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Methyltetrahydrofolate-homocysteine methyltransferase reductase gene and congenital heart defects in Down syndrome

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Abstract

Congenital heart defects (CHD) are the most common abnormalities occurring in 40% -60% of Down syndrome (DS) patients. The 5-methyltetrahydrofolate homocysteine methyl transferase reductase (*MTRR*) is one of the key regulatory enzymes involved in folate pathway. Disrupted folate pathway due to *MTRR* polymorphism could be a risk factor for CHD in DS. The aim of the study was to determine the association between polymorphism *MTRR* 66A> G and CHD in DS. Additionally, the impact of maternal endogenous factors on CHD was analyzed, intake of folate through diet, periconceptional folic acid supplementation, smoking and alcohol drinking. A total of 155 children with DS and 148 their mothers have been enrolled in this study. Genotyping was performed by PCR-RFLP. The frequency of alleles and genotypes of *MTRR* 66A> G polymorphisms was not significantly different between a group with CHD compared to a group without CHD among DS subjects as well as in their mothers. The mothers with mutated homozygous genotypes who have taken folic acid preparations from the fourth week before pregnancy to eight weeks of pregnancy were more likely to have DS-CHD+ child. The study results suggested that maternal *MTRR* 66A> G polymorphisms associated with their lifestyle habits such as folic acid intake could altered individual risk for CHD in DS child.

Introduction

Folate, vitamin B9 or pteroyl-L-monoglutamic acid is derivative found in the natural compounds in foods. In the cells, folates serve as methyl group donors for the synthesis of nucleic acids, amino acids and S-adenosyl-L-methionine (SAM). The transfer of the methyl group from SAM takes place with the methyl transferase enzyme of a particular recipient, thus producing S-adenosyl-L-homocysteine (SAH) which convert to homocysteine. Depending on the needs of the body, homocysteine can be

further degraded through transsulfuration and remethylation (Preedy 2013; Litwack 2015; Voet et al., 2016). Remethylation of homocysteine to methionine is catalyzed by 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) enzyme (EC 2.1.1.135) (Frey, 2007). It is coded by the *MTRR* gene (OMIM: 602568, HGCN ID: 7473) located on the human chromosome 5p15.31. The most common single nucleotide polymorphism (SNP) of the human *MTRR* gene is 66A> G (rs1801394)(NM_002454.3(*MTRR*):c.66A>G (p.Ile22Met) (Jacques, 2003; OMIM). Isoleucine substitution with methionine in the flavin-mononucleotide binding site of the enzyme causes a decrease in its predisposition for remethylation of homocysteine to methionine. If mutated homozygote genotype is present, methylation reactions could be disrupted (Voet et al., 2016).

Studies indicate that the *MTRR* 66 A> G SNP is most prevalent among the Caucasian population (about 20 - 38%), much less found in Asians (about 7 - 10%), while among Africans (about 6 - 8%) and Mexicans (about 6-7%) are least represented (Yadav, 2019; www.snpedia.com/index.php/Rs1801394). Evidence suggested that the *MTRR* 66 A> G polymorphism seemed to have a relationship with congenital heart defects (CHD) (Cai et al., 2014; Guo et al., 2017; Xu et al., 2018). Association with the onset of congenital heart defects in Down syndrome (DS) was also observed (Asim et al., 2017). CHD are one of the most common abnormalities occurring in 40% -60% of DS cases but the etiology is still unknown. Trisomy 21 alone was considered insufficient to develop CDH since they do not occur in all persons with DS (Hoobs et al., 2005; Rao et al., 2013; Benhaourech et al., 2016; Zaidi and Brueckner, 2017). The most common CHD in DS patient is atrioventricular septal defects (AVSD). Other septal defects such as ventricular and atrial septal defects (VSD, ASD) or tetralogy of fallot was also presented (Morrison & McMahon 2017; Pfitzer et al., 2018; Zahari et al., 2019).

