocysteine level if their folate intake is insufficient but normal if their folate intake is adequate[10].

In a recent Irish study, they found that up to half of the folate related NTDs may be explained by this single genetic variant[11]. The maternal homozygousity seems to confer a small additional risk of NTDs. Thus, the risk of NTDs might be almost completely due to TT genotype of the embryo[12]. Embryonic tissue grows extremely rapidly. There is a high requirement for methyl groups from S-adenosylmethionine, which are provided by folate-dependent homocysteine remethylation. Hence individual with homozygous (TT) genotype may need more folate to provide the sufficient amount of methyl group required for embryogenesis.

In early pregnancy, fetal DNA is hypomethylated, this relatively mild deficiency in folate metabolism may become important in pregnancy when large amount of methyl groups are required to methylate DNA[6]. Disrupted gene expression, due to the undermethylation of part of the gene, can explain how a relatively mild defect leads to severe developmental abnormalities in the embryo[6]. Both the lowered folate and increased homocysteine concentrations associated with TT genotypes can be corrected by folic acid, even in relatively small doses[11]. However, not all population established similar relationship between the MTHFR C677T polymorphism and NTDs. The association between the C677T variant in the MTHFR gene and NTDs is controversial amongst several populations worldwide. The association between maternal ethnicity and the risk of NTDs remained poorly understood. A number of studies have reported the frequency of MTHFR C677T gene mutation in European and American Caucasian[13]. Based on a pooled data from published studies, about 59% of the European population and 53% of the North American population have either CT or TT genotypes[11].

This article was accepted: 5 November 2008
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The frequency of MTHFR C677T mutation differs widely in different geographic and ethnic populations[7,10,13]. To date, there is no such report on the Malays in Malaysia. In this case control study, we studied the frequency of the gene polymorphism in 22 Malay with non-syndromic NTDs and 20 healthy unrelated Malay as controls.

MATERIALS AND METHODS

This is a case control study to ascertain the relationship of the MTHFR C677T polymorphism and incidence of NTDs in Malaysian Malay population. Saliva was collected from 22 Malay patients with NTDs who was on follow-up at the Spina Bifida Clinic, Hospital Universiti Kebangsaan Malaysia and Hospital Kuala Lumpur within the period of June 2005 and August 2006. DNA extraction from saliva specimen was performed in this study in view of the difficulty in sampling adequate blood from the mainly pediatric subjects, whose ages were between 5 and 12 years old. The detection of the MTHFR C677T polymorphism was carried out by means of polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis.

The clinical history of the 22 patients were reviewed to exclude syndromic NTDs, maternal diabetes mellitus, usage of antiepileptic drugs or anti-folate medication during pregnancy, consanguineous marriage and malnutrition. Saliva was collected from each of these patients for DNA analysis. Twenty unrelated healthy volunteers of ages less than 30 years were recruited randomly from the general population. They had no history of NTDs and never delivered NTDs-affected child. Blood samples were taken for DNA extraction. This study has been approved by the Ethical Committee of Universiti Kebangsaan Malaysia, Malaysia.

DNA extraction from saliva (for 22 neural tube defects patients)

Genomic DNA was isolated from the collected saliva using Oragene DNA Self-Collection Kit, Canada (DNA genotek). 2 ml of saliva were collected on each occasion. 500μl of the aliquot was taken from each sample for DNA extraction in accordance with the manufacturer's guidelines. Before the DNA extraction, the samples were incubated overnight in a water bath at 50°C. They were later transferred to micro-centrifuge tubes adding into each 20μl of Oragene Purifier and mixed gently by inversion. The specimens were incubated on ice for 10 minutes.

After incubation, the specimens were centrifuged for 3 minutes at 13000 rpm at room temperature. The clear supernatant from each sample was transferred into a fresh micro-centrifuge tube without disturbing the pellet. The pellet was later discarded. For the following step, 500μl of 95% ethanol at room temperature was added to the supernatant and mixed gently by inversion. The samples were kept standing for 10 minutes, following which, there were centrifuged again for 1 minute at 13000 rpm at room temperature.

