Middle-East Journal of Scientific Research 4 (2): 127-132, 2009
ISSN 1990-9233

Detection *of MTHFR C677T and A1298C* Gene Polymorphism in Congenital Heart Disease

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Abstract: Specific objectives of the study were to analyze in children with and without congenital heart disease and their mother. The prevalence of Single Nucleotide Polymorphisms (SNPs) of *MTHFR* gene (677C-T and 1298A-C) and its relation to CHD. Estimate the risk of combined genotype among cases and controls. To correlate the prevalence of the SNPs among children with maternal *MTHFR* genotype.

Key words: Nucleotide · SNP · Heart disease · Genotype

INTRODUCTION

Congenital heart disease refers to a problem with the heart's structure and function due to abnormal heart development before birth. Congenital means something already present at birth. It is the most common type of major birth defect. Congenital heart defects can disrupt the normal flow of blood through the heart. Congenital heart defects occur while the fetus is developing in the uterus and affect 8-10 out of every 1,000 children [1]. According to the American Heart Association, about 35,000 babies are born each year with some type of congenital heart defect in the United States. In the vast majority of patients, the cause of CHD is unknown. However, there are some risk factors that have been associated with a higher rate of congenital heart disease. In the vast majority of patients, the cause of CHD is unknown. However, there are some risk factors that have been associated with a higher rate of congenital heart disease. These risk factors include: Chromosomal or genetic abnormalities (especially Down syndrome) in the child. Certain medications taken during pregnancy Alcohol, drug abuse or smoking duringpregnancy.

Maternal viral infection, such as rubella (German measles) in the first trimester Maternal diabetes mellitus, even if it is gestational diabetes. Congenital heart disease will affect approximately one in every 125 babies born, so it is most common birth defect.According to a status report on CHD in India, 10% of the present infant mortality may be accounted for by CHD. According to a large hospital based study from India, the incidence of congenital heart disease is 3.9/ 1000 live births [2]. In community based studies from India the prevalence of CHD ranges from 0.8-5.2/1000 patients (Indian pediatrics,) Going by crude birth rate of 27.2/1000(2001 census data), the total live births are estimated at nearly 28 million per year. With a believed incidence rate of 8/1000 live births; nearly 180000 children are born with CHD each year in India. Of these nearly 60000 to 90000 suffer from critical CHD requiring early intervention. Approximately 10% of present infant mortality in India may be accounted for by CHD alone [3].

Homocysteine is an amino acid normally found in blood, but elevated levels have been linked with coronary heart disease and stroke. Elevated homocysteine levels may impair endothelial vasomotor function, which determines how easily blood flows through blood vessels. High levels of homocysteine also may damage coronary arteries and make it easier for blood clotting cells called platelets to clump together and form a clot, which may lead to a heart attack [4].

A deficiency of folate, vitamin B_{12} or vitamin B_6 may increase blood levels of homocysteine and folate supplementation has been shown to decrease homocysteine levels and to improve endothelial function [5]. Daily consumption of folic-acid fortified breakfast cereal and the use of folic acid supplements has

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been shown to be an effective strategy for reducing homocysteine concentrations [6].

Specific objectives of the study were to analyze in children with and without congenital heart disease and their mothers .The prevalence of Single Nucleotide Polymorphisms (SNPs) of *MTHFR* gene (677C_T and 1298A_C) and its relation to CHD. Estimate the risk of combined genotype among cases and controls. To correlate the prevalence of the SNPs among children with maternal *MTHFR* genotype.

MATERIALS AND METHODS

This case/control study included 28 subjects 7 babies with congenital heart disease and their mothers in the case arm and 7 normal babies and their mothers in the control arm. Patients were selected at random from the Pediatric ward of SAT Hospital, Medical College, Thiruvananthapuram, India. The Research Review and Advisory Committee of the Medical College had approved the study.

Isolation of Genomic DNA: For genomic DNA isolation, 3ml of blood was taken from the study subjects. The red blood cells were lysed completely using RBC lyses solution. The lysate were then treated with cell lysis solution in order to lyse the cell components. The protein content is removed by protein precipitation solution and isopropanol was used to precipitate the DNA. The precipitated DNA was suspended in 70% ethanol in order to remove the salts. The DNA was then dissolved in TE buffer and stored at 4°C .Cell lysis, protein precipitation, DNA precipitation and DNA hydration were carried out in the experiment.

Polymerase Chain Reaction: PCR is a rapid technique for *in vitro* amplification of a specific DNA fragment by use of two short single stranded primers flanking this segment. PCR reactions were used to amplify the desired regions of the gene MTHFR at 677 and 1298.The PCR reaction was done in a sterile 0.5ml tube, the reaction mix was made in the following order.

