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Functional variant in methionine synthase reductase intron-1 is associated with pleiotropic congenital malformations

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Abstract Congenital malformations, such as neural tube defects (NTDs) and congenital heart disease (CHD), cause significant fetal mortality and childhood morbidity. NTDs are a common congenital anomaly, and are typically induced by higher maternal homocysteine (Hcy) levels and abnormal folate metabolism. The gene encoding methionine synthase reductase (*MTRR*) is essential for adequate remethylation of Hcy. Previous studies have focused on the coding region of genes involved in one-carbon metabolism, but recent research demonstrates that an allelic change in a non-coding region of *MTRR* (*rs326119*) increases the risk of CHD. We hypothesized that this variant might contribute to the etiology of NTDs as well, based on a common role during early embryogenesis. In the present study, 244 neural tube defect cases and 407 controls from northern China were analyzed to determine any association (by χ^2 test) between *rs326119* and disease phenotypes. Significant increased risk of anencephaly was seen in *MTRR* variant *rs326119* heterozygote (het) and homozygote (hom) individuals [odds ratios $(OR)_{het} = 1.81$; $OR_{hom} = 2.05$)]. Furthermore, this variant was also a risk factor for congenital malformations of the adrenal gland (OR = 1.85), likely due to multiple systemic malformations in the NTDs case population. Our present data indicate that the *rs326119* non-coding variant of MTRR has a pleiotropic effect on the development of multiple tissues, especially during early stages in utero. This suggests the allelic state of *MTRR* is a significant clinical factor affecting Hcy levels and optimal folic supplementation.

Keywords Congenital malformations · Neural tube defects · MTRR · MTR · Single nucleotide polymorphism · Folic acid

Introduction

Congenital malformations such as neural tube defects (NTDs) and congenital heart disease (CHD) are the main causes of fetal death. NTDs are a set of severe congenital anomalies involving the skull, spine, and the central nervous system. Their incidence is ~2 % in some regions of North China [1].

Neural tube defects are influenced by multiple genetic and environmental factors. A number of studies have confirmed that a deficiency in folic acid in pregnant women is an important risk factor for NTDs [2, 3]. In the critical period of organ formation during pregnancy, folic acid supplementation can effectively reduce NTD risk [2, 4–6]. Homocysteine (Hcy) is an intermediate of methionine metabolism, and elevated Hcy levels are related to vitamin B12 and folic acid deficiencies [7, 8]. Elevated Hcy concentrations have been observed in families with NTDs [9–11].

Methionine synthase reductase (encoded by the *MTRR* gene) is essential for the adequate remethylation of Hcy,

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which regenerates functional methionine synthase via reductive methylation. Previous studies have indicated that *MTRR* coding region single nucleotide polymorphisms (SNPs) are associated with NTD pathogenesis [12–14], and our published data also indicate that the *MTRR* coding region polymorphism *rs1801394* is associated with risk of NTDs and multiple congenital defects in the Han Chinese population from northern China [15].

However, to our knowledge, no studies of the possible correlation between NTDs and variations in the non-coding region of *MTRR* have been published. In the present study, we have investigated polymorphisms in the non-coding region of the *MTRR* gene in 244 NTD cases and 407 unrelated region-matched healthy controls from the Han Chinese population. We identified an allelic variant, *rs326119*, in the first intron of *MTRR* that is associated with increased risk of anencephaly in NTD cases, as well as malformation of the adrenal gland, demonstrating a pleiotropic effect involved in congenital malformations.

Material and methods

Subjects

The NTD case subjects were obtained from Shanxi Province of northern China [1]. The Committee of Medical Ethics at the Capital Institute of Pediatrics (Beijing, China) approved this study. Pregnant women were enrolled in the project and NTD cases were diagnosed in utero by trained local clinicians using ultrasonography. Written informed consent was obtained from all adult participants included in this study and written informed consent was obtained from the parents on behalf of minors. In the present study, 244 NTD cases were diagnosed either prenatally or at stillbirth with anencephaly, spina bifida, or encephalocele. The gestational age of diagnosis was between 15 and 38 weeks. 407 unrelated ethnicity-matched healthy subjects were recruited as normal controls.

