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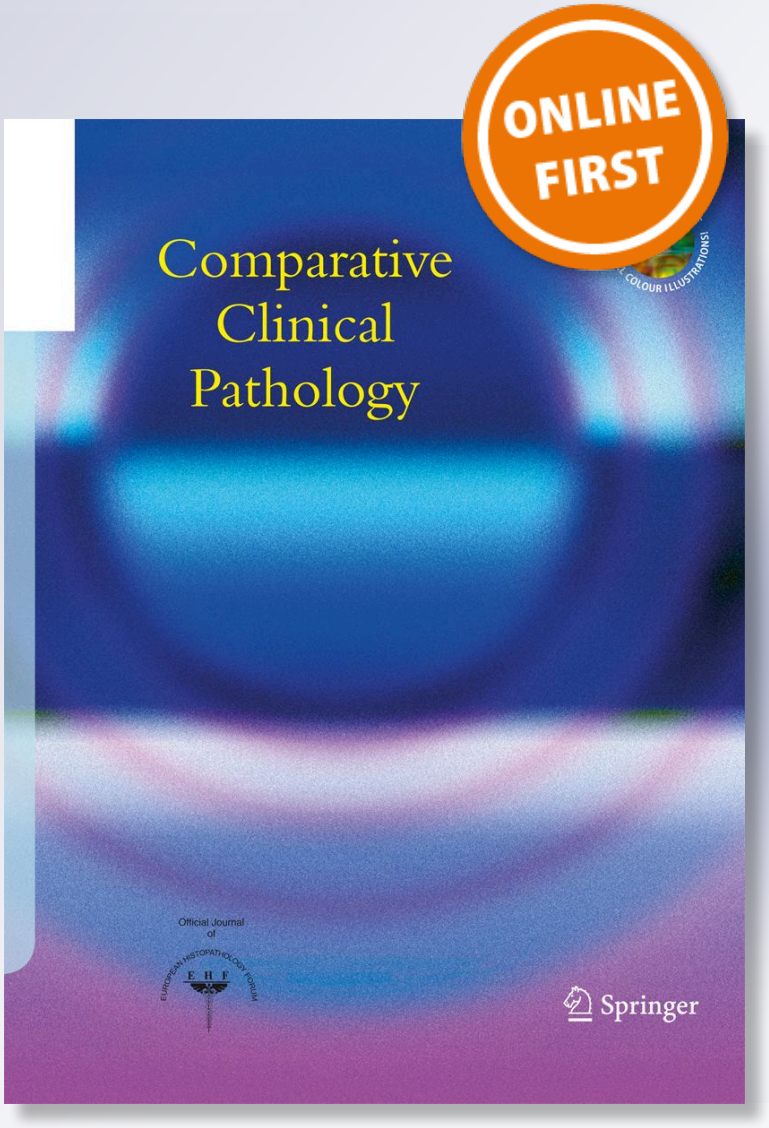

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ORIGINAL ARTICLE

Maternal and offspring methylenetetrahydrofolate reductase gene C677T polymorphism: does it influence the prevalence of congenital heart defects in Egyptian neonates?

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Abstract Congenital heart defects (CHDs) are the most prevalent heart diseases in neonates. There is evidence suggesting that the risk of CHDs may be related to the folate status as well as the genetic variants in folate-related genes. Investigating the relationship between methyltetrahydrofolate reductase (MTHFR) C677T gene polymorphism and CHDs in full-term neonates and considering the possible protective role of folate supplementation were made. This study included 26 cases, 18 controls, and their biological mothers. Echocardiography was performed to all neonates for diagnosis of the type of congenital heart disease. Mothers and their off springs were subjected to DNA analysis for MTHFR C677T using polymerase chain reaction restricted fragment length polymorphism. An association between maternal (p=0.044) and infant (p=0.001) MTHFR C677T polymorphism and transposition of great vessels (d-TGA) was found. Odds ratio (OR) for the genotypes CT and TT versus CC was 10, with 95 % confidence limits (CI) (1.05–95.23) and OR 26 with 95 % CI (2.60–259.29), respectively. Also for the genotypes CT and TT versus CC, an association was found between infant MTHFR C677T polymorphism and atrial septal defect [p=0.000; OR 36.4; 95 % CI (3.7–354.40)], ventricular septal defect [p=0.025; OR 5.2; 95 % CI (1.2–23.04)], as well as patent ductus arteriosus [p=0.000; OR 33.8; 95 % CI (3.5–330.62)]. Maternal folic acid supplementation proved protective against CHDs. MTHFR C677T polymorphism is associated with certain subgroups of CHDs.