Amplification buffer-5µl, forward primer -1µl, reverse of primer -1µl, dntps-3µl, DNA template (mthfr)-5µl, Taq DNA polymerase-0.5µl, sterile water-34.5 µl.

Pcr Conditions for *MTHFR 677* and *1298*: Initial-Denaturation-94°C for 8 Min, Denaturation-94°C for 1 Min, Annealing-63°C for 1 Min, Extension -72°C for 1 Min, Final extension-72°C for7Min4°C, Forever Repeated for 40 cycles. The primer sequences were:

Gene	Primer Sequence	Amplified Product (bp)	Restriction Enzyme
MTHFR 677	5'TGAAGGAGAAGG TGTCTGCGGGA3' 5' AGGACGGTGCG TGAGAGTG3'	198	*Hinf* I
MTHFR 1298	5'-CAAGGAGGAGCT GCTGAAGA-3' 5'-CCACTCCAGCAT CACTCACT-3'	128	*Mbo* II

Agarose Gel Electrophoresis: An agarose matrix can efficiently separate larger DNA fragments ranging from 100-50000 nucleotides. Adjusting the agarose concentration can separate DNA fragments in different size ranges. They also utilized fluorescent dye, ethidium bromide to stain DNA fragments. This sensitive technique can detect as little as 5ng of DNA.

Restriction Fragment Length Polymorphism: The amplified PCR products (MTHFR) were subjected to *Hinf* I and *Mbo II* restriction enzyme digestion at 37°C for 1hour.The PCR products subjected to enzyme digestion was visualized on 3% agarose gel stained with ethidium bromide. Gel photography was done using a Kodak digital science electrophoresis documentation and analysis system 120. For MTHFR 677, the PCR yielded a 198 bp product, which on digestion with *Hinf* I produced a 175 and 23 bp fragments for TT condition (homozygous polymorphic) and a 198,175 and 23 bp fragments for CT condition (heterozygous polymorphic). An undigested product length of 198 bp was retained by the wild types. The 23bp fragment was not retained on the gel. For MTHFR 1298 genotyping PCR conditions were same as that of MTHFR 677 and the product yielded a 128bp fragment. AA genotype (wild) on digestion with *Mbo* II produced three fragments of 28, 28 and 72, CC genotype (homozygous polymorphic) yielded two fragments of 28 and 100 and AC genotype (heterozygous polymorphic) produced 28,72 and 100bp fragments. The 28bp fragment was not retained on the gel.

The Enzymes Used Were *Hinf I and Mbo II* Source: *Hinf1* is from *Haemophilus influenzae* and *Mbo* II from *Moraxella bovis*. The following is the procedure for a typical reaction volume of 20µl.

Agarose Gel Electrophoresis: Joseph Sambrook and his research team did further advancement in DNA electrophoresis. They replaced polyacrylamide gel with agarose a highly purified form of agar. An agarose matrix can efficiently separate larger DNA fragments ranging from 100-50000 nucleotides. Adjusting the agarose

concentration can separate DNA fragments in different size ranges. They also utilized fluorescent dye, ethidium bromide to stain DNA fragments. This sensitive technique can detect as little as 5ng of DNA.

RESULTS

Isolation of Genomic DNA: White precipitated DNA was formed after the precipitation .This DNA pellets are allowed to re-hydrate and stored at 2-8°C .

Polymerase Chain Reaction: For MTHFR 677, the PCR yielded a 198 bp product (Fig 1). For MTHFR 1298 genotyping PCR conditions were same as that of MTHFR 677 and the product yielded a 128bp fragment (Fig. 3).

Restriction Fragment Length Polymorphism: MTHFR 677 (Fig. 2), which on digestion with *Hinf* I produced a 175 and 23 bp fragments for TT condition (homozygous polymorphic) and a 198,175 and 23 bp fragments for CT condition (heterozygous polymorphic). An undigested product length of 198 bp was retained by the wild types. For MTHFR 1298 (Fig 4). Genotype AA genotype (wild) on digestion with *Mbo* II produced three fragments of 28, 28 and 72, CC genotype (homozygous polymorphic) yielded two fragments of 28 and 100 and AC genotype (heterozygous polymorphic) produced 28, 72 and 100bp fragments.

