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RESEARCH PAPER

The maternal folate hydrolase gene polymorphism is associated with neural tube defects in a high-risk Chinese population

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Abstract Folate hydrolase 1 (*FOLH1*) gene encodes intestinal folate hydrolase, which regulates intestinal absorption of dietary folate. Previous studies on the association between polymorphisms rs202676 and rs61886492 and the risk of neural tube defects (NTDs) were inconclusive. A case–control study of women with NTD-affected pregnancies ($n = 160$) and controls ($n = 320$) was conducted in the Chinese population of Lvliang, a high-risk area for NTDs. We genotyped the polymorphic sites rs202676 and rs61886492 and assessed maternal plasma folate and total homocysteine (tHcy). Our results showed that in case group, plasma folate concentrations were 18 % lower compared with those of control group (8.32 vs. 6.79 nmol/L, $p = 0.033$) and tHcy concentrations were 17 % higher (10.47 vs. 12.65 μmol/L, $p = 0.047$). Almost all samples had the rs61886492 GG genotype (99.78 %). The result showed that the frequency of GG genotype in rs202676 was significantly higher in group with multiple NTDs than in controls ($p = 0.030$, OR = 2.157, 95 % CI, 1.06–4.38). The multiple-NTD group showed higher maternal plasma concentrations of tHcy (10.47 vs. 13.96 μmol/L, $p = 0.024$). The GG genotype of rs202676 had a lower maternal folate and higher tHcy concentrations than other genotypes with no significant differences. The result of structural prediction indicated that this variation might change the spatial structure of the protein. These results suggested that the maternal polymorphism rs202676 was a potential risk factor for multiple NTDs in this Chinese population. The allele G might affect maternal plasma folate and tHcy concentration.

Keywords Association study · Chinese population · *FOLH1* · Neural tube defects · Single-nucleotide polymorphism

Introduction

Neural tube defects (NTDs) are one of the most common and severe birth defects and caused by partial or complete failure of neural tube closure. NTDs occur with a high incidence of approximately 1 in 1000 worldwide (Feuchtbaum et al. 1999; van der Put et al. 2001) and 27.4 per 10,000 in China (Xiao 1989). In northern China, Shanxi Province appears to have the highest incidence, with a prevalence of 138.7 per 10,000 in 2003 (Li et al. 2006) and 199.38 per 10,000 in our recently completed survey (Gu et al. 2007). NTDs represent a major public health problem because of their mortality, morbidity, social cost, and the human suffering they cause. Therefore, much research has focused on their etiology, but the mechanism of NTDs remains elusive. One of the most promising clues to the causes of NTDs is that women who use folic acid periconceptionally have a 50–70 % reduced risk of NTD-

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affected pregnancies (Czeizel and Dudás 1992). Hence, to date, most of the research emphasis has been on evaluating folate-related genes as NTDs candidates (Molloy et al. 2009; Shang et al. 2008). The polymorphisms of partial folate-related genes have shown an association with NTDs risk in some studies (Barber et al. 2000; Botto and Yang 2000; Guéant-Rodriguez et al. 2003; Zhu et al. 2003). However, no specific folate-related gene has yet been implicated as a major determinant of NTDs risk.

The folate hydrolase 1 (*FOLH1*) gene encodes intestinal folate hydrolase, which regulates folate absorption by cleaving glutamates from dietary polyglutamyl folates. *FOLH1* is on chromosome 11p11.2 in human. A variant in this gene may be associated with impaired intestinal absorption of dietary folate. The *FOLH1* polymorphism rs202676 has been studied extensively in lung and colorectal tumor, but was not found to be associated with disease risk (Hazra et al. 2007; Liu et al. 2008). Our recent research showed that fetal rs202676 A>G polymorphism is a potential risk factor for anencephaly (Xie et al. 2012). As to the rs61886492 polymorphism which was first reported as a single-nucleotide polymorphism (SNP) of *FOLH1* that leads to reduced enzyme activity with subsequently decreased plasma folate concentration and increased total plasma homocysteine (tHcy) concentration (Devlin et al. 2000). Lievers et al. (2002) and Halsted et al. (2007) confirmed these results, and hyperhomocysteinemia is considered to be associated with increased NTDs risk. In contrast, Afman et al. (2003) found that the rs61886492 polymorphism contributed to a significantly higher plasma folate concentration. However, Morin et al. (2003) and Vieira et al. (2002) failed to find an effect of the maternal rs61886492 SNP on NTDs risk or metabolite concentration. Because of these conflicting results, we examined the association between the maternal *FOLH1* gene polymorphisms (rs61886492 and rs202676) and NTDs risk in the high-risk area of Lvliang, Shanxi Province, and determined folate and tHcy concentrations in maternal plasma. The objective was to assess the potential impact of the maternal *FOLH1* genotypes on the plasma parameters and on the risk of having an offspring with NTDs.

