Genetic Variants in the Methylenetetrahydrofolate Reductase Gene in Egyptian Children with Conotruncal Heart Defects and their Mothers

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Abstract

Aim: This study aimed at evaluation of MTHFR 677C/T and 1298A/C polymorphisms in MTHFR gene as maternal risk factors in conotruncal heart defects (CTDs).

Material and Methods: Thirty cases with CTDs and their mothers and thirty control children and their mothers were studied. Medical and nutritional histories for all mothers were taken with emphasis on folate intake. The mutations in MTHFR gene are a C to T substitution at base pair 677 and a A to C at base pair1298 were investigated by polymerase-chain reaction, followed by restriction enzyme digestion and detection by electrophoresis.

Results: Folic acid intake was below the recommended daily allowance in case mothers compared to control mothers. No significant difference between patients and control group or their mothers regarding MTHFR gene polymorphisms at exon 4. Significant difference between case and control groups and between their mothers regarding MTHFR gene polymorphisms at exon 7. Homozygous genotype frequencies of MTHFR at position 1298 was significantly higher in cases and their mothers with an odds ratio 56 (95% confidence interval 10.3-303.7).

Conclusion: Egyptian population may have high incidence of MTHFR polymorphism at exon 7 rather than at exon 4. Preconception folate intake might decrease MTHFR polymorphism at exon 7 and decrease risk of CTD.

Introduction

Congenital heart defects (CHDs) are the most common structural birth defects, affecting about 8 to 10 of every 1000 live birth. The etiology of non-syndromic CHDs is complex, involving both genetic and environmental risk factors [1]. Conotruncal heart defects
(outflow tract defect) are a serious subset of CHDs with prevalence rate about 8 per 10,000 live births. Common types are tetralogy of Fallot (TOF), transposition of great arteries (TGA), truncus arteriosus, double outlet right ventricle and interrupted aortic arch. All defects cause improper circulation of oxygenated and deoxygenated blood [2]. The association between periconceptional maternal folic acid use and a reduced risk of fetal conotruncal cardiac defects has been reported in a number of case-control studies [3-5]. It is widely accepted that the impact of folic acid intake on pregnancy outcome is modified by variants in both maternal and fetal genes that code for critical enzymes in the folate and homocysteine pathways [6].

The 5,10-methylenetetrahydrofolate reductase (\textit{MTHFR}) gene is located on chromosome 1 at 1p36.3. The complementary DNA sequence is 2.2 kilobases long and consists of 11 exons. \textit{MTHFR} catalyses the biologically irreversible reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for methionine synthesis from homocysteine [7]. Two single nucleotide polymorphisms (SNPs) in \textit{MTHFR}, 677C-T (exon 4) and 1298A-C (exon 7) are associated with decreased enzyme activity [8]. Few studies have investigated the association between \textit{MTHFR} genotypes and the risk of development of congenital anomalies including Down syndrome, oral clefts, urogenital anomalies and limb defects [9, 10].

**Patients and Methods**

**Study design**

This was a cross-sectional observational case-control study.

**Patients**

We studied 60 children and their mothers divided into two groups. Case group included thirty cases (18 males and 12 females) with conotruncal heart defects and their mothers selected from the Specialized Pediatric Cardiology Clinic, Children Hospital, Ain Shams University. Control group included thirty healthy age and sex matched infants and children (16 males and 14 females) and their mothers were included in the study as a control group.

**Inclusion criteria**

Conotruncal heart defects in the offspring and non syndromic: no clinically evident dysmorphic faces.

**Exclusion criteria**

Syndromic CHD and non conotruncal heart defects.

Informed consent was obtained from the parents of all children and the study protocol was approved by the ethical committees of the Pediatric department, Faculty of Medicine, Ain Shams University and National Research Center.