Each supernatant together with the ethanol were discarded without disturbing the DNA pellet. With the excess ethanol removed, the DNA pellets were dissolved in 100μl of standard buffer. The expected concentration of the rehydrated DNA is 10 to 100ng/μl each.

DNA extraction from blood (for 20 healthy controls)

Genomic DNA was isolated from whole blood samples using Wizard Genomic DNA Purification Kit (Promega) following the instruction given by the manufacturer. The DNA was extracted from 300μl of whole blood. 900μl of Cell lysis solution was added to each of the 300μl of blood samples in 1.5 ml micro-centrifuge tubes. The mixtures were incubated for 10 minutes at room temperature. They were then centrifuged at 13000 rpm for 20 seconds, after which, the supernatants were discarded and the remaining solution in the tube were mixed for 15 seconds. 300μl of Nuclei lysis solution was subsequently added to lyse the white blood cells within the mixtures. Subsequently, the mixture became very viscous, forming clumps. These mixtures were incubated at 37°C until the clumps were disrupted. After incubation, 100μl of Protein precipitation solution was added, vortexed for 20 seconds and then centrifuged at 13000 rpm for 3 minutes.

The supernatants obtained were each transferred to a fresh 1.5 ml micro-centrifuge tube containing 300μl of isopropanol. The mixtures were centrifuged again at 13000 rpm for 1 minute and the supernatant discarded. 300μl of 70% ethanol was added to the DNA and centrifuged again at 13000 rpm for 1 minute. The ethanol was then removed by aspirating. 100μl of DNA rehydration solution was subsequently added and the DNA obtained was incubated overnight at room temperature. The isolated DNA was stored at -20°C. The quality of the DNA was confirmed by electrophoresis using an agarose gel.

Genetic analyses / Polymerase chain reaction (PCR)

Genomic DNA was prepared from the saliva (patient) and blood (control). Polymerase chain reaction was carried out on the extracted DNA with a MyCycler Personal Thermal Cycler (Bio-rad, USA) in 50μl of reaction mixture containing 5.0μl of genomic DNA in 10X PCR buffer with 1.5μl of 25Mm MgCl2, 1.0μl dNTPs, 0.2ªm of primers and 0.5μl of AmpliTaq Gold DNA Polymerase (Applied Biosystems).

The sequence of the primers were 5'TGAAGGAGAAGGTGTCTGCGGGA-3' (exonic) and 5'GGACGGTGCGGTGAGAGTG-3' (intronic). The condition of the PCR included an initial pre-denature at 94°C for 7 minutes followed by 35 cycle of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. The amplified products were digested with 1.5μl (final concentration 15U) of restriction enzyme Hinf I (New England Biolabs Inc, USA) at 37°C for 4 hours. DNA fragments were separated by electrophoresis on an agarose gel and were visualized with ethidium bromide.

The individual with the CC (wild-type homozygous) genotype has a single band of 198 base pairs, those with the C/T (heterozygous) genotype has 2 bands of 198 base pairs and 175 base pairs, and the T/T (mutated homozygous) genotype has single band of 175 base pairs.

RESULTS

In this hospital-based study, all subjects studied suffered from lumbosacral NTDs (low and intermediate spina bifida). Of these 22 patients, 8 (36%) are males and 14 (74%) are females. Among the 20 normal controls, 16 (80%) of them are males whilst 4 (20%) are females.

Among the 22 NTDs patients and 20 control subjects, the MTHFR 677TT genotype was found to be absent in both groups. The MTHFR 677CT heterozygous genotype was also absent in the NTDs patients (Figure 1); however it was present in three of the control subjects (15%). Figure 2 shows the results from normal control patients where samples 2, 4 and 11 displayed 2 bands at 198 and 175 bp. All of our NTDs patients (100%) and 17 of the controls exhibited homozygous MTHFR 677CC genotype (85%) (Table I). Hence in this study, there was no Malaysian Malay individual with homozygous MTHFR 677TT genotype.

DISCUSSION

The etiology of most common birth defects has long been described as multifactorial and the dilemma has yet to be resolved. It is known that peri-conceptional supplementation of folic acid reduces the frequency of NTDs by up to 70%[2]. In view of this fact, many folate related genes have been investigated for any mutations altering folate metabolism. This study of MTHFR C677T may help to define some of such factors[10].