Genomic DNA extraction

Genomic DNA was isolated from muscle and extracted with the Blood and Tissue DNA Kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's instructions. The concentration and purity of DNA were determined by light absorbance at 260 and 280 nm.

SNP identification and genotyping

Single nucleotide polymorphisms were genotyped using the SNaPshot technology (Applied Biosystems, Foster City, CA). In brief, genomic DNA was amplified from individual samples using the primers listed in Table 1 and using the following cycling program: 95 °C for 5 s; followed by 11 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 40 s; then 31 cycles of 95 °C for 30 s, 54 °C for 20 s, and 72 °C for 40 s. After SAP and *Exo*I enzymatic treatment, we obtained purified templates including the target SNP site. This template, the internal primer (Table 1), and SNaPshot Multiplex were mixed and cycled according to the manufacturer's instructions using the following program: 96 °C for 1 min; followed by 28 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. These genotyping samples were run on an ABI 3730 automated sequencer (Applied Biosystems) and visualized using the Peakscan software.

Statistical analysis

Hardy–Weinberg equilibrium in controls was tested by χ^2 tests with $P > 0.05$ defined as within equilibrium. To evaluate the associations between genotypes and case risks, odds ratios (OR) and 95 % confidence intervals (CIs) were calculated by unconditional logistic regression analysis using the SNPStats website (http://bioinfo.iconcologia.net/snpstats/start.htm). Each SNP was evaluated under four genetic models: a codominant model, a dominant model, a recessive model, and a log-additive model. All statistical tests were two-tailed, with $P < 0.05$ taken as statistically significant, and performed using the SPSS software (version 15.0, SPSS, Chicago, IL).

Results

We classified the 244 NTD cases into 17 groups based on NTD phenotype and accompanying syndromes (Table 2, International Statistical Classification of Diseases and Related Health Problems, 10th Revision, 2010). Associations between variant *rs326119* and each disease phenotype were analyzed for disease phenotypes with $n > 20$.

These genetic analysis results showed that there was no statistically significant association between variant *rs326119* and the overall incidence of NTDs (Table 3). However, for the 114 NTD-associated anencephaly cases studied, both heterozygosity (het) and homozygosity (hom) for this allele increased the risk of anencephaly [$OR_{het} = 1.81$ (95 % CI 1.11–2.94) and $OR_{hom} = 2.05$ (1.10–3.80), $P = 0.022$, Table 3]. For cases of spina bifida and encephalocele, the ORs were 1.36 (0.81–2.26) and 1.46 (0.68–3.12), respectively, and not statistically different from controls (Table 3). These results suggest that the *rs326119* allele in the non-coding region of *MTRR* increased the risk of anencephaly in cases of NTDs.

Table 1 Gene data and single nucleotide polymorphism genotyping primers to evaluate the implications of the allelic variant *rs326119* of MTRR in neural tube defects

Refseq	NC_000005.9
OMIM	602568
SNP ID	*rs326119*
Chr	5
Position[a]	7870083
SNP type	Intronic
Nucleotides at SNP position	A/C
PCR primers	F: 5′TCATTATCGTTTCCACCGTTT3′
	R: 5′CCATACTCATCTAACGGCTAAAAT3′
Internal primer	5′GTTCATTCACCGAAAGCCAAG3′

SNP ID single nucleotide polymorphism identification, *Chr* chromosome

[a] In reference to NCBI build 37.3, available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp

Table 2 Summary of various anomalies presenting in the study population

Malformation	Abbreviation	Cases seen
Neural tube defect	NTD	244
Anencephaly	Ane	114
Spina bifida	SB	189
Encephalocele	Enc	72
Congenital hydrocephalus	Hyd	96
Craniofacial malformation	Cra	10
Cleft lip and cleft palate	Cle	6
Congenital malformation of the adrenal gland	CMAG	64
Congenital heart disease	CHD	18
Congenital malformations of the spine and bony thorax	CMSBT	38
Cervical fusion syndrome	CFS	49
Limb malformation	LM	67
Congenital malformation of the spleen	CMS	8
Congenital malformation of the urinary system	CMUS	21
Celoschisis	Cel	4
Congenital malformation of the respiratory system	CMRS	106
Congenital malformation of the digestive system	CMDS	5
Single umbilical artery	SUA	8

