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Interaction between *MTHFR* Polymorphisms and Maternal Age Increases the Risk of Congenital Heart Defects in Down Syndrome

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SJC and KMAH presented the concept of the study. SJC and AMA designed methodology. AHAM and MKAH did data curation. SJC wrote the manuscript. KMAH reviewed and edited the manuscript.

Key words
Congenital heart disease, Down syndrome, Methylenetetrahydrofolate reductase, Polymorphism, Maternal age, Saudi Arabia.

ABSTRACT

Congenital heart disease (CHD) is responsible for one-third of all congenital anomalies in newborns and is the most frequent cause of infant deaths. Several cohort studies show that down syndrome (DS) and CHD are associated, and maternal hyperhomocysteinemia is an independent risk factor for CHD. In Saudi Arabia CHD represents one of the most important health problems. Here we examined the association between methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms and CHD and DS co-inheritance in patients from Al Madinah, Saudi Arabia. *MTHFR* rs1801133 and rs1801131 polymorphisms were genotyped in 99 CHD patients with or without DS and 126 ethnically matched controls by allelic discrimination. Of 99 patients with CHD, 26 had DS. *MTHFR* rs1801133 and rs1801131genotypes and alleles were not significantly different between controls and CHD patients. Further, in CHD individuals, these genotypes failed to show any significant association with DS. However, maternal age increased the risk of CHD in DS (OR=5.32; 95% CIs 1.43-19.82; p=0.013). Mantel-Haenszel analysis showed that *MTHFR* polymorphisms confounded the effect of maternal age CHD in DS. *MTHFR* polymorphisms appear to be risk factors for CHD in DS.

INTRODUCTION

Congenital heart disease (CHD) comprises one-third of all congenital anomalies in newborns and is the most frequent cause of infant deaths. Long-term morbidity and mortality from CHD pose a substantial challenge to healthcare systems globally (Dolk *et al*., 2011). The exact etiology of CHD remains uncertain, the disease is heterogeneous, but it is believed to be multifactorial: environmental factors contribute (Gladki *et al*., 2015; Gorini *et al*., 2014; Huhta and Linask, 2013); and twin studies suggest familial aggregation of CHD (Kuo *et al*., 2017). Specific recurrence patterns and phenotypes also suggest that genetically-determined developmental mechanisms exert a strong influence in CHD etiology (Ellesøe *et al*., 2018).

Several cohort studies have established a relationship between Down syndrome (DS) and CHD (Ferencz *et al*., 1989; Kidd *et al*., 1993), with 4%-10% of all CHD cases associated with DS and 40%-60% of DS patients having CHD (de Rubens-Figueroa *et al*., 2003). Several lines of evidence indicate that maternal hyperhomocysteinemia is an independent risk factor for CHD (Malik *et al*., 2017), and recent population studies in humans have indicated that folic acid supplementation before and/or during pregnancy can decrease CHD risk (Czeizel *et al*., 2015; Leirgul *et al*., 2015; Li *et al*., 2013). This evidence prompted several follow-on studies examining the role of folic acid in CHD risk (Parnell and Correa, 2017; Xu *et al*., 2016; Feng *et al*., 2015).

Folates serve as cofactors for nucleotide synthesis and co-substrates of DNA methyltransferases (Stover and Field, 2011). DNA methylation plays a key role in embryonic development, and abnormally methylated genes have been detected in fetuses with both isolated and syndromic heart malformations (Serra-Juhe *et al*., 2015). Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) is a key enzyme in folate and homocysteine (Hcy) metabolism (Bhaskar *et al*., 2011). The gene encoding human *MTHFR* is known to have two important functional polymorphisms, C677T/A222V/rs1801133 and A1298C/Glu429Ala/rs1801131, which generate a thermolabile form