Folic acid is efficacious in reducing NTDs but the extent of this has not been ascertained in the Malaysian population. The frequency of the MTHFR C677T mutation differs among different ethnic populations[14]. There is no Malaysian Malay individual with homozygous MTHFR 677TT genotype (control and NTDs patient) detected in this study. In contrast to this finding, Ling *et al.* found the prevalence of the homozygous 677TT in Malays was 3.8%[15]. However, our result was similar with the findings obtained in a study performed on the Indonesian Javanese with frontoethmoidal encephalocoele which showed no genetic association as well. This is probably based on the fact that the Malaysian Malay and Indonesian populations, especially the Javanese and the Madurese are closely linked anthropologically[16].

Hence in this study, a different aetiology and pathogenesis of the development of NTDs among the Malaysian Malay as compared to the European and American Caucasian is postulated. Moreover there is a reverse ratio of spina bifida to anencephaly as compared to the Caucasian[1].

Malnutrition among pregnant women is a common social problem in developing countries. There were a few published data on serum folate level in Malaysians[17-19]. In 1983, a study by Jaafar *et al.,* where serum folate levels were measured on 104 women attending antenatal clinic at Hospital Kuala Lumpur[17]. Subsequently, Tee *et al.* found 60.9% of pregnant women in Malaysia had low serum folate levels[18]. The most recent study on Malaysian women of childbearing age by

Table I: Neural tube defects status versus MTHFR 677 Genotype of individual

Neural tube defects	Genotype of individual		Total
	CC	CT	
No (Control)	17 (85.0%)	3 (15.0%)	20 (100.0%)
Yes (Patient)	22 (100.0%)	0 (0%)	22 (100.0%)
Total	39 (92.9%)	3 (7.1%)	42 (100.0%)

$\chi^2 = 0.099$

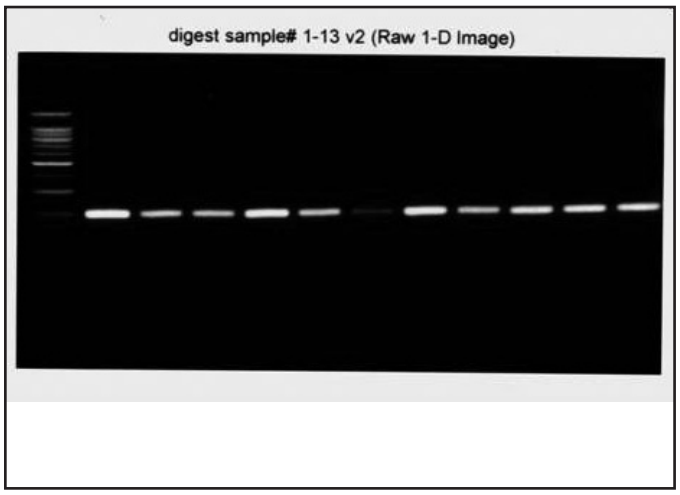

Fig. 1: Result from neural tube defect patients showed single band at 198 bp, indicating that there was no MTHFR 677CT heterozygous genotype.

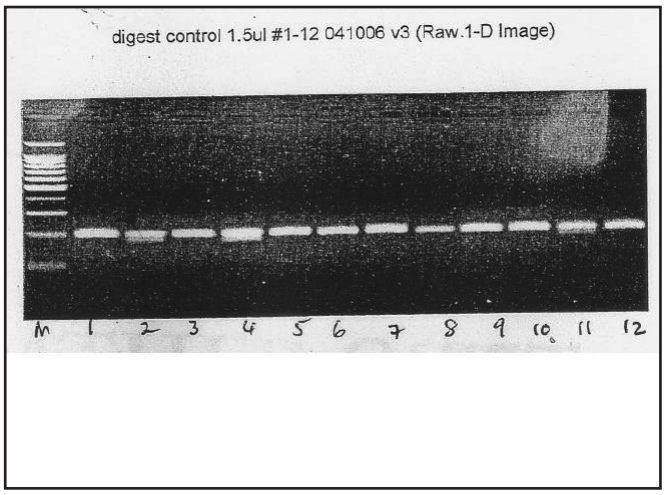

Fig. 2: Result from normal control patients where samples 2, 4 and 11 displayed 2 bands at 198 and 175 bp, indicating presence of MTHFR 677CT heterozygous genotype.

