Original Article

Association of folate metabolism genes MTHFR and MTRR with multiple complex congenital malformation risk in Chinese population of Shanxi

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Abstract: Birth defects are common, serious malformations with a complex etiology that suggests involvement of both genetic and environmental factors. Low dietary folate and polymorphisms in genes of folate metabolism can influence risk for birth defects. In the present study 250 Chinese birth defects cases who suffered 1-8 types of birth defect disease phenotypes were subjected and two genetic variants in two folate metabolism key enzymes, rs1801394 of methionine synthase reductase (MTRR) and rs1801133 of methylenetetrahydrofolate reductase (MTHFR) were genotyped by using SNaPshot method. The results indicated that rs1801394 and rs1801133 were associated with multiple birth defects. According to homology of organogenesis, the disease phenotypes were classified into ectoderm-, mesoderm-, and endoderm-developed groups. Genetic analysis results displayed that as protective factors, genetic variants of rs1801133 and rs1801394 were associated with the risk of ectoderm-, and endoderm-developed malformations, but only the variant of rs1801394 was associated with the risk of mesoderm-developed malformations. Our present study first related nutrition metabolism related gene variants to germ layers and provided a novel understanding of an implication of earlier causation of birth defects pathogenesis in humans.

Keywords: Birth defects; multiple complex congenital malformation; methionine synthase reductase (MTRR); methylenetetrahydrofolate reductase (MTHFR); single nucleotide olymorphism (SNP)

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Introduction

Birth defects are structural malformations present in a baby at or before birth affecting multiple different organs. Data from the Chinese Birth Defects Monitoring Program (CBDMP) indicated that neural tube defects (NTD) rate at birth in the whole of China is 2.7‰, but is high in Shanxi Province about 10.6‰ (1). The most common birth defects are congenital heart defects (CHD), NTD and craniofacial malformations which include cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) in USA (2). While in Chinese Shanxi the most common birth defects are anencephaly (10‰), congenital heart diseases (7.32‰) and spina bifida (6.39‰) (3). And it was reported that many birth defects cases presented as multiple congenital malformations beside single congenital malformations (4,5). The etiology of birth defects has been widely discussed but is not yet fully clarified.

Numerous studies suggest that the genetic and environmental factors are involved in birth defects information and it is reported that folate plays a pivotal role in normal embryonic development (2). Pregnant experimental animals subjected to folate deprivation often fail to deliver normal pups at term, instead of giving birth to pups with multiple malformations (6,7). Epidemiological studies have demonstrated that the benefit of folic acid supplementation in preventing NTD and other congenital abnormalities.

Folate, which acts as a one-carbon donor involved in both the synthesis of nucleotides and methyl transfer

reactions, is important for methylation of DNA, protein and lipids. Epidemiological studies provide evidence that among environmental factors, folate status plays a key role in birth defect. Polymorphic variants of genes in the folate and homocysteine pathways have well-associated with the risk of birth defects, including common variants rs1801133 (c.677C>T) of MTHFR (5, 10-methylenetetrahydrofolate reductase) and rs1801394 (c.66A>G) of MTRR (methionine synthase reductase). It is reported that rs1801133 was associated with the risk of CL/P, NTD and CHD (8-12), while rs1801394 was associated with the risk of CHD and NTD (13-15). Embryogenesis is a restrict hierarchy developing process and birth defects occur at the earlier stage. CL/P, NTD and CHD were developed from different germ layers. So we want to know that whether the two folate pathway related polymorphic variants are having? A common risk fact related to birth defects and which germ layer the two folate pathway associated polymorphic variants are related to.

Here we gathered 420 normal controls and 250 birth defects. Per case presented 1-8 types of congenital malformations and classified the phenotypes into three germ layers-developed. By genotyping of rs1801394 and rs1801133, we further identified the associations of genetic variants with the risks of birth defects and germ layers related. Our results suggest that rs1801394 and rs1801133 were associated with multiple phenotypes. And genetic variants of rs1801133 and rs1801394 were associated with the risk of ectoderm- and endoderm-developed malformations, but only the variant of rs1801394 was associated with the risk of mesoderm-developed malformations. These results indicate that birth defects are associated with the function of folate metabolism and homocysteine pathways.