Table 3 Variant *rs326119* (A → C) is associated with NTD phenotypes

Phenotype	Genotype			Codominant			Dominant	
	A/A	A/C	C/C	OR_{het} (95 % CI)	OR_{hom} (95 % CI)	*P* value	OR (95 % CI)	*P* value
Control ($n = 407$)	163 (40 %)	183 (45 %)	61(15 %)					
NTD ($n = 244$)	84 (34.4 %)	116 (47.5 %)	44 (18 %)	1.23 (0.87–1.75)	1.40 (0.88–2.24)	0.31	1.27 (0.91–1.77)	0.15
Ane ($n = 114$)	30 (26.3 %)	61 (44.7 %)	23 (20.2 %)	1.81 (1.11–2.94)	2.05 (1.10–3.80)	0.022	1.87 (1.18–2.97)	0.0062
SB ($n = 189$)	65 (34.4 %)	91 (48.1 %)	33 (17.5 %)	1.25 (0.85–1.83)	1.36 (0.81–2.26)	0.39	1.27 (0.98–1.83)	0.18
Enc ($n = 72$)	22 (30.6 %)	38 (52.8 %)	12 (16.7 %)	1.54 (0.87–2.71)	1.46 (0.68–3.12)	0.30	1.52 (0.89–2.60)	0.12

Ane anencephaly, *SB* spina bifida, *Enc* encephalocele, *het* heterozygote, *hom* homozygote

Interestingly, we found that this variant was also closely associated with congenital malformation of the adrenal gland (Table 4, OR = 1.85, $P = 0.035$). Considering neural tissue originates from the ectoderm and adrenal gland tissue from both the ectoderm and the mesoderm, our data indicate that the *rs326119* variant may have an

impact on malformations in multiple tissues during embryogenesis.

Discussion

CHD and NTDs are common and detrimental congenital disorders that initiate during early embryogenesis. Epidemiological studies indicate that their etiologies have a genetic component. For example, recent research shows that specific alleles of methylenetetrahydrofolate reductase (*MTHFR*) [16, 17], methionine synthase (*MTR*) [12, 18], and *MTRR* can all increase the risk of CHD and NTDs. Recent meta-analysis data indicated that distinct metabolic subpathways in the one-carbon pathway (including purine and pyrimidine synthesis, and Hcy recycling to methionine) are associated with increased incidence of spina bifida in an ethnic-specific risk signature [19].

Methionine synthase reductase (*MTRR*) is transcriptionally expressed in various tissues during embryogenesis (EBI database). Knocking out the murine *MTRR* gene results in embryonic lethality, while a hypomorph with reduced *MTRR* activity created by gene trap technology showed adverse effects on reproductive outcomes, particularly in higher incidence birth defects [20, 21]. It was also reported that *MTRR* plays a key role in regulating the activity of NADPH-dependent MTR [22]. Reduction of *MTRR* activity can decrease the concentration of active MTR [14]. These studies suggest that the level of *MTRR* expression could potentially affect early embryogenesis.