Khor *et al*. showed 15.1% had plasma folate deficiency with Indian subjects having highest prevalence[19]. In this study, the mean serum folate was 5ηg/ml and 28% were found to have folate level to be below normal. Gonzalez-Harrera *et al*., in his study in the state of Yucatan, Mexico also suggested that the maternal C677T mutation is not a risk factor for a mother to have a NTDs offspring[12].

The abundance of fruits and vegetables in Malaysia throughout the year suggests that most women in Malaysia have higher folate level as compared to women in temperate countries where randomized controlled trials were done. This raises the doubt in the efficacy of folate supplementation in the prevention of NTDs.

Despite the small number of subjects recruited in this study, there are several reasons for the absence of the C677T mutation among the Malaysian Malays. MTHFR C677T gene mutation is associated with 2 to 3 fold increase in recurrent early pregnancy loss in some community[8,12,20]. This is probably due to hyperhomocysteinemia in the absence of folate supplementation. Hyperhomocysteinaemia is known to be a risk factor in women with unexplained recurrent early pregnancy loss[8,14]. Insufficient intake of folate by pregnant women is considered to be a survival disadvantage for fetuses homozygous for the T alleles[14].

According to a study performed by Volcik *et al*, the frequency of NTDs with homozygous C677T genotype was greatest in those infants with high level spina bifida defects as compared to the low level spina bifida and the risk is further added if the mother did not use peri-conception folate[21,22]. Since all of our NTDs subjects have intermediate (involving L3 to L5) and low level (sacral) defects (lumbosacral spinal bifida), this may have some bearing on the absence of homozygous MTHFR C677T in this study.

Both the lower folate and increased homocysteine concentrations associated with heterozygous MTHFR CT and homozygous MTHFR TT genotypes can be corrected by folic acid, even in relatively small doses[11]. This is the basis for food fortification targeted at all women of child bearing age to prevent neural tube defects. Since the prevalence of the MTHFR C677T polymorphism in our study is low, we are doubtful of the success of this approach in the Malaysian Malay population. However a more extensive study is needed in evaluating the effects of folic acid supplementation in the prevention of NTDs in Malaysian population. The high frequency of the C677T allele found in some populations might play a role in increasing the overall susceptibility to NTDs[12].

Indeed, the controversy on the association of the C677T variant and NTDs among worldwide population may be explained by the possible multifactorial etiology of NTDs. MTHFR represents only one factor amongst many that might contribute to the appearance of such a malformation. Complex interactions may occur among genes, between alleles of the same genes, and between certain genotypes and environmental factor[4].

The full potential of folic acid to prevent NTDs should be explored further. Despite fortification of cereal products with folic acid and recommendation by their Institute of Medicine and Public Health Service that women who are at reproductive age group should consume 400μg of folic acid daily, potentially preventable disabilities continue to occur, while the underlying cause of NTDs still remained unknown in most cases[4].

Study limitation

In this preliminary study, only 22 neural tube defects samples were available over a period of 15 months. This leads to failure to achieve a statistically significant number of cases. Spina bifida occulta, the mild form of spinal dysraphism is not included in this study as it is often undetected and its relation to overt spina bifida is still uncertain[4].

CONCLUSION

In conclusion, we found that there is absence of T allele in the Malaysian Malay NTDs and we suggest that NTDs in the Malaysian Malay population is not associated with homozygousity for the C677T mutation in the MTHFR gene. However a larger study would be required to further confirm our findings.

ACKNOWLEDGEMENT

The authors would like to thank Universiti Kebangsaan Malaysia for providing the fund for this study, and Wirda Indah Binti Farouk and Faridah Abd. Rahman for their expert help in performing the polymerase chain reactions.

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