**Methods**

**Clinical Assessment**

A comprehensive history was taken from all study participants, included pedigree analysis, family history of any genetic or non genetic disorders, family history of CHD, pregnancy and obstetric history. A dietary history and food frequency questionnaire were completed for all mothers with special emphasis on folic acid intake in food or supplements during pregnancy. Adequacy of the diet was assessed by comparing the energy and nutrient intake of mothers with their recommended daily allowances [11]. Comparison between the requirements of each mother according to Food and Agriculture Organization (FAO) and the nutritive value of her food intake within 24 hours was done using Food Composition Tables of the National Nutrition Institute of Egypt [12].

A thorough clinical assessment was performed for all participating children. Data recorded included weight, height. Pulse oximetric saturation, vital measurements, presence of extracardiac congenital anomalies and a complete cardiovascular assessment were done.

**Investigations**

The diagnosis of conotruncal heart disease was verified by two dimensional and Doppler echocardiography according to the segmental sequential approach described by Snider et al. (1997) [13], and further information obtained from plain chest X rays and 12 lead Electrocardiogram.

Chest X-Ray. Posteroanterior plain X-rays were analyzed for cardiothoracic ratio (for cardiomegaly), cardiac configuration, mediastinal width and bronchovascular markings.

**Blood sampling and genetic analysis**

Venous blood samples were collected on EDTA from all cases and their mothers and preserved at 4ºC till
assayed. Total genomic DNA was extracted from whole blood using salting out technique.

**Polymerase Chain Reaction Amplification:** Amplification was performed in 50 μl reaction mixture containing approximately 100 ng genomic DNA, 200 μM dNTPS, 50 picomole each of forward 5'-TGA AGG AGA AGG TGCTG CGG GA-3' and reverse 5'-AGG ACG GTG CGG TGAGAG TG-3' primers, and 2 units of Taq DNA polymerase (Qiagene, Frankfurt, Germany) in a 1 X buffer supplied with the enzyme containing 1.5 mM MgCl₂. For the polymorphic site (C-->T) at 677 bp, primer sequences used were derived from intron sequence bracketing exon (4) according to Kowa et al. (2000) [14].

The primer sequences used for amplification of exon (7) were derived from inside exon (7) surrounding the polymorphic site (A-->C) at position 1298. The primer design was performed using Primer3 Output Software (primer3 www results.cgi) and the sequences were as follows:

**Forward primer:** '5 GAA GAG CAA GTC CCC CAA AG 3'

**Reverse primer:** '5 ACA GGA TGG GGA AGT CAC AC 3'

**Thermal cycling and product detection:** Initial denaturation for one cycle at 95ºC for 3 min followed by 35 cycles each consisted of denaturation at 95ºC for 30 seconds, annealing at 60º C for 45 seconds and extension at 72ºC for one min. Amplification protocol was ended up with a final extension step at 72ºC for 10 minutes. The PCR products were analyzed on 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The detection of a fragment of 198 bp indicates a successful amplification of exon 4 of human MTHFR gene and of a 221 bp band indicates amplification of exon 7

**Mutational analysis (Restriction fragment length polymorphism) of the digested PCR products:** The digested products were resolved on 20% poly acrylamide gel. The poly acrylamide gel was stained with ethidium bromide and visualized by UV trans illuminator. The normal pattern (wild type) (CC) for exon (4) provides a single duplet band at 198 bp after digestion with Hinf I. While the heterozygous pattern (CT) creates a Hinf I restriction site leading to the digestion of the 198 bp, providing three bands at 198, 175, and 23 bp. The 23 bp, last band is undetectable and ran out of the gel. The mutant pattern (TT) provided 2 bands at 175 and 23 bp. Only, one band at 175 bp is detectable while that of 23 bp ran out of gel

The 221 bp of exon (7) is reduced to 209 bp due to the presence of another MboII recognition site within the sequence of the forward primer which does not interfere with polymorphic site at 1298 bp, and that is used to detect whether the enzyme used is working or not. The normal pattern (wild type), (AA) for exon (7) provides 3 bands at 151, 30, and 28 bp, respectively after digestion with MboII. Only 151 bp band is visualized on 20% polyacrylamide gel while the other two bands ran out of the gel. While, the heterozygous pattern (AC), (contains both mutant and wild type allele) provides 4 bands at 179, 151, 30, and 28 bp. The only two bands visualized on gel were 179 and 151 bp while the other 2 bands ran out, as they are very small in size. The mutant pattern (CC) provided 2 bands at 179 and 30 bp where only band at 179 bp was visualized on the gel [15].