Keywords Congenital heart defects · Folic acid · Methyltetrahydrofolate reductase gene polymorphism MTHFR C677T

Introduction

Congenital heart defects (CHDs) are the most prevalent heart diseases in infants and children. They include mainly atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA), tetralogy of Fallot, and other types. With the exception of a few types of CHDs induced by single gene mutation and chromosome aberration, the majority of CHDs are polygenic diseases affected by both genetic and environmental factors. Periconceptional supplement of multiple vitamins could significantly reduce the incidence of CHDs and neural tube defects (Botto et al. 1996; Berry et al. 1999).

Homocysteine (Hcy) is a type of thio alcohol amino acid, which is the metabolic product of methionine remethylation and transsulfuration. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is the key metabolic enzyme of Hcy metabolism; it catalyses 5,10-methylenetetrahydrofolate reduction to 5-methyltetrahydrofolate which as a methyl donor induces Hcy remethylation to methionine. The MTHFR gene C677T variation could influence enzyme activity (Junker et al. 2001; Wenstrom et al. 2001; Liu et al. 2002). Deficiency of MTHFR could reduce synthesis of 5-methyltetrahydrofolate,

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Table 1 MTHF genotype distribution in the two studied groups

Laboratory findings		Cases $N=52$	Control $N=36$	p value
Neonatal genotypes	CC	(7/26) 27 %	(13/18) 72 %	0.005*
	CT	(12/26) 46 %	(5/18) 28 %	
	TT	(7/26) 27 %	–	
Maternal genotypes	CC	(10/26) 38 %	(9/18) 50 %	0.567
	CT	(15/26) 58 %	(9/18) 50 %	
	TT	(1/26) 4 %	–	

*significant p value

interrupt the Hcy remethylation to methionine, and induce hyperhomocysteinemia (Storti et al. 2003; McBride et al. 2004; Hobbs et al. 2006).

Elevated Hcy increases the risk for neural tube, neural crest, craniofacial, and congenital heart defects in the offspring (Boot et al. 2003; Tang et al. 2004; Huhta and Hernandez-Robles 2005). The association with elevated Hcy is particularly strong for specific outflow tract defects, including pulmonary valve stenosis, coarctation of the aorta, and aortic valve stenosis Huhta and Hernandez-Robles (2005). Experimental studies performed by Tang and colleagues observed that reduced availability of folate by inactivating the folate transporter, Folp1, in mice leads to an extensive reduction of migrating cardiac cells(Tang et al. 2004). Folic acid (FA) intake around the time of conception reduces the risk of neural tube defects in the newborn (De Wals 2007; Pitkin 2007). Measures to increase intake of FA in this period include multivitamin supplementation and fortification of grain products such as flour and pasta (Honein et al. 2001).

A study performed by Ionescu and coworkers in 2009 reported that fortification of grain products with folic acid in Canada was followed by a significant decrease in the birth prevalence of severe congenital heart defects. The average prevalence of severe congenital heart defects in the 9 years before fortification was higher than the average birth prevalence in the period after fortification. The time trend analysis showed that there was no change in the birth prevalence of severe congenital heart defects in the period before fortification, while the period after the mandatory fortification of flour and pasta with FA was accompanied by a 6.2 % decrease per year in birth prevalence.