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of MTHFR that subsequently increases total plasma Hcy and decreases serum folate levels (Goyette *et al*., 1994). A recent computational modeling study demonstrated that the rs1801133 polymorphism causes conformational changes in the tertiary structure of MTHFR by reducing its FAD-binding ability (Abhinand *et al*., 2016). However, results from studies examining the relationship between *MTHFR* rs1801133 and rs1801131 polymorphisms and CHD risk have been inconsistent (Zhang *et al*., 2018; Asim *et al*., 2017; Yu *et al*., 2017; Guo *et al*., 2017). We recently highlighted the high burden of DS and CHD in the Al Madinah region of Saudi Arabia (Abdulhadi *et al*., 2016). Here we investigated associations between *MTHFR* polymorphisms (rs1801133 and rs1801131) and the risk of CHD and DS co-occurrence in a Saudi population.

PATIENTS AND METHODS

Ethical statement

The Institutional Ethics Committees of Maternity and Children Hospital and Centre for Genetics and Inherited Diseases (CGID), Taibah University, Al Madinah, Kingdom of Saudi Arabia granted ethical approval. As many of the subjects in the CHD group were minors (<18 years old), written informed consent was obtained from the parents or legal guardians of the children enrolled in this study as necessary.

Study population

Ninety-nine CHD patients with (n=26) or without (n=73) DS attended the Pediatric Cardiology Clinic at the Maternity and Children Hospital, Al Madinah, Kingdom of Saudi Arabia were enrolled in this study. Participants with mosaic, translocation of chromosome 21, non-cardiac congenital anomalies were excluded from this study. The main inclusion criteria for DS was cases had full trisomy 21 confirmed by karyotype and/or Fluorescent *in situ* Hybridization (FISH) for chromosome 21. CHD and CHD in DS samples were collected after checking the family history, physical and clinical examination, electrocardiograms (ECG), chest x-rays, 2D-echocardiography, Karyotypes or FISH data from the patient's case sheet. One hundred and twenty-six healthy subjects without CHD or a family history of CHD were recruited as controls. All participants were of Saudi Arabian ethnicity.

Genotyping

Two ml of whole peripheral blood was collected from each participant after obtaining informed consent. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Two *MTHFR* SNPs [rs1801133: C___1202883_20 and rs1801131: C____850486_20] were genotyped using primers and probes purchased from Applied Biosystems, Foster City, CA. Reactions were carried out in a final volume of 5 µL (2.5 µL TaqMan PCR Master Mix, 0.125 µL TaqMan Genotyping Assay Mix, 1.375 µL nuclease free water, and 1 µL of 10 ng/µL DNA) in a 96-well optical microplate (Applied Biosystems). At least two no-template controls without any DNA were included on each plate. The PCR conditions were initial denaturation at 95°C for 10 min followed by 40 denaturation cycles (95°C for 15 s) and annealing/extension (60°C for 1 min). PCR amplification was performed using Applied Biosystems' StepOnePlus Real-Time PCR system, and fluorescence was also measured using its

Table I.- The distribution of *MTHFR* genotypes in control and CHD subjects.

SNP	Genotype	Control (n=126)	CHD (n=99)	OR (95% CI)	*p*-value
rs1801133	CC	88 (69.8)	69 (69.7)	Reference	1.0*
	CT	33 (26.2)	26 (26.3)	0.90 (0.48-1.75)	
	TT	5 (4.0)	4 (4.0)	0.85 (0.21-3.46)	
	CT+TT Vs. CC	38 (30.2)	30 (30.3)	0.93 (0.52-1.67)	0.810
	TT Vs. CC+CT	5 (4.0)	4 (4.0)	1.01 (0.26-3.97)	0.990
	MAF	17.1	17.2		
	HWp	0.402	0.445		
rs1801131	AA	57 (45.2)	51 (51.5)	Reference	0.618*
	AC	52 (41.3)	35 (35.4)	0.73 (0.40-1.33)	
	CC	17 (13.5)	13 (13.1)	0.82 (0.34-1.94)	
	AC+CC Vs. AA	69 (54.7)	48 (48.5)	1.29 (0.75-2.20)	0.360
	CC Vs. AA+AC	17 (13.5)	13 (13.1)	1.03 (0.44-2.12)	0.920
	MAF	34.1	30.8		
	HWp	0.357	0.089		

OR, odds ratio; CI, confidence interval; MAF, minor allele frequency; [a]HWp, Hardy-Weinberg *p* value.