The distribution of polymorphic variants among cases and controls. As can be seen, the polymorphic variants are more prevalent among cases, whereas the

PCR ANALYSIS OF MTHFR CODON 677 GENOTYPE

Fig. 1

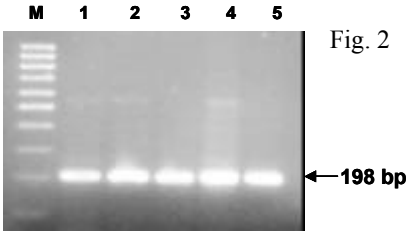
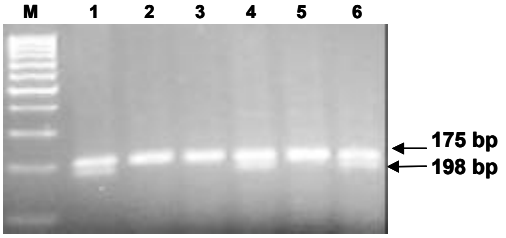


Fig. 2



M – DNA Molecular Weight Marker
Lanes 1, 4 & 6 – Heterozygous polymorphic
Lanes 2,3 & 5 – Homozygous Wild Type

Fig. 1: PCR product before Hinf I RFLP
Fig. 2: Hinf I RFLP analysis of MTHFR 677

PCR ANALYSIS OF MTHFR CODON 1298 GENOTYPE

Fig. 3

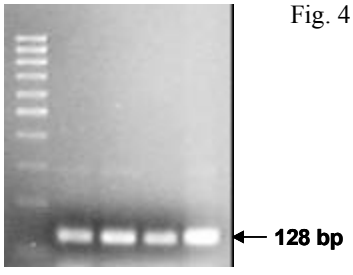
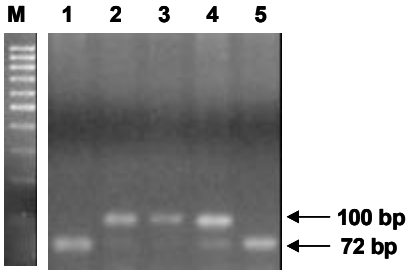


Fig. 4



M – DNA Molecular Weight Marker
Lanes 1 & 5 – Homozygous Wild Type
Lanes 2 & 3 – Homozygous Polymorphic
Lane 4 – Heterozygous Polymorphic

Fig. 3: PCR product before Mbo II RFLP
Fig. 4: Mbo II RFLP analysis of MTHFR 1298

Table 1: Distribution of polymorphism among mothers and babies in the study group

	Mothers			Babies		
Gene Variant	Condition	Case	Control	Total	Case	Control	Total
MTHFR 677	Wild	4	6	10	1	5	6
	Polymorphic	3	1	4	6	2	8
MTHFR 1298	Wild	3	4	7	3	5	8
	Polymorphic	4	3	7	4	2	6

Table 2: Distribution of babies after combining the genotypes

MTHFR 677 or MTHFR 1298	Case	Control	Total
Both wild	3	0	3
One of them polymorphic	4	4	8
Both polymorphic	0	3	3
Total	7	7	14

Table 3: Distribution of MTHFR 677 polymorphism among mothers and babies

MTHFR 677	Case	Control	Total
Both mother and baby wild	4	1	5
Mother is wild and baby is polymorphic	2	3	5
Mother is polymorphic and baby is wild	1	0	1
Both mother and baby are polymorphic	0	3	3
Total	7	7	14

Table 4: Distribution of MTHFR 1298 polymorphism among mothers and babies

MTHFR 677	Case	Control	Total
Both mother and baby wild	2	1	3
Mother is wild and baby is polymorphic	2	0	2
Mother is polymorphic and baby is wild	2	1	3
Both mother and baby are polymorphic	1	5	6
Total	7	7	14

Table 5: Distribution of mothers and babies after combining the genotypes

MTHFR 677	Case	Control	Total
Both mother and baby wild	1	0	1
Mother is wild and baby is polymorphic	3	1	4
Mother is polymorphic and baby is wild	2	0	2
Both mother and baby are polymorphic	1	6	7
Total	7	7	14

wild type was more commonly seen among controls. For MTHFR 677, among cases, 4 mothers and one baby were seen to be wild while the rest of the group was polymorphic. Among controls, six mothers and five babies were wild and only one mother and 2 babies were polymorphic. As for the case of MTHFR 1298, three mothers and 3 babies were wild and four mothers and four babies were polymorphic among cases. Among controls, four mothers and five babies were wild and 3 mothers and 2 babies carried the polymorphic variant. Statistical analysis showed a significant P value of 0.05 (Fisher's exact test) (Table 1).

Understand the collective effect of the polymorphism among babies with congenital heart disease, we combined the genotype variants and formed three groups, viz. babies with both variants as wild, those with either 677 or 1298 as polymorphic and finally babies with both 677 and 1298 as polymorphic. All seven cases had one of the genotypes or both as polymorphic while among controls, none of the babies were polymorphic for both gene variants. Statistical analysis showed a significant P value of 0.04 (Table 2).