Methods

Subjects

The study was conducted in the Lvliang mountain area of Shanxi Province in northern China, which has a NTDs prevalence of 199.38 per 10,000 pregnancies, based on local epidemiologic surveillance data from January 2002 to December 2004 (Gu et al. 2007). Nine hospitals in Zhongyang, Jiaokou, Liulin, and Lin counties in the Lvliang area participated, including Zhongyang People's Hospital, Zhongyang Maternal and Child Health Hospital, Jiaokou People's Hospital, Jiaokou Maternal and Child Health Hospital, Liulin People's Hospital, Liulin Red Cross Hospital, Lin County Hospital, Lin County Traditional Chinese Medical Hospital, and Lin County Maternal and Child Health Hospital.

A case–control study was designed to investigate the relationship between *FOLH1* polymorphisms and NTDs occurrences. From March 1, 2004, to April 30, 2009, we recruited pregnant women to NTD-affected pregnancy group and normal pregnancy group (case:control = 1:2), from women who were receiving prenatal health care or had delivered in the above hospital and agreed to participate in our study. All participants were Han Chinese. For case group, women who were diagnosed with NTD fetus by B-type ultrasonic inspection were recruited as cases. And the diagnosis was ascertained after the pathological diagnosis was made. The diagnosis was made by experienced pathologists according to the International Classification of Disease Tenth Revision Codes of Q00 anencephaly, Q05 spina bifida, and Q01 encephalocele. According to the Nakatsu et al.'s (2000) proposed multisite closure model for human embryos, we stratified our cases into two groups: single NTDs referred to cases whose neural tube was unclosed at one initiation site, and multiple NTDs were cases whose neural tube was unclosed at more than one initiation site. Almost all mothers of affected pregnancies in the nine hospitals were invited to participate in the study. For control group, women who had a live-born infant with no identified structural malformation after 1-year follow-up and who aborted for nonmedical reasons were ascertained as controls. The aborted fetus also underwent the pathological anatomy. Any fetuses displaying pathological malformations or intrauterine growth retardation were excluded from the control group. When pregnant women were recruited, the medical staffs collected clinical information about the subjects.

Fasting blood samples from pregnant women were collected just before abortion. Fasting blood samples of controls that had live-born babies were collected when they delivered. We followed a previous approach (Wang et al. 2010). Generally, 2 ml venous blood was collected into purple-top evacuated tubes (without anticoagulant; BectonDickinson). Blood samples were immediately centrifuged at 2,500 rpm for 10 min, and the separated plasma was aliquoted (without reducing agent) and stored at −20° in local hospitals before shipping, on ice, to the study laboratories. Samples were not thawed until analysis. This study was reviewed and approved by the ethic evaluation committee of Capital Institute of Pediatrics. All participants provided written informed consent.

DNA extraction

Genomic DNA was extracted from frozen blood samples using the Blood and Tissue DNA Kit (Qiagen, Germany) according to the manufacturer's instruction and was subsequently used for genotyping. DNA concentration and purity were determined by absorbance at 260 and 280 nm.

Biochemical measures

We followed the protocol of our previous study (Wang et al. 2010) to detect the plasma concentration of folate and tHcy. Briefly, Plasma folate was measured by using a competitive receptor binding immunoassay (Chemiluminescent Immunoenzyme Assay Access Immunoassay system II; Beckman Coulter, Krefeld, Germany). 200 µl plasma was used for detecting the plasma folate level. tHcy was measured with a Hitachi Model 7170A automatic analyzer (Hitachi, Tokyo, Japan) with the homocysteine assay kit (Jiu-Qiang Company, Beijing, China), and 200 µl plasma was needed. The intraassay coefficient of variability (CV) for folate and tHcy was 3.8–6.5 and 2.6–4.0 %, respectively. We selected 64 subjects from cases and controls, respectively. None of the subjects who undertook biochemical detection had taken periconceptional folic acid supplements. The population of these selected subjects was similar to the original groups.