Table II.- The distribution of *MTHFR* genotypes and other risk factors among CHD patients and their association with Down syndrome.

Gene	Genotype	CHD		Univariate OR (95% CI)	*p*-value
		Without DS (n=73)	With DS (n=26)		
rs1801133	CC	52	17	Reference	
	CT	18	8	2.38 (0.58-9.66)	
	TT	3	1	1.03 (0.08-13.24)	0.831
rs1801131	AA	38	13	Reference	
	AC	27	8	1.43 (0.39-5.19)	
	CC	8	5	4.64 (0.86-25.06)	0.542
Sex	Female	35	13	Reference	
	Male	38	13	1.12 (0.42-3.54)	0.707
Paternal age	< 40 y	51	9	Reference	
	≥ 40 y	22	17	2.45 (0.66-9.19)	0.182
Maternal age	< 35 y	61	11	Reference	
	≥ 35 y	12	15	5.32 (1.43-19.82)	0.013

OR, odds ratio; CI, confidence interval.

Sequence Detection Software (SDS) v. 2.3. A genotype call rate over 99% was considered for the analysis. As a quality control measure, 10% of samples selected at random were included for duplicate genotyping.

Statistical analyses

Allele frequencies of both polymorphisms in CHD and control groups were calculated by the gene counting method. The genotype distribution for each polymorphism was evaluated for Hardy–Weinberg equilibrium. The strength of the association between *MTHFR* gene polymorphisms and CHD was evaluated using the χ^2 test and odds ratios (OR) with 95% confidence intervals (CI). The influence of different genotypes on the relationship between DS and maternal age and their interaction was examined using the Mantel-Haenszel stratified analysis. All statistical analyses were performed in SPSS v. 14.0 (IBM Statistics, Chicago, IL).

RESULTS

Of 99 patients with CHD, 26 (26.3%) had DS. Among DS patients, atrioventricular septal defect (AVSD) was the most common cardiac defect followed by ventricular septal defect (VSD) and atrial septal defect (ASD). The distributions of *MTHFR* rs1801133 and rs1801131 genotypes and alleles in CHD and control groups are shown in Table I. The *MTHFR* rs1801133 (p=0.402) and rs1801131 (p=0.357) genotype distributions in the control group followed HardyWeinberg equilibrium (p=0.359).

Genotypes and alleles were not statistically different between controls and individuals with CHD (Table I). *MTHFR* rs1801133 and rs1801131 polymorphisms were not associated with CHD risk (Table I) and, in CHD individuals, *MTHFR* rs1801133 and rs1801131 genotypes failed to show any significant association with DS (Table II).

Univariate analysis showed that increased maternal age (≥35 years) contributed to CHD risk in DS patients (OR: 5.32, 95% CI: 1.43-19.82; p=0.013), while increased paternal age (≥40 years) had no effect on CHD in DS patients (OR: 2.45, 95% CI: 0.66-9.19; p=0.182) (Table II). Significant heterogeneity in the effect of maternal age on DS was observed among different genotypes of *MTHFR* rs1801133 and rs1801131 polymorphisms. The M-H combined OR for maternal age and rs1801133 (p<0.001) was 6.33 and for maternal age and rs1801131 was 7.42 (p<0.001) (Table III).

Table III.- Association between CHD with or without down syndrome and maternal age stratified by *MTHFR* genotypes.