Van Beynum et al. in 2010 found that additional periconceptional folic acid use reduces CHD risk in infants. The use of periconceptional folic acid supplements was related to approximately 20 % reduction in the prevalence of any CHD. Hernández-Díaz in 2000 found that the use of folic acid antagonists in early pregnancy may increase the risk of cardiovascular defects, oral clefts, and urinary tract defects, particularly among the infants of women who do not use a multivitamin containing FA. This could be explained by the fact that FA induces cardiac neural crest formation and migration. Hyperhomocysteinemia, which result from unavailability of folic acid, repress and delay the induction of Hex and Islet-1. Those genes are associated with the induction of cardiogen.

Aim of the work

The purpose of this work was to study the possible association between maternal and offspring MTHFR gene C677T polymorphism and the risk of congenital heart defects. Also, it aimed at detecting the protective role of FA against CHDs in the presence of that polymorphism.

Subjects and methods

This prospective study included 26 full-term neonates suffering from congenital heart disease and their biological mothers, as well as 18 full-term apparently healthy controls and their biological mothers. Cases were recruited from the neonatal intensive care unit of the Cairo University Children's Hospital and of the Cairo University Obstetrics and Gynecology Hospital. Control subjects were recruited from the well baby clinic of the Cairo University Children's Hospital, Center for Preventive and Social Medicine. The study was approved by the ethical committee of the Cairo University Children's Hospital. Exclusion criteria are the following: neonates with congenital heart disease associated with chromosomal anomalies and genetic syndromes, premature infants (<37 weeks gestation), and maternal diabetes, malabsorption, wasting syndromes, or any condition associated with folate deficiency were all excluded from this study.

Cases and controls were subjected to detailed history taking including: maternal age, parity, medical illnesses, supplemental mineral or vitamin intake, any performed tests of fetal well-being, premature rupture of membranes, mode of delivery, and intrapartum monitoring.

Table 2 Frequency distribution of MTHFR C677T alleles in studied groups

MTHFR C677T alleles		Cases	Controls	p value	OR (95 % CI)
Neonatal	*C* allele ($n=57$)	26 (45.6 %)	31 (54.4 %)	0.000*	6.2 (2.085–18.437)
	T allele ($n=31$)	26 (84 %)	5 (16 %)		
Maternal	*C* allele ($n=62$)	35 (57 %)	27 (43 %)	0.437	–
	T allele ($n=26$)	17 (65.4 %)	9 (34.6 %)		

*significant p value

Table 3 Frequency distribution of patient genotype CT+TT versus CC in study group

Genotypes		Cases n=26	Controls n=18	OR (95 % CI)	p value
Patient	CT and TT	19/26 (73 %)	5/18 (27.7 %)	7.057 (1.835–27.144)	0.004*
	CC	7/26 (27 %)	13/18 (72.3 %)		

*significant p value

They were further examined for general appearance and features of chromosomal abnormalities or genetic syndromes. Full anthropometric measurements (weight, length, and skull circumference) were recorded as well as gestational age calculation using the new Ballard score. Cases were subjected to clinical evaluation including cardiac, chest, abdominal, and neurological examinations on admission to NICU and daily afterwards. The final diagnosis of the type of congenital heart disease was reached by full cardiac examination, routine chest X-ray, and echocardiographic study. Lab investigations included infection workup, complete blood picture, C reactive protein, and arterial blood gases measurement.