Previous studies have indicated that SNPs in the coding regions of *MTRR* are associated with NTD pathogenesis [12–14]. A recent study also demonstrated that the allelic variant *rs326119* in the first intron of *MTRR* (a non-coding region) increases the risk of CHD in the Han Chinese population [23]. This study showed that this allelic variant specifically alters the binding site for the transcription factor C/EBPα that plays vital roles in cortical dendrite differentiation by regulating the recruitment of brain-derived neurotrophic factors to immediate-early genes critical to early neuronal development. The *MTRR* variant *rs326119* potentially results in low levels of methionine and hyperhomocysteinemia by dysfunction of MTR-catalyzed Hcy recycling to methionine. It was reported that hyperhomocysteinemia can induce increased concentrations of S-adenosylmethionine (SAH) and global DNA hypomethylation, and reduce the concentration of S-adenosylhomocysteine (SAM) and the SAM:SAH ratio. These results are consistent with the data showing that northern Chinese NTD cases were associated with tissue-specific global DNA hypomethylation [24–26]. Zhang et al. suggest that this hypomethylation is likely due to disturbance of maternal folate and homocysteine concentrations [27]. It is known that DNA methylation is pivotal to the regulation of gene expression [28], including the regulation of genes of the planar cell polarity pathway, which play important roles in embryonic development [29]. Thus, we suggest that the functional genetic variant *rs326119* of *MTRR* is likely to stimulate hyperhomocysteinemia, which can further induce DNA hypomethylation of planar cell polarity pathway genes. Taken together with data from these previous studies, our data showed that NTD-related anencephaly was strongly correlated with *MTRR* function and suggests that the function of genes involved in Hcy recycling to methionine is associated with the development of congenital neuronal tissue malformations. Interestingly, we also found that this allelic variant was correlated with malformation of the adrenal gland, demonstrating a pleiotropic role in congenital malformations. In summary, this study is the first to report that a functional non-coding

Table 4 Variant *rs326119* (A → C) is associated with some disease phenotypes

Phenotype	Genotype			Codominant			Dominant	
	A/A	A/C	C/C	OR_{het} (95 % CI)	OR_{hom} (95 % CI)	*P* value	OR (95 % CI)	*P* value
Control ($n = 407$)	163 (40 %)	183 (45 %)	61 (15 %)					
Hyd ($n = 96$)	40 (41.7 %)	42 (43.8 %)	14 (14.6 %)	0.94 (0.58–1.51)	0.94 (0.48–1.84)	0.96	0.94 (0.60–1.47)	0.77
CMSBT ($n = 36$)	9 (25 %)	20 (55.6 %)	7 (19.4 %)	1.98 (0.88–4.47)	2.08 (0.74–5.83)	0.19	2.00 (0.92–4.37)	0.068
LM ($n = 67$)	19 (28.4 %)	39 (58.2 %)	9 (13.4 %)	1.83 (1.02–3.29)	1.27 (0.54–2.95)	0.11	1.69 (0.96–2.98)	0.064
CFS ($n = 49$)	15 (30.6 %)	25 (51 %)	9 (18.4 %)	1.48 (0.76–2.91)	1.60 (0.67–3.85)	0.42	1.51 (0.80–2.87)	0.91
CMUS ($n = 21$)	6 (28.6 %)	10 (47.6 %)	5 (23.8 %)	1.48 (0.53–4.17)	2.23 (0.66–7.56)	0.44	1.67 (0.63–4.39)	0.28
CMAG ($n = 64$)	17 (26.6 %)	33 (51.6 %)	14 (21.9 %)	1.73 (0.93–3.22)	2.20 (1.02–4.73)	0.086	1.85 (1.02–3.33)	0.035
CMRS ($n = 106$)	34 (32.1 %)	50 (47.2 %)	22 (20.8 %)	1.31 (0.81–2.13)	1.73 (0.94–3.19)	0.21	1.41 (0.90–2.23)	0.13

Hyd congenital hydrocephalus, *CMSBT* congenital malformations of the spine and bony thorax, *LM* limb malformations, *CFS* cervical fusion syndrome, *CMUS* congenital malformation of the urinary system, *CMAG* congenital malformations of the adrenal gland, *CMRS* congenital malformation of the respiratory system, *het* heterozygote, *hom* homozygote

allelic variant of *MTRR* is correlated with defects of early embryogenesis, specifically with congenital neural and adrenal gland malformations. These data may have an important impact on our understanding of Hcy remethylation, folate metabolism, and maternal folate supplementation during pregnancy.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Committee of Medical Ethics at the Capital Institute of Pediatrics (Beijing, China) and with the 1964 Helsinki declaration and its later amendments.

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