The aim of this study was to determine whether the single nucleotide polymorphism of the 66A> G *MTRR* gene is a risk factor for the onset of CHD in Down syndrome. In addition, the influence of endogenous maternal factors on the development of CHD, such as folate intake by diet, periconceptional intake of folate preparations, alcohol and smoking, was examined.

Material and methods

The study was conducted on a sample of 146 mothers and 155 of their children with DS. One child had a mosaic type of DS, five children had a translocation form of DS, while the other 149 had a regular form of trisomy 21. In 78 children, a CHD was present and this was a group of subjects, while in other 77 children CHD was absent and therefore served as a control group. Furthermore, to examine maternal risk factors for CHD, two groups of mothers were compared; mothers of DS child with CHD (DS-CHD+) (N=72) and mothers of DS child without CHD (DS-CHD-) (N=74). The dietary intake of folate in the mother (diet and synthetic folate) as well as their life habits (cigarette smoking and alcohol consumption) were studied. All participants in this research were from the territory of the Republic of Croatia.

Genomic DNA was extracted from EDTA-treated blood or saliva using the QIAamp DNA blood FlexiGene DNA Kit or QIAamp DNA Micro Kit QIAamp® (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The *MTRR* 66A> G polymorphism (rs1801394) was evaluated by PCR-RFLP according to publishing protocol (Jacques, 2003). Briefly, genomic DNA was amplified using primers targeting the gene of interest (Table 1). Cycling conditions were as follows: 92°C/2 min, followed by 35 cycles of 92°C/60'', 56°C/60'', 72°C/90''. A final, 7-minute elongation step was performed at 72°C/7 min. The positive PCR products from the reaction (10 μl) were digested with restriction enzyme Alf III (0,1 U) at 37°C/3 hours. Fragments were separated on a 1% agarose gels that containing ethidium bromide (0.5 μg/ml) and photographed under UV light with a gel documentation system.

Table 1. Primers used in this study

Primer	Sequences 5'→ 3'
MTRR A66G-f	CAGGCAAAGGCCATCGCAGAAGACAT
MTRR A66G-r	CACTTCCCAACCAAAATTCTTCAACG

Statistical analyses were performed using statistical software package for Windows (StatSoft, Inc. (2011) STATISTICA (Data Analysis Software System, Version 10. Tulsa, OK) and MedCalc Software (Version 12.4.0; last modified January 2, 2017, 1993–2017 MedCalc Software, Ostend Belgium). P value <0.05 was considered as statistically significant.

Results and Discussion

Statistically significant differences in the incidence of congenital heart defects with respect to the genotypes and alleles of the *MTRR* 66A> G polymorphism in children with DS or in their mothers were not found (Table 2, Table 3). Also, statistically significant differences in the incidence of congenital heart defects in DS with respect to the codominant genetic pattern (AA, AG and GG genotypes), dominant genetic pattern (AA vs. AG + GG genotype) and recessive genetic pattern (AA + AG genotype vs. GG genotype), or allelic genetic pattern (alleles A and G) were not found (P>0.05).

Table 2. Frequencies of alleles and genotypes of the single-nucleotide polymorphism *MTRR* 66A> G of children with DS (N= 155) in relation to the incidence of congenital heart defect

SNP *MTRR* 66A>G	Congenital heart defect in Down syndrome child		p OR (95 % CI)
	Yes N (%)	No N (%)	
Genotype			
AA	14 (18.18)	21 (26.92)	Referent genotype
AG	42 (54.54)	36 (46.15)	0.223 1.750 (0.779-3.932)
GG	21 (27.27)	21 (26.92)	0.491 1.500 (0.605-3.716)
AG+GG	63 (81.82)	57 (73.08)	0.249 1.658 (0.771-3.564)
Allele			
A	70 (45.45)	78 (50.00)	Referent allele
G	84 (54.54)	78 (50.00)	0.429 1.200 (0.768-1.875)

OR - appearance ratio, 95% CI - 95 percent confidence interval of aspect ratio, p-values were determined by Pearson's chi-square test.