Materials and methods

Subjects

Stillborn birth defects case subjects were obtained from Shanxi Province of Northern China (16). This study was approved by the Committee of Medical Ethic in the Capital Institute of Pediatrics, Beijing, China; and written informed consent was received by the adult subjects and written informed consent from the parents on behalf of the minors. The enrolled pregnant women were diagnosed by local trained clinicians and ultrawave, and then registered in a database. The surgical details were described in our previous paper (17). In the present study 250 birth defects subjects were employed who were diagnosed with CHD, NTD and craniofacial malformations covering gestational ages more than 13 weeks. A total of 420 unrelated region-matched healthy subjects were recruited as normal control.

SNP identification and genotyping

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

SNPs were genotyped using the SNaPshot analysis (ABI). In brief, the genomic DNA was individually amplified by using the primers listed in *Table 1*, After SAP and Exo I purification, obtained purified template which include target SNP site. According to manufacturer's instructions, extent primer (as shown in *Table 1*) and SNaPshot Multiplex were added into the template and the thermal cycling reaction was done. Then the genotyping samples were run on ABI 3730 automated sequencer and analyzed by Peakscan software.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) in control was tested by χ^2 test and the P values are larger than 0.05. To evaluate the associations between genotypes and case risks, OR and 95% CIs were calculated by unconditional logistic regression analysis with adjustment for age using the SNP Stats 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 $P<0.05$ regards as statistically significance, and performed by SPSS 15.0 software (SPSS, Chicago, IL, USA).

Results

Description and classification of disease phenotypes

To characterized the present group of birth defect cases, we described the phenotypes of birth defects and complications according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) Version for 2010 (*Figure 1A*). Then, the presented phenotypes were sorted into ectoderm-, mesoderm- and endoderm-developed (*Figure 1B*). Multiple birth defects are defined as two or more unrelated major structural malformations

