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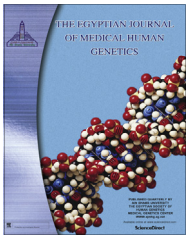

ORIGINAL ARTICLE

Maternal *MTHFR* C677T genotype and septal defects in offspring with Down syndrome: A pilot study

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KEYWORDS

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Abstract *Background:* While abnormal folate/homocysteine metabolism has been implicated as an etiology for the development of both CHD and DS, recent studies and meta-analyses did not consider *MTHFR* C677T genotype as a maternal risk factor for either of these conditions alone.

Aim of work: To investigate if methylenetetrahydrofolate reductase (MTHFR) C677T genotype is a maternal risk factor for the development of congenital heart disease (CHD) only in children with Down syndrome (DS).

Subjects and methods: Molecular analysis of *MTHFR* C677T and serum folic acid was done for sixty-one consecutive mothers of children with CHD in the form of septal defects (26 with DS and 35 without DS) and another 61 mothers of apparently healthy children (without DS or CHD).

Results: The frequency of CT genotype was significantly higher in mothers of children with AV canal (whether in DS or non-DS) when compared to ASD and in mothers of DS with AV canal when compared to controls. The frequency of TT genotype was higher in mothers of DS with ASD than controls but statistically insignificant. In non-DS mothers, the distribution of the genotypes did not differ in relation to the type of CHD. The mean folic acid level did not differ between different study groups.

Conclusions: *MTHFR* 677CT genotype could be implicated as a maternal risk factor for septal defects especially in children with DS. Carriers of this genotype may have more risk of development of AV canal in their children. A major limitations of this study was the small sample size and so further studies on a larger sample of patients and their mothers in addition to measurement of

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homocysteine level in this population is needed to investigate this theory and to clarify the actual role of MTHFR polymorphism and the risk of development of CHD in DS.

1. Introduction

Methylenetetrahydrofolate reductase (MTHFR) is one of the most important enzymes in folate/homocysteine metabolism. It is responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and regulates the intracellular flow of folate through the conversion of homocysteine to methionine for nucleotide synthesis. C677T and A1298C polymorphisms in the *MTHFR* gene affect this pathway by reducing enzyme activity and subsequently increase total plasma homocysteine and decrease serum folate levels [1,2]. In 1999, James et al. hypothesized that the MTHFR 677CT polymorphism might be a risk factor for maternal meiotic nondisjunction and Down syndrome (DS) in young mothers [3]. Since then, several studies on MTHFR polymorphism have been carried out on different populations including Egyptians [4,5]. Results were controversial, while James et al. [3], Scala et al. [6] and Rai et al. [7] reported a significant increased risk, Coppede et al. did not report a significant difference [8]. Actually, despite 10 years of active research, the question is still unsolved. Overall, both in vitro and in vivo studies indicate that an impaired folate/homocysteine metabolism can result in chromosome 21 nondisjunction; however, the birth of a DS child seems to be the result of the interplay of several factors of genetic, epigenetic, environmental, and stochastic origin, making it difficult to discriminate the single contribution of each of them [8].

MTHFR gene polymorphisms have been also implicated in the etiology of congenital heart defects (CHD) [1,9]. Previous studies showed that the overall risk of heart defects can be reduced by multivitamin supplements (including folic acid) especially in some septal defects (ventricular septal defect (VSD)) and some conotruncal anomalies (teratology of Fallot and transposition of great arteries) [10,11].

Recently, the relationship between *MTHFR* genotype and the development of CHD in DS has been investigated and data were controversial in different populations [12,13]. In fact, several genes encoding for the reduced folate carrier (*SCL19A1* or *RFC1*) and the cystathionine beta synthetase are located on chromosome 21 for which trisomies increase folate demand in the developing DS fetus. DS cases with atrioventricular septal defects (AVSD) shared significantly more alleles across *SCL19A1* than expected suggesting an association between variation of this gene and AVSD [14].

The aim of this study was to assess the relationship between the maternal *MTHFR* C677T genotype and the development of certain types of CHD as septal defects in their children with DS in our Egyptian population.

2. Subjects and methods

The study included 61 consecutive Egyptian mothers of children with septal defects (with or without DS) from June 2009 to June 2010 who did not receive folic acid supplementation in the periconceptional period (Folic acid food fortification is not a current policy yet in Egypt). All patients and mothers were recruited from the Ain Shams University Hospital which is a tertiary governmental hospital in which most of the patients are low economy class with poor diet. The hospital is the second largest hospital in Cairo and receives patients from all governorates all over Egypt. Although it is a homogenous population, it is known that the Egyptians have a mixed genetic background with an ethnic heterogeneity with most of the population of Mediterranean or Arabic origin. The study group was in Hardy–Weinberg equilibrium. The population was not chosen from any isolates or members from the same family.