Gene	Genotype	OR (95% CI)	*p*-Value*
Maternal age			
rs1801133	CC	11.78 (3.29-42.14)	<0.001
	CT	3.50 (0.59-20.68)	
	TT	-	
	M-H combined	6.33 (2.37-16.93)	
rs1801131	AA	9.96 (2.37 -41.86)	<0.001
	AC	5.75 (1.00-32.95)	
	CC	4.47 (0.30-73.38)	
	M-H combined	7.42 (2.67-20.64)	

CHD, congenital heart disease; M-H, *Mantel-Haenszel.* *Homogeneity test *p* value.

DISCUSSION

The pathogenesis of CHD is complex and remains poorly understood due to its multifactorial etiology. Individuals with DS show several common cardiac malformations such as VSDs, atrioventricular canals, and the tetralogy of Fallot (Tandon and Edwards, 1973). As folate serves as a DNA methyltransferase co-substrate, reduced folate causes hypomethylation and consequently abnormal segregation of chromosomes or chromosome nondisjunction (Fenech, 2011; James *et al*., 1999; Blom and Smulders, 2011). One common factor in these folate abnormalities is *MTHFR* rs1801133 polymorphisms, with decreased enzyme activity causing impaired re-methylation of Hcy to methionine and subsequent hyperhomocysteinemia (Yigit *et al*., 2013).

However, the association between maternal genetic polymorphisms in folate metabolism genes and DS risk is still controversial (Yang *et al*., 2013; Brandalize *et al*., 2009, 2010). Significantly elevated Hcy levels in mothers of children with CHD suggest that maternal hyperhomocysteinemia is an independent risk factor for CHD (Malik *et al*., 2017; Lu *et al*., 2011; Verkleij-Hagoort *et al*., 2007). Due to the high prevalence of CHDs in neonates born with DS and DS survivors in Al Madinah, CHDs are considered as an important health problem in Saudi Arabia, a problem further exacerbated by widespread consanguinity in this region (Abdulhadi *et al*., 2016). Thus, maternal supplementation with folic acid is likely to be associated with a reduced risk of CHD in DS. Given this perspective and to inform management, we considered the association between *MTHFR* polymorphisms and CHD in DS susceptibility in a Saudi population.

We did not identify any significant association between *MTHFR* polymorphisms and CHD risk. Both polymorphisms had a confounding effect on the relationship between CHD in DS and maternal age. The presence of the maternal *MTHFR* rs1801133 "T" allele was associated with an increased risk of DS in a Brazilian population (Brandalize *et al*., 2009), while another study of a Croatian population reported no association between maternal *MTHFR* polymorphisms and CHD in DS (Bozovic *et al*., 2011; Elsayed *et al*., 2013). *MTHFR* rs1801131 was over-transmitted in patients with CHD in DS ASDs (Locke *et al*., 2010). A meta-analysis showed that *MTHFR* rs1801131 polymorphisms reduced the risk of CHD in patients without DS (Yu *et al*., 2017), while another suggested that the maternal *MTHFR* rs1801133 "T" allele was a risk factor for DS-affected pregnancies (Rai *et al*., 2014). Finally, a recent study showed that the *MTHFR* gene was twice as likely to be promoter methylated in mothers of children with DS with CHD than mothers of children with DS without CHD (Asim *et al*., 2017).

The following strengths and limitations must be taken into consideration when interpreting our results. Significant strengths include the inclusion of subjects with CHD diagnosed by echocardiography and clinical cardiac examination and DS by karyotype or FISH for chromosome 21. However, the study also has several limitations: plasma Hcy levels were not determined and correlated with DS-related CHD or *MTHFR* variants, and maternal *MTHFR* gene polymorphisms were not tested. Further, the study is relatively small and retrospective, which may impact on the validity of the results.

CONCLUSION

In conclusion, polymorphisms in *MTHFR* may interact with other confounding variables and contribute to the increased risk of CHD in DS.

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Statement of conflict of interests

The authors have no conflicts of interest.

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