**Statistical analyses**

Odds ratios and 95% confidence intervals (95% CI) were calculated to estimate the risk of the different genotypes. Allele frequencies and genotype frequencies were calculated and the differences between mothers of children with conotruncal heart defects and control mothers were determined using chi-square test. Expected genotype frequencies were calculated from the allele frequencies under the assumption of Hardy-Weinberg equilibrium. All statistical analyses were done with SPSS soft ware, version 9.0. P values were two tailed, and P was considered statistically different P <0.05.

**Results**

There was no significant differences between the two groups in any of the demographic features.

Table 1: Descriptive analysis of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Cases (n= 30)</th>
<th>Control (n=30)</th>
<th>Statistical test Value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (months)</td>
<td>31.5 ± 40.5</td>
<td>32.37 ± 33.03</td>
<td>Z:0.96</td>
<td>0.57</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>9.7 ± 6</td>
<td>12.13 ± 6</td>
<td>Z:1.07</td>
<td>0.29</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>29.3 ± 6.6</td>
<td>29.7 ±5</td>
<td>Z:0.18</td>
<td>0.85</td>
</tr>
<tr>
<td>Male</td>
<td>18 (60%)</td>
<td>16 (53.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (40%)</td>
<td>14 (46.7 %)</td>
<td>X²:0.136</td>
<td>0.713</td>
</tr>
</tbody>
</table>

Nonparametric Z test was used. Pearson chi square test (X²) was used for qualitative data.

Table 2 showed the cardiac diagnoses of the children with conotruncal heart defects.

As regard dietary findings of patients and control mothers, only 4 patients’ mothers (13.3%) used the recommended periconceptional folate intake adequately
and intake of folate in 26 patients’ mothers (86.7 %) was below recommended daily allowance.

Table 2: Main dietary findings of case and control mothers.

<table>
<thead>
<tr>
<th>Folic acid intake from supplement or food</th>
<th>Mothers</th>
<th>Folic acid intake from supplement or food</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 400 µg/d</td>
<td>(n=30)</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>&lt; 400 µg/d</td>
<td>(n=30)</td>
<td>23 (76.7%)</td>
</tr>
</tbody>
</table>

There was no statistically significant difference between patients and controls as regards the presence of homzygous or heterozygous gene polymorphism of the MTHFR gene at exon 4 (P= 0.341). In the patient group, 40% had no polymorphism (CC), 46.7% had heterozygous polymorphism (CT) and 13.3% had homzygous polymorphism at exon 4 (TT). In the control group, 66.7% had no polymorphism (CC), 26.6% had heterozygous polymorphism (CT) and about 6.7% of them had homzygous polymorphism at exon 4 (TT).

Table 4: Genotype frequencies of C677 T in exon 4 among case and control groups.

<table>
<thead>
<tr>
<th>Exon-4</th>
<th>Cases (n=30)</th>
<th>Control (n=30)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>x²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>12 (40%)</td>
<td>20 (66.7%)</td>
<td>1.0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>14 (46.7%)</td>
<td>8 (26.6%)</td>
<td>2.42</td>
<td>0.82-7.1</td>
<td>2.152</td>
<td>0.341 (NS)</td>
</tr>
<tr>
<td>TT</td>
<td>4 (13.3%)</td>
<td>2 (6.7%)</td>
<td>2.1</td>
<td>0.36-12.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Similarly, we found no significant statistical difference between patients and controls as regards maternal MTHFR gene polymorphism at exon 4 C677T (p= 0.260). 40% of the patients’ mothers had no polymorphism (CC), 53.3% had heterozygous polymorphism (CT) and 6.7% had homzygous polymorphism at exon 4 (TT). At the same time, 67.7% of the mothers of the control group had no polymorphism (CC), 33.3% of them had heterozygous polymorphism (CT) and none of them had homzygous polymorphism at exon 4 (TT).