Genotyping using direct sequencing

Genotyping was conducted by the experienced technician who was blinded to the diagnosis. PCR primers were designed based on the human *FOLH1* genomic sequence, to amplify a fragment containing the polymorphic regions (rs61886492: forward primer: TGTGAAGATGTGATGTC ATA and reverse primer: CAGGAAACTACACTCTGAG A; rs202676: forward primer: ACTCCTGCTCTAAACC TCTGTAAT and reverse primer: ATCTCGTTTACACCC ATTAGTTG). Reactions for rs61886492 were: 94 °C for 10 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 15 s, and 72 °C for 15 s, then 72 °C for 10 min, and cooling to 4 °C. Reactions for rs202676 were: 94 °C for 10 min, followed by 35 cycles of 94 °C for 20 s, 57 °C for 20 s, and 72 °C for 30 s, then 72 °C for 10 min, and cooling to 4 °C. PCR products were subjected to direct sequencing using an ABI3700 sequencer (Applied Biosystems). Sequencing results were exported to the Mutation Surveyor, version 3.25 (Softgenetics, State College, PA; http://www.softgenetics.com), for genotype analysis. To ensure genotyping consistency, 10 % of samples were regenotyped.

Statistical analysis

Lifestyle and sociodemographic characteristics of case and control subjects were compared using a chi-square (χ^2) test for categorical variables. Hardy–Weinberg equilibrium was tested by χ^2 test. *FOLH1* gene genotype or allele frequency differences between case and control groups were tested by χ^2 test or Fisher's exact test. Odds ratios (OR) with a 95 % confidence interval (CI) were calculated to estimate the risks related to polymorphisms and NTDs. Plasma folate and tHcy concentrations were skewed and normalized by logarithmic transformation for all analyses. An independent T test and linear regression model were used to compare biochemical parameters between the groups. Analyses were performed using R 2.11.0. All p values were two-sided, and $p < 0.05$ was considered significant.

Results

We recruited 160 cases and 320 matched controls. Among the control subjects, five declined participation and three were excluded for pathological malformations or intrauterine growth retardation, for a study participation rate of 98.33 %. For rs61886492, we obtained 466 satisfactory DNA samples (160 cases; 306 controls) for genotyping and 156 cases and 300 controls were successfully genotyped. For the polymorphism rs202676, 148 cases and 299 controls were successfully genotyped. The genotyping call rate was 96.89 % across all samples. Regenotype results showed 100 % concordance. Among the 456 genotyped subjects for rs61886492, 455 samples gave a homozygous GG genotype. Only one heterozygote GA occurred in the case group whose fetus phenotype was anencephalus; therefore, we did not proceed with further analysis. The characteristics of the study subjects for rs202676 are in Table 1. The gestational week of controls was lower than those in cases because some pregnant women aborted for nonmedical reasons during early pregnancy. The age, education level, gravidity, parity, and periconceptional folic acid used were not different between cases and controls.

For results of the biochemical detection, using an independent T test, we found that the NTD group had a significantly lower level of plasma folate ($p = 0.033$) and a higher level of tHcy ($p = 0.047$) as shown in Table 2. We stratified the NTD group into single-NTD group and multiple-NTD group and compared the two groups with the control group. We found that the plasma folate concentration from the single-NTD group was significantly lower than in the control group ($p = 0.042$), and the plasma tHcy level in the multiple-NTD group was significantly higher ($p = 0.024$) than in the control group (Table 2). As

Table 1 Characteristics of study participants

Characteristic	Case (148[a]) *n* (%)[b]	Control (299[a]) *n* (%)[b]	p^c
Age (year)	144	298	0.096
<20	9 (6.25)	22 (7.38)	
20–29	112 (77.78)	203 (68.12)	
>29	23 (15.97)	73 (24.50)	
Educational level	138	269	
<Middle school graduation	14 (10.14)	25 (9.29)	0.157
Middle school graduation	106 (76.81)	188 (69.89)	
>Middle school graduation	18 (13.04)	56 (20.81)	
Gravidity (*n*)	137	269	
1	9 (6.57)	27 (10.04)	0.507
2	15 (10.95)	29 (10.78)	
≥3	113 (82.48)	213 (79.18)	
Parity (*n*)	137	269	
0	14 (10.22)	27 (10.04)	0.530
1	10 (7.30)	29 (10.78)	
≥2	113 (82.48)	213 (79.18)	
Periconceptional folic acid use[d]	123	254	
No	116 (94.31)	246 (96.85)	0.265
Yes	7 (5.69)	8 (3.15)	
Gestational week	145	291	
<21	82 (56.55)	203 (69.76)	0.016
21–29	41 (28.28)	63 (21.65)	
≥30	22 (15.17)	25 (8.59)	