Exclusion criteria included women who started to use folate supplements after known that they were pregnant, mothers of children with other congenital anomalies, a recognized genetic syndrome or chromosome anomalies other than DS and mothers receiving antiepileptic drugs (valproic acid, phenytoin, carbamazepine) since all can alter folic acid metabolism and increase the risk of congenital anomalies.

The CHD included only septal defects: atrial septal defects (ASD), venricular septal defect (VSD), ASD + VSD, and atrioventricular (AV) canal. Diagnosis of septal defects was made clinically and confirmed by echocardiography done by an expert pediatric cardiologist. They were subdivided into 2 groups:

- *Group 1 (mothers of children with DS)*: mothers of karyotypically ascertained non-disjunction DS children with septal defects from the Genetics Clinic, Children's Hospital, Ain Shams University ($n = 26$)
- *Group 2 (mothers of non-DS children)*: mothers of children with septal defects from the Cardiology Clinic, Children's Hospital, Ain Shams University ($n = 35$).

Sixty-one women serving as controls were mothers of apparently healthy children (without DS or CHD) who did not receive folic acid supplementation in the periconceptional period. The study was approved by the ethical committee of the institute and informed consent was obtained from all mothers. The work was carried out in accordance with the code of ethics of the World Medical Association (declaration of Helsinki) (http://www.wma.net/en/30publications/10policies/b3/index.html).

3. Methods

3.1. Molecular method

Genomic DNA was extracted using QIAamp DNA extraction kit (Cat ≠ 51104). Polymerase chain reaction (PCR) was performed according to the method described by Frosst et al. [1]: Two primers were used for analysis of the C-T mutation.

MTHFR F: 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and MTHFR R: 5′-AGG ACG GTG CGG TGA GAG TG-3′:

One μL of genomic DNA was amplified in a 50 μL reaction volume containing 5 μl of 10× buffer (100 mM; Tris–HCl, 500 mM KCL, $(NH_4)_2SO_4$, 15 mM; $MgCL_2$, pH = 8.7), 0.2 mM each dNTPs, 0.4 μM primers, and one unit of Taq DNA polymerase enzyme (Qiagen, cat#20123). The PCR consisted of initial denaturation at 94 °C for 150 s followed by 35 cycles at 94 °C for 30 s, 57 °C for 60 s, 72 °C for 120 s and a final elongation step at 72 °C for 3 min. The PCR product of 198 bp was analyzed using 100 bp ladder on 1.5% agarose gel electrophoresis. Twenty μl of PCR product was mixed with 10 units of Hinf1 (Promega, Madison) enzyme and incubated at 37 °C overnight. The substitution creates a Hinf1 recognition sequence that is digest of 198 bp into 175-bp and 23-bp. To ensure quality control, the presence of C > T polymorphism was performed with blinding to case/control status, non-template control and positive/amplification genotype control was included within each experimental PCR run. Ten percent sample cases and controls were genotyped twice and reproducibility was 100%.

3.2. Serum folic acid level

Whole blood was collected after overnight fast for all subjects in a vacutainer tube without anticoagulant. Samples were left undisturbed at 4–8 °C until complete clot formation was observed. Clotted samples were then centrifuged at 2000*g* for 10 min. Serum was collected and aliquottes were stored at −70 °C until analyzed. The level of serum folic acid was quantified using competitive chemiluminescent immumoassay (IMMULITE Analyzer, Cat#LKFO1, SIMENS), [15].

3.3. Statistical methods

Data were expressed as mean ± SD (range) or as number (%) of cases. Comparison of proportions and means between both groups was made by using the chi-square test and independent *t*-test, respectively. The Fisher's exact test was used when applicable. ANOVA (Analysis of variance) was used to test the difference about mean values of folic acid among groups, multiple comparisons between pairs of groups were performed using Bonferroni test (Post hoc range test). Results were presented as mean and SD. Analysis was performed by using the Statistical Package for the Social Sciences (SPSS, version15). The level $P < 0.05$ was considered the cut-off value for significance.

4. Results

The mean age of DS mothers was 30.5 ± 6.39 years (range: 20–40), non-DS mothers 29.4 ± 5.94 years (range: 22–42) and controls was 28.9 ± 6.69 years (range: 18–45). Septal defects included: ASD in 14 children (7 DS and 7 non-DS), VSD in 19 (3 DS and 16 non-DS), ASD + VSD in 14 (7 DS and 7 non-DS), AV canal in 10 (5 DS and 5 non-DS) and 4 patients with PFO (2 DS and 2 non-DS).