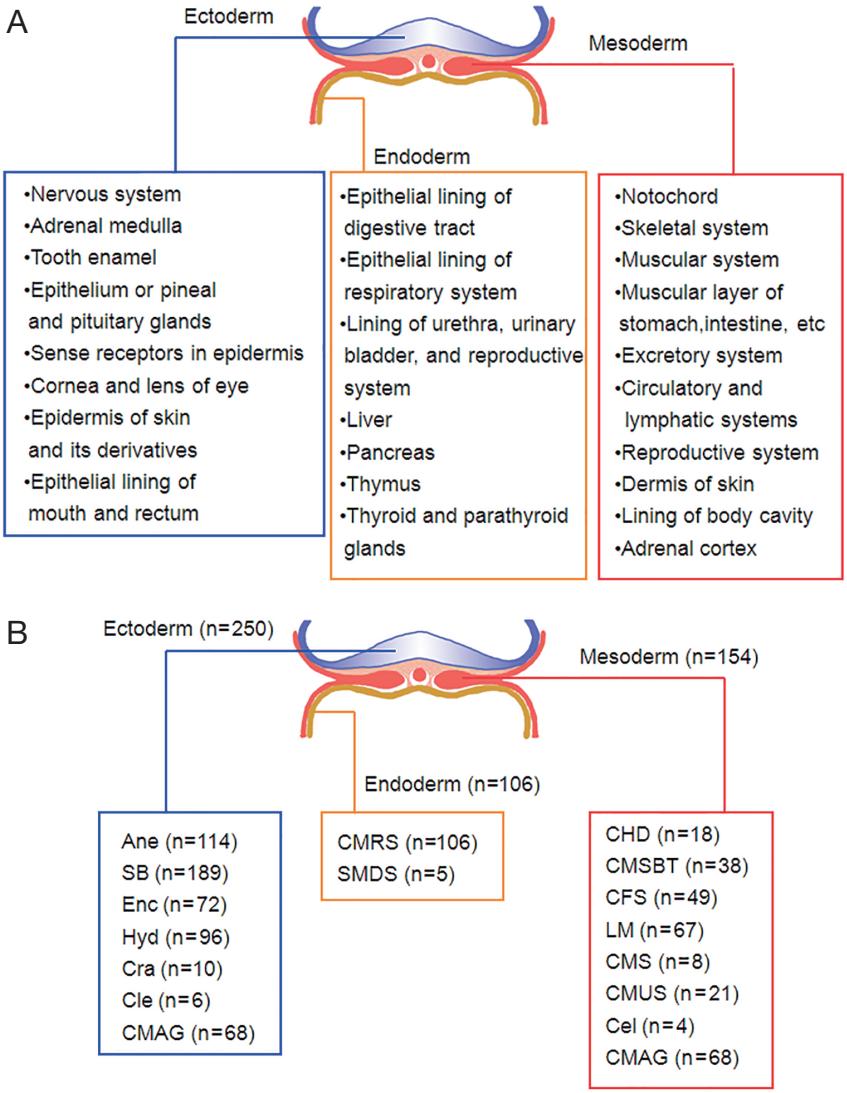

Figure 1 Schematic of derivatives of the three germ layers and classifications of birth defects cases. (A) Derivatives of the three germ layers in humans; (B) classifications of the present case subjects according to the disease phenotypes and their homology. Abbreviations: Ane, anencephaly; SB, spina bifida; Enc, encephalocele; Hyd, congenital hydrocephalus; Cra, craniofacial malformation, including macrophthalmos, agenesis and underdevelopment of nose or accessory nose and microtia; Cle, Cleft lip and cleft palate; CAMG, congenital malformations of adrenal gland; CHD, congenital heart disease, including congenital malformation of cardiac chambers and connections; CMSBT, congenital malformations of spine and bony thorax, including scoliosis and congenital bony malformation; CFS, cervical fusion syndrome; LM, Limb malformation, including talipes equinovarus, polydactyly, syndactyly, congenital complete absence of upper limb and elongated limbs; CMS, congenital malformation of spleen; CMUS, congenital malformation of the urinary system, including renal agenesis, cystic kidney disease, renal pelvis malformation; Cel, celoschisis; CMRS, congenital malformation of the respiratory system, including lung agenesis and deformity. CMDS, congenital malformation of the digestive system, including hepatomegaly; SUA, single umbilical artery. According to the homology of organogenesis, disease phenotypes were sorted into ectoderm-, mesoderm- and endoderm-developed.

that cannot be explained by an underlying syndrome or sequence (4,18). *Figure 2* presented the sample characters in our cases, it shows that multiple birth defects were chose.

Associations of SNPs with risks of various disease phenotypes in cases

To identify whether the SNPs rs1801394 and rs1801133 were associated with the risk of birth defects and the germ layers, SNaPshot method was used to genotype both of SNPs sites. Considering the presentation of diseases, some congenital malformations were given up to be analyzed if the case amounts were less than 30. The results indicated that the odds ratios (OR) and 95% confidence intervals (CIs) of recessive homozygote of rs1801394 were 0.33 (0.15-0.74) in anencephaly (Ane), 0.36 (0.19-0.67) in spina bifida (SB), 0.36 (0.16-0.85) in congenital hydrocephalus (Hyd), 0.20 (0.06-0.66) in encephalocele (Enc), 0.15 (0.03-0.64) in Limb malformation (LM) and 0.38 (0.17-0.85) in congenital malformation of the respiratory system (CMRS) (*Tables 2,3*). The values of recessive homozygote of rs1801133 were 0.41 (0.21-0.82) in Ane, 0.54 (0.32-0.91) in SB, 0.30 (0.10-0.92) in cervical fusion syndrome (CFS) and 0.47 (0.24-0.92) in CMRS (*Tables 4,5*). The data displayed that rs1801394 is associated with the risks of six types of disease phenotypes while rs1801133 is associated with of four types.

Association of SNPs with risks of germ layer-developed tissue defects

Since rs1801394 and rs1801133 are associated with the risks of multiple phenotypes in the same cases population, this promotes us to consider whether the etiologies of multiple malformations presented on one population are resulted from a common developmental defects. Therefore, the associations of SNPs with the risks of three germ layers-developed tissues defects (as shown in *Figure 1B*) were analyzed. The results indicated that in ectoderm- and endoderm-developed groups, the OR and 95% CI of recessive homozygote of rs1801133 were 0.58 (0.36-0.94) and 0.47 (0.24-0.92); and of rs1801394 were individually 0.33 (0.19-0.60) and 0.38 (0.17-0.85) (*Tables 6,7*). However, in mesoderm-developed group, only the OR and 95% CI of rs1801394 were 0.26 (0.12-0.57) (*Tables 6,7*).