On the other hand, there was a highly significant statistical difference in MTHFR gene polymorphism at exon 7 between patients and controls (p= 0.000). 6.7% of the patients had no polymorphism (AA), 13.3% had heterozygous polymorphism (AC) and 80% had homozygous polymorphism at exon 7 (CC), whereas 40% of control group had no polymorphism (AA), 53.3% had heterozygous polymorphism (AC) and only 6.7% of them had homozygous polymorphism at exon 7 (CC).

Table 6: Genotype frequencies of A1298C in exon 7 among case and control groups.

<table>
<thead>
<tr>
<th>Exon-7</th>
<th>Cases (n=30)</th>
<th>Control (n=30)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>x²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>2 (6.7%)</td>
<td>12 (40%)</td>
<td>1.0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>4 (13.3%)</td>
<td>16 (53.3%)</td>
<td>0.13</td>
<td>0.04-0.48</td>
<td>16.479</td>
<td>0.000</td>
</tr>
<tr>
<td>CC</td>
<td>24 (80%)</td>
<td>2 (6.7%)</td>
<td>56.0</td>
<td>10.3-303.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Furthermore, assessment of maternal MTHFR gene polymorphism at exon 7 also revealed that there was a highly significant difference between mothers of patients and mothers of the control group (p=0.000). 6.7% had no polymorphism (AA), 13.3% had heterozygous polymorphism (AC) and 80% of them had homozygous polymorphism at exon 7 (CC) On the other
hand, 46.7% of control mothers had no polymorphism (AA) and 46.7% had heterozygous polymorphism (AC) and only 6.7% had homozygous polymorphism at exon 7(CC).

**Discussion**

The etiology of non-syndromic CHD is a multifactorial complex and results from interaction between genetic susceptibility and environmental stimulus [16, 17]. As the mother is the environment of the child in utero, maternal environmental exposures, such as the intake of vitamins, medicines, and smoking, influence the organ development of the unborn child as well [18]. The impact of folic acid intake on pregnancy outcome is modified by variants in both maternal and fetal genes that code for critical enzymes in the folate and homocysteine pathways.

Folate acts as a cofactor for enzymes involved in DNA and RNA synthesis and in the supply of methyl group to the methylation cycle. Thus, folate deficiency can lead to defective cell proliferation and cell death [19].

Several studies have been carried out to find the association between conotruncal heart defects and several single nucleotide polymorphisms (SNPs) [3, 4, 18]. Consequently, the $MTHFR$ polymorphism, one cause of hyperhomocysteinemia and decreased plasma folate level, was associated with neural tube defect (NTD) [20]. As neural crest cells contribute to the outflow tract separation of the heart, folic acid intake is postulated to have a preventive effect on CHD development [21, 22].

The present study focused on two common SNPs in the $MTHFR$ gene, 677C-T (exon 4) and 1298A-C (exon 7) in both children with conotruncal heart defect, and their mothers.

In this work, we found no statistically significant difference between cases and controls as regards the presence of homozygous or heterozygous gene polymorphism of the $MTHFR$ gene at exon 4, or maternal $MTHFR$ gene polymorphism at exon 4 C677T. These results were in agreement with those reported by Shaw et al. (2005)[4] and Goldmuntz et al. (2008) [23] who reported non significant difference between patients and controls as regards the presence of homozygous or heterozygous gene polymorphism of the $MTHFR$ gene at exon 4. Storti et al. (2003) [3] also reported no association between maternal $MTHFR$ gene polymorphism at exon 4 C677T $MTHFR$ genotype and conotruncal heart defects.

On the other hand Junker et al. (2001) [24] in similar study concluded that there was a risk between maternal homozygous polymorphism at exon 4 (TT) and occurrence of conotruncal heart defects, and Goldmuntz et al. (2008) [23] also reported that maternal $MTHFR$ polymorphism at exon 4 was associated with a moderate increase in the risk of CTD.