Although the frequencies of *MTHFR* C677T genotypes did not show statistically significant difference between different study groups, none of the mothers of patients with non-DS had the TT genotype, (Table 1).

The mean folic acid level did not show significant difference between mothers of DS (13.87 ± 4.71 ng/ml) and the controls (13.75 ± 5.32 ng/ml) or mothers of non-DS (12.35 ± 4.06 ng/ml). It did not also show a significant difference in relation to *MTHFR* C677T genotypes, (Table 2).

In comparing all mothers to controls, mothers of patients with ASD did not show a significance difference regarding the CT or the TT genotypes compared to controls ($P = 0.10$ (OR, 0.25, 95%CI: 0.05–1.25) and $P > 0.99$, (OR, 0.86, 95%CI: 0.15–4.82), respectively). The same was found in mothers of patients with VSD , ASD + VSD, and AV canal who showed non-significant difference of CT genotype compared to controls ($P = 0.60$, 0.77 (OR, 1.39, 95%CI: 0.49–3.96), (OR, 1.25, 95%CI: 0.39–4.06) and $P = 0.08$ (OR, 5, 95%CI: 0.97–25.8), respectively). Interestingly, it was noted that the 80% of patients with AV canal has the CT genotype (whether in DS or non-DS). It was significant when compared only to mothers of ASD ($P = 0.008$, OR 20, 95% CI: 2.29–175.04). This means that those mothers with CT genotype have more risk to have a baby with AV canal than to have a baby with ASD, (Table 3).

Table 1 Comparison of frequencies of *MTHFR* C677T genotypes between different study groups.

	MTHFR C677T genotype		
	CC[a]	TT	CT
Study groups			
Mothers of DS, *N* (%)	11 (42.3)	3 (11.5)	12 (46.2)
Mothers of non-DS, *N* (%)	19 (38.8)	0 (0)	16 (40)
Controls, *N* (%)	30 (49.2)	7 (11.5)	24 (39.3)
DS versus controls			
OR (95%CI)		1.17 (0.26–5.34)	1.36 (0.51–3.63)
P		>0.99	0.62
Non-DS versus controls			
OR (95%CI)		0.81 (0.69–0.95)	1.05 (0.45–2.47)
P		0.08	>0.99
DS versus non-DS			
OR (95%CI)		0.79 (0.60–1.03)	0.77 (0.27–2.22)
P		0.07	0.79

[a] Reference category, Chi-square test, Fisher's Exact Test.

Table 2 Comparison between *MTHFR* C677T genotypes in relation to the level of folic acid in different studied groups.

MTHFR C677T genotype					
Folic acid (ng/ml) Mean ± SD	CC Mean ± SD	TT Mean ± SD	CT Mean ± SD	*F*	*P*
Mothers of DS	13.08 ± 4.64	13.20 ± 3.81	14.88 ± 5.31	0.34	0.72
Mothers of non-DS	11.33 ± 3.14		14.23 ± 5.11	1.58	0.13
Controls	11.36 ± 3.68	20.23 ± 7.55	14.98 ± 4.91	6.95	0.003

The differences in mean level in groups were tested using one-way analysis of variance. Multiple comparisons were performed using Bonferroni test.

Table 3 *MTHFR* C677T genotypes in relation to different septal defect types in all mothers in the study group.

		All mothers, *N* (%)				Controls, *N* (%)
		ASD (14)	VSD (19)	ASD + VSD (14)	AV canal (10)	
MTHFR C677T genotype	CC[a]	10 (71.4)	9 (47.4)	7 (50)	2 (20)	30 (49.2)
	TT	2 (14.3)	0 (0)	0 (0)	0 (0)	7 (11.5)
	CT	2 (14.3)	10 (52.6)	7 (50)	8 (80)	24 (39.3)

[a] Reference category. Chi-square test with Bonferroni correction (level of significance $\leqslant 0.01$).

Table 4 *MTHFR* C677T genotype in relation to type of septal defect in mothers of children with DS.

		Type of Septal defects, *N* (%)				Controls, *N* (%)
		ASD (7)	VSD (3)	ASD + VSD (7)	AV canal (5)	
MTHFR C677T genotype	CC	3 (42.9)	2 (66.7)	4 (57.1)	0 (0)	30 (49.2)
	TT	2 (28.6)	0 (0)	0 (0)	0 (0)	7 (11.5)
	CT	2 (28.6)	1 (33.3)	3 (42.9)	5 (100)	24 (39.3)

Chi-square test with Bonferroni correction (level of significance $\leqslant 0.01$).