Those results are in line with some similar researches systematized in published paper by Coppede (2015). Conversely, some studies indicate that a *MTRR* 66A> G polymorphism may be associated with DS and the rate of CHD. Namely, a study conducted by Asim et al. (2017) indicates a higher incidence of CHD in DS with heterozygous genotype of the *MTRR* 66A> G polymorphism and the same effect was observed in the dominant genetic pattern of the subjects.

Table 3. Frequencies of alleles and genotypes of the single-nucleotide polymorphism *MTRR* 66A> G of mothers of children with DS (N=146) in relation to the incidence of congenital heart defects

SNP *MTRR* 66A>G	Congenital heart defect in Down syndrome child		p OR (95 % CI)
	Yes N (%)	No N (%)	
Genotype			
AA	16 (22.22)	16 (21.62)	Referent genotype
AG	36 (50.00)	33 (44.59)	1.000 1.091 (0.472-2.523)
GG	20 (27.77)	25 (33.78)	0.651 0.800 (0.322-1.985)
AG+GG	56 (77.78)	58 (78.38)	1.000 0.966 (0.441-2.115)
Allele			
A	68 (47.22)	65 (43.91)	Referent allele
G	76 (52.77)	83 (56.08)	0.638 0.875 (0.552-1.388)

OR - appearance ratio, 95% CI - 95 percent confidence interval of aspect ratio, p-values were determined by Pearson's chi-square test.

The *MTRR* 66A> G polymorphism in humans has been associated with elevated blood plasma homocysteine values, which are thought to cause CHD in offspring. Namely, the researches indicate that homocysteine can be harmful to the cell because it causes oxidative stress due to the production of oxygen free radicals, binding to nitric oxide, or through the accumulation of SAH as its precursor that suspends transmethylation (Verkleij-Hagoort et al., 2006; Surmiak et al., 2017). In addition, studies indicate a possible correlation of elevated homocysteine and a higher incidence of CHD in offspring due to changes in methylation parameters. However, the global hypomethylation indicates an association with DS and CHD, respectively (Serra-Juhe et al., 2015; Muka et al., 2016; Božović et al., 2019; Vraneković et al., 2019). Evidence offered contradictory results of the role of maternal *MTRR* 66A> G polymorphism in the onset of CHD in their child (Coppede, 2015; Xu, 2018). Results of this study support the study conducted by Locke (2010) who indicates that there are no statistically significant differences in the incidence of CHD, or their individual forms, in DS children regarded to the frequency of maternal genotypes of the *MTRR* 66A> G polymorphism. Wang et al. (2018) observed

that mothers who have the mutant homozygous genotype *MTRR* 66A> G polymorphism were significantly less likely to have a child with Fallot tetralogy and pulmonary artery or a ventricular septal defect. Guo (2017) showed that an independent association of the *MTRR* 66A> G polymorphism and the higher incidence of the ventriculo septal defects in the offspring.