Specimen collection

Two milliliters of venous blood was collected, from the neonates and their mothers, on EDTA using a sterile vacutainer tube. The EDTA samples were stored in the same vacutainer at −70 °C to be used for performing the polymerase chain reaction (PCR) for MTHFR gene study. Genomic DNA was extracted from peripheral blood leucocytes by Genomic DNA Purification Kit (Fermentas, 798 Cromwell Park Drive, Glen Burine Maryland 21061-2594, USA)

The C677T polymorphism was genotyped by PCR–restriction fragment length polymorphism analysis. Primers, supplied by Bioneer (1000 Atlantic avenue, Alameda CA94501 USA), were designed to include two potential restriction sites in the amplified fragment: the site with the SNP and an invariant site, which was used as an internal control for the enzymatic digestion. Amplification was carried out with the primer pair 5′-GGAGCTTTGAGGCT GACCTGAA-3 (F) 5-AGGACGGTGCGGTGAGAGTG-3 (R) The PCRs were performed in the following sequence: 12.5 μl (reaction buffer, $MgCl_2$, dNTPs), 1 μl of each primer, 0.5 U of Taq DNA polymerase, 2.5 μl genomic DNA, and 7.5 μl distilled water. Cycling conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 63 °C for1 min, and extension at 72 °C for 1 min. Final elongation was performed at 72 °C for 10 min. The digestions were done with Hinf I and the fragments were separated by electrophoresis in 3 % agarose gel. After digestion, normal allele had the length of 198 bp, while mutated allele had the length of 175 bp. Concerning the bands, the subject was considered to carry:

- Normal MTHFR gene if only the band develops at 198 bp.
- Homozygous mutation of the MTHFR gene (mutant gene) if only the band develops at 175 bp.
- Heterozygous mutation of the MTHFR gene if both bands develop.

Data analysis

All results were analyzed using SPSS statistical package (version 15). Qualitative data were expressed as frequency and percentage. Association between qualitative variables was done using Pearson Chi-square and Fisher's exact test. Risk estimate was done by odds ratio. p value was considered significant at 0.05.

Results and discussion

The results of this study revealed the following: out of a total number of 26 full-term neonates, 15 neonates had VSD, 14 had PDA, 11 had transposition of the great arteries (TGA), 4 had pulmonary stenosis, 3 had coarctation of the aorta, 2 had double inlet right ventricle, and only 1 had each of the following: aortic stenosis, tricuspid regurgitation, common atrioventricular canal, and total anomalous pulmonary venous return.

There was a significant association between the neonatal genotype and the presence of CHD in general (p=0.005) (Table 1). The neonatal allele frequency also revealed a highly significant association for the T allele with an odds ratio (OR) (95 % CI)=6.2 (2.0–18.4) and a p value of 0.00 (Table 2). Further analysis of patients' genotype revealed CT and TT frequency to be 73 %, OR (95 % CI)=7.057 (1.83–27.14), with a highly significant p value (0.004) (Table 3).

Table 4 Frequency distribution of different CHDs according to maternal genotype

	Maternal genotype CC	CT and TT	p value	OR 95 % CI
ASD	3 (20 %)	12 (80 %)	0.074	
VSD	5 (33 %)	10 (66.7 %)	0.335	
PDA	3 (21 %)	11 (79 %)	0.098	
TGA	1 (9 %)	10 (91 %)	0.044	10(1.0–95.2)

Table 5 Frequency distribution of different CHD according to neonatal genotypes

	Neonatal genotype		*p* value	OR (95 % CI)
	CC	CT and TT		
ASD (*n*=15)	1 (6.7 %)	14 (93.3 %)	0.000*	36.4 (3.7–354.409)
VSD (*n*=15)	5 (33.3 %)	10 (66.7 %)	0.025*	5.2 (1.2–23.043)
PDA (*n*=14)	1 (7 %)	13 (93 %)	0.000*	33.8 (3.5–330.622)
TGA (*n*=11)	1 (9 %)	10 (91 %)	0.001*	26 (2.607–259.295)