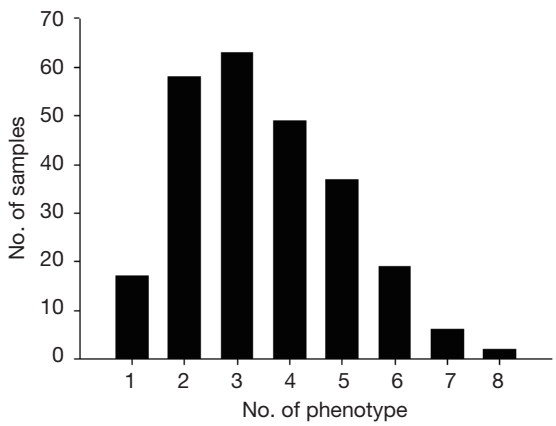

Figure 2 Sample amount of different phenotype per case. X-axis showed the amount of sample; Y-axis showed the number of phenotype in one case.

Table 1 SNPs genotyped in the present subjects to evaluate their implication in NTDs and complications genetic susceptibility

Gene	MTRR	MTHFR
Refseq	NC_000005.9	NC_000001.10
OMIM	602568	607093
SNP ID	rs1801394	rs1801133
Chr	5	1
Position*	7870973	11856378
SNP type	Missense (I22M)	Missense (A222V)
Alleles	A/G	C/T
PCR primer	F: GGAAACACAGATTCAAGCCCAA	F: TCAGCGAACTCAGCACTCCA
	R: CCCAACCAAAATTCTTCAAAGC	R: TCTTCATCCCTCGCCTTGAA
Extent primer	AAAGGCCATCGCAGAAGAAAT	GCTGCGTGATGATGAAATCG

SNP ID, single nucleotide polymorphism identification; Chr, chromosome; UTR, untranslated region. *According to NCBI build 37.3 available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp.

Table 2 rs1801394 genotype distribution in case and control

SNP	Genotype	Control (n=401) (%)	Ane (n=114) (%)	SB (n=189) (%)	Hyd (n=96) (%)	Enc (n=72) (%)	LM (n=67) (%)	CMRS (n=106) (%)
rs1801394	A/A	156 (38.9)	55 (48.2)	90 (47.6)	44 (45.8)	35 (48.6)	31 (46.3)	48 (45.3)
	G/A	177 (44.1)	51 (44.7)	85 (45.0)	45 (46.9)	34 (47.2)	34 (50.8)	50 (47.2)
	G/G	68 (17.0)	8 (7.0)	14 (7.4)	7 (7.3)	3 (4.2)	2 (3.0)	8 (7.5)

Ane, anencephaly; SB, spina bifida; Hyd, hydrocephalus; Enc:encephalocele; LM, limb malformation; CMRS, congenital malformation of the respiratory system.

Table 3 rs1801394 were most associated with six disease phenotypes

Phenotype	Codominant			Dominant		Recessive		Log-additive	
	OR (95% CI)$_{het}$	OR (95% CI)$_{hom}$	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Ane (n=114)	0.82 (0.53-1.27)	0.33 (0.15-0.74)	0.012	0.68 (0.45-1.04)	0.075	0.37 (0.17-0.79)	0.0046	0.66 (0.48-0.91)	0.0084
SB (n=189)	0.83 (0.58-1.20)	0.36 (0.19-0.67)	0.0028	0.70 (0.49-0.99)	0.046	0.39 (0.21-0.72)	0.001	0.67 (0.52-0.87)	0.0025
Hyd (n=96)	0.90 (0.56-1.44)	0.36 (0.16-0.85)	0.035	0.75 (0.48-1.18)	0.22	0.39 (0.17-0.87)	0.011	0.70 (0.50-0.98)	0.035
Enc (n=72)	0.86 (0.51-1.44)	0.20 (0.06-0.66)	0.0056	0.67 (0.41-1.11)	0.12	0.21 (0.07-0.70)	0.0015	0.61 (0.42-0.90)	0.01
LM (n=67)	0.97 (0.57-1.65)	0.15 (0.03-0.64)	0.0026	0.74 (0.44-1.24)	0.26	0.15 (0.04-0.63)	6e-04	0.63 (0.42-0.93)	0.018
CMRS (n=106)	0.92 (0.58-1.44)	0.38 (0.17-0.85)	0.034	0.77 (0.50-1.19)	0.24	0.40 (0.19-0.86)	0.0099	0.72 (0.52-0.98)	0.036

Ane, anencephaly; SB, spina bifida; Hyd, hydrocephalus; Enc, encephalocele; LM, limb malformation; CMRS, congenital malformation of the respiratory system; het, heterozygote; hom, homozygote.