[a] Referes to the number of subject

[b] Percentages may not equal 100 because of rounding

[c] Chi-square test was used to calculate the *p* values

[d] The "periconceptional" refers to the month before conception and the first 3 months after conception

Table 3 Genotype distributions and allele frequencies of rs202676 in *FOLH1* between cases and controls

Genotype/ allele	Cases (%)[a]	Controls (%)[a]	OR (95 % CI)	p^b
	148	299		
AA	58 (39.19)	118 (39.46)		0.679
AG	69 (46.62)	147 (49.16)	0.96 (0.62,1.46)	
GG	21 (14.19)	34 (11.37)	1.26 (0.67,3.34)	
A	185 (62.50)	383 (64.05)		0.658
G	111 (37.50)	215 (35.95)	1.07 (0.80,1.43)	

[a] Percentages may not equal 100 because of rounding

[b] Chi-square test was used to calculate the *p* values

maternal age is an underlying factor affecting plasma folate and tHcy concentrations, we performed a linear regression model to adjust the factor. The difference in plasma folate and tHcy between cases and controls was of borderline significance statistically (plasma folate: $p = 0.052$; OR = 0.157; 95 % CI, 0.024–1.025; plasma tHcy: $p = 0.075$; OR = 5.073; 95 % CI, 0.850–30.266). The difference in biochemical result between controls and either single-NTD group or multiple-NTD group was also of borderline significance.

The rs202676 polymorphism was in Hardy–Weinberg equilibrium (data not shown), and the allele and genotype distribution showed no significant differences between the NTD and control groups (Table 3). Table 4 shows the plasma folate and tHcy levels for the different genotypes. Compared with the other genotypes, mothers with the GG genotype had the lowest plasma folate and the highest tHcy levels, although this difference was not significant.

We further analyzed the potential associations to compare the genotype frequency based on the number of initiation sites involved. We found that the frequency of the GG type was significantly higher in the multiple-NTD group than in the control group (Table 5).

The crystal structure of *FOLH1* (PDB ID 3BI1) was downloaded from Protein Data Bank (PDB) (RCSB) and analyzed (Rose et al. 2011). The molecular structure was

Table 2 Plasma concentration of folate and tHcy in NTDs subtypes and controls

Group	Folate (nmol/L)		tHcy (μmol/L)	
	Mean ± SD (n^a)	P_5–P_{95}^b	Mean ± SD (n^a)	P_5–P_{95}^b
Control	8.32 ± 1.68 (64)	3.97,24.66	10.47 ± 1.73 (54)	3.97,24.66
NTDs	6.97 ± 1.49[c] (64)	3.23,10.89	12.65 ± 1.61[d] (64)	6.70,35.60
Single NTDs	6.81 ± 1.49[e] (40)	3.19,16.44	11.75 ± 1.61 (39)	6.57,38.80
Multiple NTDs	7.25 ± 1.45 (24)	3.22,13.71	13.96 ± 1.56[f] (25)	6.10,35.55

[a] *n* refers to the number of subjects

[b] P_5–P_{95}, 5th–95th percentile

[c, d, e, f] Significantly different compared with the controls (Student's *t* test): $p = 0.033$; $p = 0.047$; $p = 0.042$; $p = 0.024$

Table 4 Plasma concentration of folate and tHcy according to rs202676 genotypes

Genotypes	Folate (nmol/L)		tHcy (μmol/L)	
	Mean ± SD (n^a)	P_5–P_{95}^b	Mean ± SD (n^a)	P_5–P_{95}^b
AA	7.46 ± 1.08 (51)	3.42, 16.88	11.71 ± 1.57 (48)	5.06, 24.78
AG	7.78 ± 1.61 (66)	3.53, 18.48	11.44 ± 1.81 (60)	3.81, 34.92
GG	7.37 ± 1.32 (11)	4.59, 10.72	12.02 ± 1.37 (10)	7.20, 18.50