The distribution of MTHFR 677 and MTHFR 1298 polymorphisms among mothers and babies. It was observed that for MTHFR 677, three out of seven cases were in the group pertaining to both mother and baby being carriers of polymorphic variant. Fisher's exact test revealed a non-significant P value of 0.!55. In the case of MTHFR 1298, five out of seven cases belonged to the fourth group ie. both mother and baby being polymorphic for MTHFR 1298. Again the P value was not significant despite having the majority of cases in the high risk group (P value=0.19) (Table 3 and 4).A combination of both genotypes among mothers and babies were analyzed and the results are detailed in (Table 5). The four categories allotted were

The two genotypes were wild for both babies and their mothers. The two genotypes were wild for mothers and one or both were polymorphic for the babies. One of the genotypes or both were polymorphic for mothers and both were wild for the babies. Both mother and babies polymorphic for both genotypes.

A statistically significant P value of 0.04 was observed for this category. Among cases, six out of seven mother's of babies with CHD were carriers of polymorphic variants (either both genotypes or at least one were polymorphic), but among controls, only one pair of mother and baby had polymorphic variants.

DISCUSSION

Congenital heart defects (CHDs) are a common variety of birth defect, with a prevalence of confirmed defects of approximately 1:100 living births, although

this varies throughout the world. They account for approximately one third of all congenital anomalies and are the single largest contributor to infant mortality attributable to birth defects. CHDs mainly result from incomplete development of the heart during the first 6 weeks of pregnancy [7].

Genetic insufficiency of folate results due to the inefficiency of the enzymes in the folate metabolism cycle and the most important one being Methylene tetrahydrofolate reductase or MTHFR. Two common polymorphism are seen in MTHFR gene namely; C677T and A1298C [8]. The C677T (Ala-to-Val) transition, which produces thermo ability and somewhat reduced enzyme activity in vitro, was first described by [9]. Individuals homozygous for the C677T mutation have moderately increased concentrations of fasting plasma homocysteine especially in the presence of low (<15.4 mol/L) plasma folate [10]. The second prevalent polymorphism (A1298) is associated with decreased enzyme activity in vitro. This genetic variant consists of an A¬C transversion at nucleotide 1298, which produces a Glu-to-Ala substitution [11].

We conducted this pilot study to analyze the influence of polymorphisms of the MTHFR gene observed at the 677 and 1298 nucleotides in the gene sequence among a study population of 28 subjects, viz. 7 babies with congenital heart disease and their mothers and 7 apparently healthy babies and their mothers [12]. We observed that polymorphic variants of the MTHFR gene were more predominant among cases than controls which was substantiated by a significant P value of 0.05.Our results have also shown that combined polymorphic variants of MTHFR 677 and 1298 are more prevalent among babies with CHD than among those without the disease [13].

Though we combined the genotype status of mother and baby to analyze the effect of MTHFR 677 and 1298 separately, it did not reveal any statistically significant results, which could be because of the reduced sample size. But there are reports on the importance of these polymorphisms in the etiology of CHDs [14]. Studied the association between MTHFR 677C>T variants and CHD risk. The interaction with periconceptional folate supplementation was also investigated. They concluded that the maternal MTHFR 677C>T variants are a risk factor for CHD in offspring, confined to conotruncal heart defects [15]. Observed a higher frequency of the 677TT genotype among children with a CHD. The results we observed after combining the genotypes of mothers and babies as well as the genotype status of MTHFR gene, was promising. Six out of seven cases and one out of seven controls were carriers of either one or both of the polymorphic variants, which emphasizes the importance of polymorphic allele in the development and progression of CHD [16].

Studies of [17] have shown that women who used medications that are folic acid antagonists had an increased risk of having babies with heart defects but that this risk was reduced for women who also took multivitamin supplements containing folic acid. Our results suggest a major role of MTHFR polymorphism in CHD patients as well as a strong suggestion to maternal genotype. One of the major drawbacks of our study was the limited sample size, which was so small that, it was not even sufficient to conduct proper statistical tests to improve the power of the results observed. Though there was no clinical data to supplement the molecular data, it was clear that combined polymorphic variants of MTHFR 677 and MTHFR 1298 among mothers and babies could be an independent risk factor in the development of CHDs among babies [18].

To conclude, our study suggests a possible genetic mechanism in the development and progression of congenital heart disease, in relation to maternal genotype of folate metabolizing enzyme, MTHFR. In the application level we can say that, mothers with polymorphic variants for MTHFR C677T and/or MTHFR A1298C have an increased chance of carrying babies with congenital heart disease and that the aberrant genotype maybe carried forward, which could further aggravate the baby's chances of developing the disease. By identifying the mother's genotype and if the mother is a carrier of variant allele, periconceptual supplementation of folate will be a better strategy to reduce the incidence of CHDs among babies.

ACKNOWLEDGEMENT

The authors are very thankful to The Director, SAT Hospital and staff members of Pediatric ward of SAT Hospital, Medical College, Thiruvananthapuram, India,is highly acknowledged.

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