Table 4 rs1801133 genotype distribution in case and control

SNP	Genotype	Control (n=402) (%)	Ane (n=114) (%)	SB (n=189) (%)	CFS (n=49) (%)	CMRS (n=106) (%)
rs1801133	T/T	122 (30.4)	45 (39.5)	72 (38.1)	19 (38.8)	40 (37.7)
	T/C	195 (48.5)	56 (49.1)	90 (47.6)	26 (53.1)	53 (50)
	C/C	85 (21.1)	13 (11.4)	27 (14.3)	4 (8.2)	13 (12.3)

Ane, anencephaly; SB, spina bifida; CFS, cervical fusion syndrome; CMRS, congenital malformation of the respiratory system.

Table 5 rs1801133 were most associated with four disease phenotypes

Phenotype	Codominant			Dominant		Recessive		Log-additive	
	OR (95% CI)$_{het}$	OR (95% CI)$_{hom}$	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Ane (n=114)	0.78 (0.49-1.22)	0.41 (0.21-0.82)	0.028	0.67 (0.43-1.03)	0.069	0.48 (0.26-0.90)	0.014	0.68 (0.50-0.92)	0.011
SB (n=189)	0.78 (0.53-1.15)	0.54 (0.32-0.91)	0.059	0.71 (0.49-1.02)	0.063	0.62 (0.39-1.00)	0.043	0.74 (0.58-0.95)	0.018
CFS (n=49)	0.86 (0.45-1.61)	0.30 (0.10-0.92)	0.056	0.69 (0.37-1.27)	0.24	0.33 (0.12-0.95)	0.018	0.64 (0.41-0.99)	0.042
CMRS (n=106)	0.83 (0.52-1.32)	0.47 (0.24-0.92)	0.073	0.72 (0.46-1.12)	0.15	0.52 (0.28-0.98)	0.032	0.72 (0.52-0.98)	0.033

Ane, anencephaly; SB, spina bifida; CFS, cervical fusion syndrome; CMRS, congenital malformation of the respiratory system; het, heterozygote; hom, homozygote.

Table 6 rs1801394 and rs1801133 genotype distribution in three germ layers

SNP	Genotype	Control (%)	Ectoderm (n=250) (%)	Mesoderm (n=154) (%)	Endoderm (n=106) (%)
rs1801133	T/T	122 (30.4)	91 (37.0)	54 (36.7)	40 (37.7)
	T/C	195 (48.5)	116 (47.5)	69 (46.9)	53 (50.0)
	C/C	85 (21.1)	37 (15.0)	24 (16.3)	13 (12.3)
rs1801394	A/A	156 (38.9)	117 (48.0)	70 (47.3)	48 (45.3)
	G/A	177 (44.1)	110 (45.1)	70 (47.3)	50 (47.2)
	G/G	68 (17.0)	17 (7.0)	8 (5.4)	8 (7.5)

Table 7 Associations of SNPs with the risks of disease organ originated from different germ layers

Germ layer	SNP ID	Codominant			Dominant		Recessive		Log-additive	
		OR (95% CI)$_{het}$	OR (95% CI)$_{hom}$	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Ectoderm	rs1801133	0.80 (0.56-1.14)	0.58 (0.36-0.94)	0.075	0.73 (0.52-1.02)	0.07	0.67 (0.44-1.02)	0.057	0.77 (0.61-0.97)	0.024
(n=250)	rs1801394	0.83 (0.59-1.16)	0.33 (0.19-0.60)	4e-04	0.69 (0.50-0.95)	0.024	0.37 (0.21-0.64)	2e-04	0.66 (0.52-0.84)	6e-04
Mesoderm	rs1801133	0.80 (0.52-1.22)	0.64 (0.37-1.11)	0.26	0.75 (0.50-1.12)	0.16	0.73 (0.44-1.20)	0.2	0.80 (0.61-1.05)	0.1
(n=154)	rs1801394	0.87 (0.58-1.29)	0.26 (0.12-0.57)	7e-04	0.70 (0.48-1.03)	0.067	0.28 (0.13-0.60)	2e-04	0.64 (0.48-0.86)	0.0021
Endoderm	rs1801133	0.83 (0.52-1.32)	0.47 (0.24-0.92)	0.073	0.72 (0.46-1.12)	0.15	0.52 (0.28-0.98)	0.032	0.72 (0.52-0.98)	0.033
(n=106)	rs1801394	0.92 (0.58-1.44)	0.38 (0.17-0.85)	0.034	0.77 (0.50-1.19)	0.24	0.40 (0.19-0.86)	0.0099	0.72 (0.52-0.98)	0.036