[a] *n* refer to the number of subjects

[b] P_5–P_{95}, 5th–95th percentile

Table 5 The distribution of NTDs subtypes in different rs202676 genotypes

Genotype	Control	Single NTDs (%)	Multiple NTDs(%)
AA + AG	265 (88.63)	80 (90.91)	47 (78.33)
GG	34 (11.37)	8 (9.01)	13 (21.67)[a]
Total	299	88	60

[a] GG genotype is significantly associated with multiple NTDs compared with AA + AG group; chi-square test: $p = 0.030$, OR = 2.157, 95 % CI [1.059, 4.378]

calculated and visualized by UCSF Chimera (Pettersen et al. 2004). Based on the structure (Fig. 1), residue Y75 is located at the region of alpha helix (in the red box) and also located at the surface of the protein (solvent accessibility: 50 %; data from PDB). The G allele of rs202676 resulted in amino acid change of neutral tyrosine (pI = 5.66) to basic histidine (pI = 7.59), which would change the number of the alpha helix (from 266 to 259), extended strand (from 110 to 105), random coil (from 307 to 318), and beta turn (from 36 to 37) and then lead to the variation in the secondary structure as well as tertiary structure and thus affect its binding ability to polyglutamyl folates.

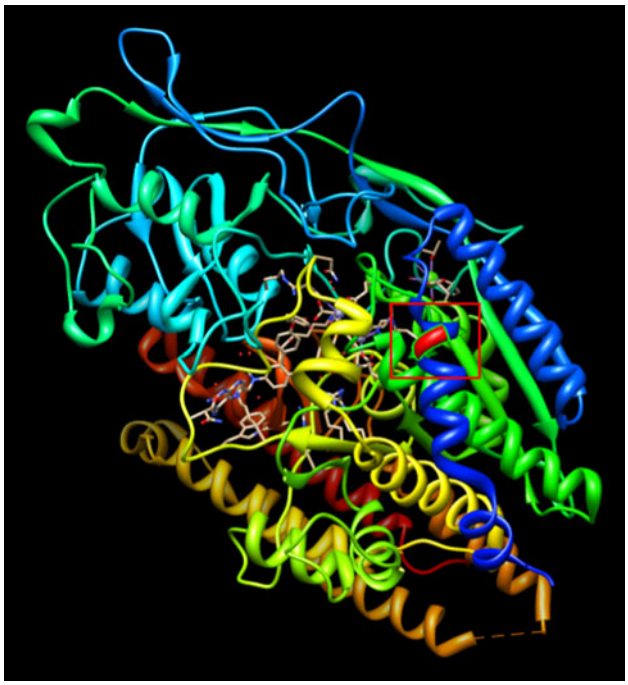

Fig. 1 The molecular structure of *FOLH1*. The residue Y75 (marked in *red*) is located at the region of alpha helix

Discussion

Folate has been established as essential for normal development of the fetal nervous system (Zhang XM et al. 2009), and this study was conducted in a rural mountain area with long-term poor nutritional conditions. The local residents seldom take folate supplements, and the percentage of periconceptional folic acid usage was low, with 5.7 % in cases and 3.2 % in controls. Using an independent *T* test, we observed significantly lower plasma folate and higher tHcy levels in the NTD group compared with the control group, which was consistent with the previous studies of our group in this region (Zhang T et al. 2009), suggesting that folate insufficiency was involved in NTD-affected pregnancies in this Chinese Shanxi mountain area with a high-risk population. The difference was of borderline significance after adjusting for the maternal age, possibly due to the small sample size for biochemical measurement. Folate is an important factor for a number of metabolic pathways in cells, which provides the one-carbon units required for purine and thymidylate syntheses and for methylation of a wide variety of essential biological substances, including phospholipids, proteins, DNA, and neurotransmitters; any disturbance in the above processes may affect normal embryonic development (Jacob 2000). The results were also similar with other regions, such as Dublin and northern UK (Sutton et al. 2011; Relton et al. 2004). Plasma folate is a marker of recent folate intake, which is significantly influenced by fasting; whole blood (intracellular) folate would have been not only a better marker for intake, but also a functional biomarker for folate function and may have reflected total plasma homocysteine findings. Due to the experimental limitations, fasted venous plasma was used for biochemical detection in our study. We plan to improve our protocol in the future.