Studies have found that impaired folate and homocysteine metabolism affects neural crest cells formation and migration leading to defect in trunco-conal septum and mal alignment of outflow tract, resulting in CTDs [25]. Since $MTHFR$ polymorphism affects folate and homocysteine metabolism, the presence of such polymorphism can result in impaired folate metabolism, and a resultant defect in neural crest cell formation and migration, and subsequent CTD formation.

Contrary to our results, Storti et al., (2003) and Goldmuntz et al. (2008) [3, 23], found non significant association between A1298C genotype and conotruncal heart defects. They also reported that although there was no association between C677T $MTHFR$ genotype or A1298C genotype and conotruncal heart defects, the frequency of C677T polymorphism was much higher than the A1298C polymorphism. Moreover the Italian population demonstrated a higher frequency of C677T polymorphism than some other European countries.

Nutritional history, as a possible risk factor, was considered in details to evaluate folate status of patients mothers in relation to control mothers. Our results showed that folic acid intake was significantly lower than the Recommended Daily Allowance, which is 400 $\mu$g/d in case mothers compared to control mothers. This observation supports the hypothesis that an impaired folate and/or homocysteine metabolism interferes with the developing heart, possibly by affecting neural crest cells. In vitro studies found that an impaired folate and homocysteine metabolism affect the neural crest cells formation and migration [1, 26].

Tang et al.( 2004) [27] observed that reduced availability of folate by inactivating the folate transporter, Folbp1, in mice leads to an extensive reduction of migrating cardiac neural crest cells. The authors observed neural crest cell-associated CHD, including the improper septation, persistent truncus arteriosus, and double-outlet right ventricle.

Castro et al.,(2004) [28] suggested that the 1298CC $MTHFR$ genotype, independently of folate
availability, might be potential risk factors for disease states associated with DNA hypomethylation status including cancer. Moreover, the involvement of the 1298A>C variant in the development of neural tube defects has only been considered in a few studies which report a tendency towards a higher frequency in neural tube defective children [29].

To summarize, our results showed that there was an association between MTHFR polymorphism at exon 7 and occurrence of CTDs in cases and their mothers but there was no such association at exon 4. Our findings differ from other studies (ethnic variations) who either reported an association between maternal MTHFR polymorphism at exon 4 and the occurrence of CTD [24, 23] or no association between CTD and exon 4 or exon 7 MTHFR gene polymorphism [3].

This marked variation of previous results can be explained by the work of Wilcken et al. (2003) [30] who found differences in geographic distribution of the C677T polymorphism. The TT genotype was low among newborns of African ancestry, intermediate among newborns of European origin, and high among newborns of American Hispanic ancestry. On the other hand a study by Meguid et al. (2008) [31] on Egyptian mothers with Down syndrome revealed that the frequency of A1298C polymorphism was more than that of C677T. These findings suggested that the existence of marked variation in gene polymorphism was related to race.

The effect of such MTHFR polymorphism can also be affected by many factors such as maternal life style, nutritional status, and drug intake in pregnancy which means gene-environment interaction and also gene-gene interaction [22, 32].

These factors can explain the differences in MTHFR polymorphism between different ethnic groups and geographical distribution. Thus we recommended that each community should study its own pattern of polymorphism.

This complexity and debates involved means that large studies are recommended to determine whether MTHFR gene polymorphism is independent risk factor for occurrence of CHD and CTD or is dependant on gene- environment interaction.

Conclusion

In conclusion, our study revealing a gene–environment interaction between the maternal MTHFR 677C.T polymorphism and periconceptional folate supplementation on the risk of CHD in offspring. We reported a significant difference between case mothers and control mothers and children regarding MTHFR polymorphism at exon 7. The question whether the MTHFR polymorphism might be per se an independent contributor to cardiovascular risk is debated. We emphasized that folate fortification as a population measure may influence the genetic selection of a potentially deleterious genotype.

References