het, heterozygote; hom, homozygote.

Discussion

The present study indicated that genetic variants of MTRR and MTHFR were associated with risks of multiple disease phenotypes presented on Chinese North population. Further, they conferred protective factors of ectoderm- and endoderm-developed tissue malformations. However, only MTRR genetic variation was a protective factor of mesoderm-developed tissue malformations.

Our data presented an implication that causation of birth defect and complications in humans could be resulted from the nutrition gene factors in mammals, some previous studies supported this viewpoint. During gastrulation the visceral endoderm defects were formed because of the mutation of the Slc40a1 gene encoding the iron transporter ferroportin1; and further the mutant embryo were developmentally delayed and exhibited exencephaly, microphthalmia and generalized edema (19), Moreover, Slc40a1 knockout mice results in embryonic lethality at E7.5 (20). Similarly, the failure of distal visceral endoderm migration and primitive streak formation were brought on deletion of mouse prickle1 gene, one of the core components of planar cell polarity signaling (21). Importantly, in humans a homozygous mutation PRICKLE1 caused an autosomal recessive progressive myoclonus epilepsy-ataxia syndrome, their further experiment validated that the mutation could reduce the effect of overexpression of wild type prickle1 and decreased gastrulation defects (22). Taken together, these lines of evidence demonstrate that in early stages of embryogenesis, mild mutation may result in the embryonic defects. In addition, the roles of both genes in visceral endoderm developing may be a clue for the present study in which ectoderm-developed defect seems to has a closer association to endoderm- than mesoderm-developed defect, because during gastrulation visceral endoderm and yolk sac provide for the nutritional support (including the uptake of folate) and exchange of waste products between the maternal circulation and the developing embryo (23).

In the present study the genetic variants rs1801394 and rs1801133 as important biological markers hint us the etiologies of NTDs and other malformations in various stages of development. During the embryogenesis, MTRR is transcriptionally expressed during metaphase I of oocyte to blastocyst in the pre-implantation development (24), and distributed in the neural tube and other tissues (25). MTRR knockout results in embryonic lethality and a hypomorph, with reduced MTRR activity by gene trap technology, adversely impacts reproductive outcomes and birth defects (25,26). The variant rs1801394 locus is in flavordoxin domain of MTRR gene and the mutation affects the affinity of MTRR to MTR (27). These indicated that rs1801394 is a functional genetic variant and potentially affects the early embryogenesis. MTHFR is expressed from metaphase I oocyte to senescence. Mild MTHFR deficiency caused by c.677C→T encodes a thermolabile enzyme with reduced activity (28). MTHFR knockout significantly decreased S-adenosylmethionine levels and increased S-adenosylhomocysteine levels with global DNA hypomethylation, and further show developmental retardation with cerebellar pathology and abnormal lipid deposition (29), which are consistent with our previous studies that in the NTDs population in Shanxi Province area, global DNA are hypomethylated (17,30,31). Taken together, these hint us that MTRR and MTHFR function spanning the embryogenesis, and their functional genetic variants potentially impact the embryo development at the early stage of embryogenesis, hence, result in complicated disease phenotypes.

In summary, our present study first reports an overall view about a subset of birth defect cases in Chinese northern population of Shanxi. And upon their complicated phenotypes, we use two genetic variants of MTRR and MTHFR, both of which play roles in early embryogenesis, to discover the probability of common tissue pathogenesis. The implication could be a reminder for our consideration about the etiology of birth defects, furthermore, the present study offers an experimental support for the supplementary of folic acid in the early stage of embryogenesis.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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