*significant *p* value

However, there was no association between both maternal genotype and allele frequency regarding the presence of congenital anomaly in their offspring (p=0.567and p=0.437) (Tables 1 and 2). This comes in harmony with the study performed by Wintner et al. (2007) who documented the frequencies of the MTHFR genotypes (CC, CT, and TT) in mothers of cases with CHDs which matched the MTHFR genotype distribution in a healthy population, CC=52.3 %, CT=36.4 %, and TT=11.2 %. This also comes in accordance with the work done by Li et al. (2009) where 44.24 % of those having CHDs had the TT genotype, 40.38 % had the CT genotype, and 15.38 % had the CC genotype. In the control group, TT genotype comprised 18.75 %, the CT genotype 54.81 %, and the CC genotype 26.44 %. A significant genotype distribution difference was identified between the case and control group (p<0.0001). On the other hand, Van Driel et al. (2008) stated that MTHFR C677T polymorphism is not a strong risk factor for CHDs and explained their findings by the possibility that selective survival might have diluted their results.

Results revealed a significant association between neonatal CT and TT genotypes with each of the following: ASD (p=0.000), VSD (p=0.025), PDA (p=0.000), and TGA (p=0.001) (Tables 4 and 5). Similar results were obtained by Zhu et al. (2006) who found significant association between patient C677T mutations of MTHFR enzyme and ASD. They also found no significant association between maternal genotype TT, when compared to CC and CT, and occurrence of ASD in their offspring. In contrast Junker et al. (2001) found no significant association between patient C677T mutations of MTHFR enzyme and ASD; however, they did not study periconceptional folic acid supplementation.

Neonatal MTHFR CT and TT genotypes were significant risk factors for VSD occurrence (OR 5.2; 95 % CI 1.2–23.04; p=0.025). But regarding maternal genotype, the results did not reveal that CT and TT as compared to CC were significant risk factor for VSD (p=0.335). Similarly, Czeizel et al. (2004) and Botto et al. (2000) stated that VSD are folate-related as they found that VSD can be prevented by periconceptional folic acid supplements.

Neonatal MTHFR CT and TT genotypes were significant risk factor for the occurrence of PDA (OR=33.8; 95 % CI 3.5–330.622; p=0.000). However, maternal genotype results did not reveal statistical significant difference between the three types (p=0.098).

Similar results were obtained by Zhu et al. (2006) who found also that CT and TT mutations of MTHFR enzyme are risk factors for PDA. However, unlike the results of this study, they found that maternal TT genotype was associated with the occurrence of PDA in their offspring. That could be explained by a small number of maternal TT genotype in the current study. Neonatal and maternal MTHFR CT and TT genotypes were significant risk factor for the occurrence of TGA (OR 26; 95 % CI 2.6–259.2; p=0.001) and (OR 10; 95 % CI 1.0–95.2; p=0.044) respectively.

Similar results were obtained by Van Beynum et al. in 2006 who found that maternal MTHFR C677 T variants are risk factors for CHD in offspring, confined to conotruncal heart defects including tetralogy of Fallot, complex heart defects, outlet VSDs, pulmonary atresia and VSD, truncus arteriosus, TGA, and aortopulmonary window. They reported that maternal MTHFR 677 CT and TT genotypes in combination with non-use

Table 6 Frequency distribution of neonatal genotypes in cases and control regarding FA

Mothers *N*=44	Neonatal type	Cases	Controls	*p* values	OR (95 % CI)
No FA supplement	Mutated genes CT and TT *N*=9	9 (100 %)	–	0.00*	2.6 (1.4–5.0)
	Wild CC *N*=16	6 (37.5 %)	10 (62.5 %)		
FA supplement	Mutated genes CT and TT *N*=15	10 (66.7 %)	5 (33.3 %)	0.17	6 (0.4–73.4)
	Wild CC *N*=4	1 (25 %)	3 (75 %)		

*significant *p* value

of periconceptional folate supplements were associated with a threefold to sixfold increased risk for conotruncal heart defects in offspring.

Also, Goldmuntz et al. (2008) found that the maternal MTHFR 677TT genotype was associated with a moderate increase in the risk of conotruncal heart defects among offsprings. This could be explained by that MTHFR gene polymorphism C677T results in a thermo labile enzyme with reducing function, which leads to decreased availability of FA and accumulation of homocysteine.