On comparing mothers of DS to the controls, the frequency of CT genotype was also significantly higher in mothers of DS with AV canal ($P = 0.01$, OR: 1.21, 95%CI: 1.02–1.43). On the other hand, the frequency of the CT genotype did not show a significant difference in mothers of DS with ASD, VSD, and ASD + VSD compared to controls ($P > 0.99$ (OR, 0.83, 95%CI: 0.13–5.40), $P > 0.99$ (OR, 0.63, 95%CI: 0.05–7.31) and (OR, 0.94, 95%CI: 0.19–4.60), respectively).

This statistically insignificant relation was also noted in the frequency of TT genotype but an interesting high OR value was noted in mothers of DS and ASD (2.86) so we may say that DS mothers with this genotype had more risk to have children with ASD, although this relation is weak as CI is only 0.4 ($P = 0.28$, OR: 2.86, 95%CI: 0.4–20.5), (Table 4). On the other hand, in mothers of non-DS, the frequency of *MTHFR* 677CT genotype did not differ in relation to the type of septal defects (data not shown).

5. Discussion

The precise role of folate during cardiac morphogenesis remains unclear. Biochemically, folate is a one-carbon donor, as such involved in many important cellular reactions, including the synthesis of nucleotides and methyl transfer reactions important for methylation of DNA, proteins, and lipids [16]. In 1999, Jill James and colleagues reported that a particular variant (polymorphism) of *MTHFR* might be a maternal risk factor for having a child with DS and that a significant increase in plasma homocysteine levels exists in mothers of these children. This genotype is known to decrease the activity of *MTHFR* enzyme [3]. Since then, several studies have been carried out on DS children from different populations including Egypt which supported an independent contribution of the *MTHFR* C677T allele to DS risk [4]. Whether these patients had CHD or not was not reported. Precise data about the frequency and the type of CHD were not reported as well in other studies (as researchers included all types of CHD collectively) [3,6,7,17].

Our study included only mothers of children with septal defects (DS and non-DS). It showed that mothers of DS had insignificant higher frequencies of both *MTHFR* 677 CT and TT genotypes compared to mothers of non-DS and higher frequencies of CT genotype compared to controls. Brandalize et al. reported an influence of *MTHFR* polymorphism on the occurrence of CHD in children with DS although they did not specify the type of CHD that affected 37.6% of their patients [12]. However, Bozovic et al. could not support these data in Croatian DS population [13].

Because children with DS in this study had septal defects, it could be suggested that *MTHFR* genotype could be an important risk factor in this particular type of children (DS + septal defect). The implicating factor could be more than simply the folic acid level as mean serum level was comparable in all groups including the controls. It is known that serum/plasma folate level is a very useful indicator to diagnose deficient or excessive status and to estimate the needed additional folate intake to reach specific cutoff points associated with reducing

abnormalities in populations. The 15.9 nmol/L (7. 016 ng/ml) level was suggested as a cut off value required to reduce neural tube defect occurrence above the plateau rate for "normal" populations [18]. Only one DS mother had this low level (6.3 ng/ml) that had *MTHFR* CC genotype. Differences in the level of susceptibility of individuals to potentially adverse effects of environmental influences due to *MTHFR* genotype may be an important contributor to the presence or absence of this gene-disease association. Beside the chromosomal anomaly, maternal risk factors may interact with susceptible genotype resulting in more pronounced malformations in some individuals [19].

Several genes participating in folate/homocysteine metabolism are located on chromosome 21 and therefore over-expressed in DS. This includes *SCL19A1* gene which was found to be associated with AVSD [14].

We also noted that 80% of mothers of all patients with AV canal had the CT genotype but it was statistically significant only on DS mothers (all mothers had the CT genotype). Reutter et al. reported a mother and her child who had DS and AV canal, both were homozygous to *MTHFR* 677TT genotype [20]. These interesting findings should be further investigated by a larger scale study on more children with DS and various CHD especially that AV canal is a neural crest related defect, but ASD is not related to neural crest [21,22] which contradicts the previous belief that only neural crest related disease is affected by folic acid metabolism [23].

A major limitation of this work was the lack of assessment of fetal *MTHFR* C677T genotypes. It is recognized that the maternal genetic effect can be confounded by the fetal genetic effect and that models considering one should adjust for the other.

6. Conclusions

MTHFR 677CT genotype could be implicated a maternal risk factor for septal defects especially in children with DS. Carriers of this genotype may have more risk of development of AV canal in their children. A major limitations of this study was the small sample size and so further studies on a larger sample of patients and their mothers in addition to measurement of homocysteine level in this population is needed to investigate this theory and to clarify the actual role of MTHFR polymorphism and the risk of development of CHD in DS.