Statistically significant differences in the incidence of CHD in DS regards mothers' life habits such as cigarette smoking and alcohol consumption were not found. Multiple binary logistic regressions were performed to determine whether the incidence of CHD alters in DS children with respect to the maternal *MTRR* 66A> G polymorphism genotype, daily folate intake, folic acid intake, and diet during pregnancy. Statistically significant differences in the incidence of CHD in DS regarded to the maternal intake of folic acid and *MTRR* 66GG genotype were observed ($\chi 2$ (3, N = 110) = 7.753, p = 0.039, V = 0.500). Namely, mothers with *MTRR* 66GG genotype who were taking folic acid preparations from the fourth week before pregnancy to the eighth week of pregnancy, more often have a DS-CHD+ child (z = 2.2, p = 0.028) than those who have never taken folic acid preparations (z = - 2, 4, p = 0.015). These results support similar studies, which showed that taking folic acid preparations before and during pregnancy did not reduced the incidence of CHD in the offspring (Elizabeth, 2017; Verkleij-Hagoort, 2008; Nobakht, 2018; Øyen, 2019). In contrast, other studies have suggested the possible contribution of folic acid preparation before and during pregnancy to reducing the incidence of CHD in offspring (Sarmah, 2016; Obeid, 2019). When interpreting those results the complexity of the possible interaction of nutrients as epigenetic factors, should certainly be taken into account. The cardiovascular system of the human embryo develops from about the third to the tenth week of pregnancy and the period from the fifth to eighth weeks of pregnancy is considered the most sensitive for the development of CHD (Yagel, 2018). In recent years, due to the increasing introduction of food fortification, which is often legally required for some countries, many research studies investigate the safety and justification of the use of folic acid. In addition, it is believed that people ingest folic acid several times a day, especially since products are now more and more enriched with this supplement (Scaglione, 2014; Field, 2018). It is hypothesized that unconverted folic acid could be the cause of frequent conflicting results of studies about the incidence of CHD. Due to the polymorphism decreased function of some enzymes were observed and may contributes to the larger amounts of unconverted folic acid in bloodstream (Vijayan, 2016). Incomplete conversion of folic acid finally can have various adverse effects on human health (Plumptre, 2015; Pfeiffer, 2015). Studies indicate that intake of daily amounts of folic acid less or greater 200 μg is not a risk for unconverted form. Since quantities more than 400 μg/day may very likely remain in unconverted form due to the possible insufficient effect of the enzymes those amounts are recommended for women before and during pregnancy (Obeid, 2019; Field, 2018). It is believed that unconverted folic acid in the mother bloodstream or the fetus bloodstream is most likely to serve as a source of the methyl group for methylation, which may eventually have harmful effects on fetal development (Obeid, 2019; Pfeiffer, 2015). Such epigenetic activity can be transmitted through multiple generations and finally resulting in various birth defects such as CHD or neural tube abnormalities (Verkleij-Hagoort, 2008). For that reason, more and more countries are now replacing folic acid preparations with the more biologically effective form of folate (methyltetrahydrofolate form) which is also effective in the presence of folate genes polymorphisms (Bayes, 2019). However, in order to make reliable conclusions, more research studies are needed to determinate the unconverted folic acid in blood plasma as well as the efficacy of the enzyme due to specific polymorphisms.

This research have several limitations. The sample of respondents in this survey covers only 9.27% of the total number of patients with Down syndrome in the Republic of Croatia, which significantly contributes to the weakening of the statistical power of the analyzes performed. The post hoc power analysis showed that the performed analyzes did not have sufficient power to reveal the statistical significance of the effects obtained (β = 0.210), which should certainly be kept in mind when generalizing the results of this study and which have been shown to be not statistically significant. Likewise, limited sample size may lead to larger differences in results than similar studies. Because

CHD is a disease of multiple etiologies, the possible interaction of genes and genes with the environment cannot be ruled out. In addition, in mothers of DS children values of L–homocysteine and unconverted folic acid in blood before and during pregnancy were not determinate, while daily folate intake values were determined using a short simple self-completion questionnaire on the frequency of food intake and beverages to evaluate the daily dietary intake of folate of healthy pregnant women in the Republic of Croatia that met all validation and reproducibility criteria. Biochemical determination of the levels of L-homocysteine, 2-methylpropanoic acid, folate and unconverted folic acid in the blood plasma would contribute significantly to the achievement of some specific objectives of this study.

Conclusion

Considering all above mentioned limitations of the study, the obtained results may support the hypothesis that the maternal *MTRR* 66A> G polymorphism in combination with folic acid intake modulate individual risk factors contributing to the higher incidence of CHD in DS child.

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