Multiple initiation closure sites of the neural tube have been observed in mice (Copp et al. 2003). Nakatsu et al. observed a substantial number of normal neurulation processes in humans and confirmed that the neural tube in human embryos also initiates at multiple sites. Based on observations of human embryos with fusing neural tubes (CS 10–12) (Nakatsu et al. 2000), Nakatsu et al. proposed three initiation closure sites: the future cervical region (Site A), the boundary between the mesencephalon and rhombencephalon (Site B), and the rostral end of the neural groove (Site C). From the initiation site, fusion extends both rostrally and caudally, reaching the anterior and caudal neuropore of the embryo and then completing the neural tube closure. We classified NTDs based on the Nakatsu classification and compared the plasma folate and tHcy levels between single NTDs, multiple NTDs, and controls and found lower folate level and higher tHcy levels in the multiple-NTD group (Table 2). Possibly because of the sample size of our study, we did not find the statistically significant difference.

This is the first analysis of the maternal *FOLH1* gene polymorphisms rs61886492 and rs202676 conducted in a Chinese population in the Lvliang area, which has a high risk of NTDs. Our results showed that the GG genotype of rs202676 was significantly associated with multiple NTDs compared with AA + AG, indicating the *FOLH1* gene rs202677 polymorphism was a potential risk factor for multiple NTDs, and the effect of the risk allele had a recessive inheritance pattern. Pregnant women who were homozygous for the risk allele were significantly more susceptible to multiple-NTD-affected pregnancies than controls, suggesting a possible association between NTDs severity and the maternal *FOLH1* polymorphism. Since the multiple NTDs manifested the phenotype with malformations at two or more initiation sites, they were more severe than the single NTDs, which may imply a different mechanism of susceptibility. We attempted to further analyze the relationship between the polymorphisms and specific initiation sites and failed to find an association (data not shown). We hope to explore site-specific associations in future investigations. On the other hand, *FOLH1* plays a role in N-acetylated-alpha-linked-acidic dipeptidase activity, and this polymorphism A>G may cause disorder in glutamate metabolism. In our previous study, we found that the fetal *FOLH1* rs202676 A>G is a potential risk factor for anencephaly. The result suggested the *FOLH1* rs202676 A>G might disturb the development of neural tube by influencing the glutamate release and folate absorption, resulting in NTDs.

To further understand how the polymorphisms affected gene function, we obtained the crystal structure of *FOLH1* from PDB, and the predictive secondary structure showed that Y75H mutation would change the spatial structure as well as the charge of the protein surface, and thus would affect its binding potential to the ligand and cause misregulation of folate absorption, resulting in low blood folate and consequent hyperhomocysteinemia. In this study, although nonsignificant, the GG genotype showed the lowest folate and the highest tHcy concentration. We proposed that this might be related to the dysfunction of the folate hydrolase from the rs202676 polymorphism. A larger sample size as well as the functional experiment was needed to confirm the presumption. On the other hand, FOLH1 plays a role in hydrolysis of N-acetyl-l-aspartyl-l-glutamate (NAAG) and the release of glutamate. This polymorphism A>G may cause decreasing hydrolysis of NAAG and the release of glutamate (Xie et al. 2012). We suggested the FOLH1 rs202676 A>G might disturb the development of neural tube by influencing the maternal folate absorption and glutamate release in fetal brain, resulting in NTDs.

In 1998, Falconer and Carter described a relationship between genetic predisposition, environmental factors, and NTDs in a model called the threshold model of multigenic inheritance (Gos and Szpecht-Potocka 2002). According to that model, poor environmental conditions act on a person with a specific genetic predisposition, possibly leading to threshold infringement, after which a specific effect arises, in this case a NTD. In our study of a population with folate insufficiency, *FOLH1* may act as the genetic predisposition that contributes to the occurrence of NTDs. Since many possible pathogenic genes have been found so far, and *FOLH1* is only one, we hypothesized that in the Lvliang mountain area, poor environmental conditions are a part of a cumulative effect of several interchangeable genetic predispositions, leading to the extreme high incidence of NTDs in that area. The poor environmental conditions may include poor diet, low education level, and low income. The genetic predisposition of the area requires further exploration. We also noted the complex relationship between genetic and environmental factors that must be determined to find the real mechanism of the NTDs in that area.

In conclusion, this study supports the model that lower plasma folate concentrations are involved in NTD-affected pregnancies and the polymorphism rs202676 is a potential risk factor for multiple NTDs in the Shanxi mountain area, which has a population with a high risk of NTDs. The rs202676 G risk allele may affect protein contact with ligand. A study with a larger sample size is needed to further confirm the relationship between the polymorphism and specific initiation site defects.

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Conflict of interest The authors declare no conflict of interests.

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