Conflict of interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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References

[1] Frosst P, Blom H, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.

[2] van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998;62:1044–51.

[3] James SJ, Pogribna M, Pogribny IP, Melynk S, Jean Hine R, Gibson JB. Abnormal folate metabolism and mutation in the MTHFR gene may be maternal risk factors for Down syndrome. Am J Clin Nutr 1999;70:495–550.

[4] Abdel Meguid N, Dardir AA, Khass M, El Hossieny L, Ezzat A, El Awady MK. MTHFR genetic polymorphism as a risk factor in Egyptian mothers with Down syndrome children. Dis Markers 2008;24:19–26.

[5] Shawky RM, Elsobky E, Elsayed SM, Kama H, Eðin Y, Akar N. Maternal risk factors in young Egyptian mothers of Down syndrome. Egypt J Med Hum Genet 2009;10:144–52.

[6] Scala I, Granese B, Sellitto M, Salomè S, Sammartino A, Pepe A, et al. Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring. Genet Med 2006;8:409–16.

[7] Rai AK, Singh S, Mehta S, Kumar A, Pandey LK, Raman R. MTHFR C677T and A1298C polymorphisms are risk factors for Down's syndrome in Indian mothers. J Hum Genet 2006;51:278–83.

[8] Coppede F. The complex relationship between folate/homocysteine metabolism and risk of Down syndrome. Mutat Res 2009;682:54–70.

[9] Bayley LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. Am J Clin Nutr 2005;81:1213S–7S.

[10] Czeizel AE. Periconceptional folic acid containing multivitamin supplementation. Eur J Obstet Gynecol Reprod Biol 1998;78:151–61.

[11] Botto LD, Mulinare J, Erickson JD. Occurrence of congenital heart defects in relation to maternal multivitamin use. Am J Epidemiol 2000;151:878–84.

[12] Brandalize AP, Bandinelli E, Dos Santos PA, Roisenberg I, Schüler-Faccini L. Evaluation of C677T and A1298C polymorphisms of the MTHFR gene as maternal risk factors for Down syndrome and congenital heart defects. Am J Med Genet A 2009;149A:2080–7.

[13] Božović IB, Vraneković J, Cizmarević NS, Mahulja-Stamenković V, Prpić I, Brajenović-Milić B. MTHFR C677T and A1298C polymorphisms as a risk factor for congenital heart defects in Down syndrome. Pediatr Int 2011;53:546–50.

[14] Locke AE, Dooley KJ, Tinker SW, Cheong SY, Feingold E, Allen EG, et al. Variation in folate pathway genes contributes to risk of congenital heart defects among individuals with Down syndrome. Genet Epidemiol 2010;34:613–23.

[15] Turgut B, Kaya M, Arslan S, Demir T, Güler M, Kaya MK. Levels of circulating homocysteine, vitamin B6, vitamin B12 and folate in different types of open-angle glaucoma. Clin Intervent Aging 2010;5:133–9.

[16] Rosenblatt DS. Inherited disorders of folate transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. Disorders of transsulfuration. McGraw-Hill: New York; 1995, pp. 3111–3128.

[17] Coppedè F, Marini G, Bargagna S, Stuppia L, Minichilli F, Fontana I, et al. Folate gene polymorphisms and the risk of Down syndrome pregnancies in young Italian women. Am J Med Genet A 2007;143A:1015–7.

[18] Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. Folate levels and neural tube defects. Implications for prevention. JAMA 1995;274:1698–702.

[19] Shapiro BL. Down syndrome – A disruption of homeostasis. Am J Med Genet 1983;14:241–69.

[20] Reutter H, Betz RC, Ludwig M, Boemers TM. *MTHFR* 677TT genotype in a mother and her child with Down Syndrome,

atrioventricular canal and exstrophy of the bladder: implications of a mutual genetic risk factor? Eur J Pediatr 2006;165:566–8.
[21] Kirby ML, Turnage KL, Hays B. Characterization of conotruncal malformations following ablation of cardiac neural crest. Anat Rec 1985;213:87–93.
[22] Besson WT, Kirby ML, Van Mierop LHS, Teabeaut II JR. Effects of the size of lesions of the cardiac neural crest at various embryonic ages on incidence and type of cardiac defects. Circulation 1986;73:360–4.
[23] Czeizel AE, Dobo M, Vargha P. Hungarian cohort-controlled trial of periconceptional multivitamin supplementation shows a reduction in certain congenital abnormalities. Birth Defects Res A 2004;70:853–61.