The result of this study revealed that if a mother did not receive folic acid supplementation during the periconceptional period (which is defined as a month before and 3 months after conception), her offspring with CT or TT mutation is about 2.6 times more likely to have CHDs ($p<0.01$). Therefore, mutated genes (CT and TT) were found to be a significant risk factor for CHDs when mothers did not receive FA. However, for mothers who received periconceptional folic acid supplementation, mutant genes of their offspring (CT and TT) were not significantly associated with having CHDs ($p=0.177$) proving the protective role of FA and its importance to women during the child bearing period.

In harmony with this result and results obtained by Li et al. (2009), the low levels of FA intakes and the MTHFR 677TT genotype had a positive adding effect in the occurrence of congenital heart disease. They found that there is an interaction between folate intake and the MTHFR 677TT genotype, and that increasing the intake of folate among MTHFR 677TT genotype people might decrease the incidence rate of congenital heart disease (Table 6).

Results showed a predominance of males among the cases of 69.2 %, also, a lower proportion of C677T homozygote among females of 28.6 % compared to 71.4 % among males was encountered. Rozen et al. in 1999 similarly reported a lower proportion of C677T homozygote among newborn females than among newborn males.

A significant association between neonatal CT and TT polymorphism was found regarding history of recurrent abortions and similar conditions in siblings (p value 0.043 and 0.031, respectively). However, this association was absent between the maternal genotype and the antenatal history (Tables 7 and 8).

Similarly, a study performed by Callejón et al. in 2007 on 342 fetal tissue samples selected from spontaneous abortion (SA) revealed that the wild CC genotype of the C677T polymorphism showed a strong protective effect against abortion (0.03 in SA versus 0.47 in 1950s and 0.43 in 1980s) ($p<0.001$). They concluded that fetal viability is directly related to the CC genotype

Table 7 Frequency distribution of antenatal history data among different neonatal C677T genotypes

Antenatal history		Neonatal genotype		p value
		CC	CT and TT	
Abortion	None ($n=33$)	19 (57.6 %)	14 (42.4 %)	0.043*
	Once ($n=4$)	0	4 (100 %)	
	Twice ($n=6$)	1 (16.7 %)	5 (83.3 %)	
	Thrice ($n=1$)	0	1 (100 %)	
IUFD	None ($n=39$)	19 (48.7 %)	20 (51.3 %)	0.261
	Once ($n=3$)	0	3 (100 %)	
	Twice ($n=2$)	1 (50 %)	1 (50 %)	
Similar conditions	None ($n=37$)	20 (54.1 %)	17 (45.9 %)	0.031*
	Once ($n=2$)	0	2 (100 %)	
	Twice ($n=5$)	0	5 (100 %)	

*significant p value

Table 8 Frequency distribution of antenatal history data among different maternal C677T genotypes

Antenatal history		Maternal genotype		p value
		CC	CT and TT	
Abortion	None ($n=33$)	18 (54.5 %)	15 (45.5 %)	0.055
	Once ($n=4$)	1 (25 %)	3 (75 %)	
	Twice ($n=6$)	0	6 (100 %)	
	Thrice ($n=1$)	0	1 (00 %)	
IUFD	None ($n=39$)	18 (46.2 %)	21 (53.8 %)	0.293
	Once ($n=3$)	0	3 (100 %)	
	Twice ($n=2$)	1 (50 %)	1 (50 %)	
Similar conditions	None ($n=37$)	18 (48.6 %)	19 (51.4 %)	0.216

Conclusion

The MTHFR C677T polymorphism was associated with certain subgroups of CHDs. The infant MTHFR C677T polymorphism was associated with each of ASD, VSD, and PDA. Maternal or infant MTHFR C677T was associated with occurrence of TGA in the infants. Periconceptional folic acid supplementation is protective against CHDs.

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