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An epidemiologic study of mitochondrial membrane transporter protein gene polymorphism and risk factors for neural tube defects in Shanxi, China****☆

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Abstract

The present study involved a questionnaire survey of 156 mothers that gave birth to children with neural tube defects or had a history of pregnancy resulting in children with neural tube defects (case group) and 156 control mothers with concurrent healthy children (control group) as well as detection of mitochondrial membrane transporter protein gene [uncoupling protein 2 (UCP2)] polymorphism. The maternal UCP2 3' untranslated region (UTR) D/D genotype and D allele frequency were significantly higher in the case group compared with the control group (odds ratio (OR) 3.233; 95% confidence interval (CI) 1.103–9.476; $P = 0.040$; OR: 3.484; 95% CI: for neural tube defects 2.109–5.753; $P < 0.001$). Univariate and multivariate logistic regression analysis of risk factors for neural tube defects showed that a maternal UCP2 3' UTR D/D genotype was negatively interacted with the mothers' consumption of frequent fresh fruit and vegetables ($S = 0.007$), positively interacted with the mothers' frequency of germinated potato consumption ($S = 2.15$) and positively interacted with the mothers' body mass index ($S = 3.50$). These findings suggest that maternal UCP2 3' UTR gene polymorphism, pregnancy time, consumption of germinated potatoes and body mass index are associated with an increased risk for neural tube defects in children from mothers living in Shanxi province, China. Moreover, there is an apparent gene-environment interaction involved in the development of neural tube defects in offspring.

Key Words: neural tube defects; uncoupling protein 2; genetic polymorphisms; risk factors; interaction

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INTRODUCTION

Neural tube defects (NTDs) are common and severe congenital malformations which result from the failure of neural tube closure during embryogenesis in any area of the rostrocaudal axis, and their incidence differs among various populations^[1-2]. The incidence of NTDs in Shanxi province in northern China is the highest in the world^[3-5]. Numerous studies of NTD development have implicated socioeconomic class, nutritional status and other predisposing factors such as teratogens, maternal diabetes, obesity, family history of NTDs and genes involved in folate metabolism. In addition, there is considerable evidence that genetic and environmental factors contribute toward the etiology of NTDs^[6-9]. Uncoupling protein 2 (UCP2) is a member of a family of mitochondrial transporter proteins that are responsible for uncoupling the transport of protons across the inner

mitochondrial membrane from electron transport and the synthesis of adenosine triphosphate from adenosine diphosphate, resulting in the generation of heat rather than energy. A number of recent case-control association studies have shown significant results regarding the association of UCP2 gene polymorphisms with fat metabolism, obesity and type 2 diabetes^[10-28]. Furthermore, there has been speculation that diabetic and obese women have numerous metabolic abnormalities that may increase the risk of NTDs. A variation in uncoupling proteins may lead to an accumulation of reactive oxygen species, thereby affecting the expression of transcription factors involved in the regulation of neural tube development^[25, 29]. Because of the roles that UCP2 variants play in energy metabolism, body weight regulation and preventing the accumulation of reactive oxygen species, which have been implicated as risk factors for NTDs, these UCP2 variants are potential

candidates for the screening of NTDs because these variants may increase susceptibility to obesity and diabetes. Volcik *et al*^[30] reported an association between a UCP2 dual genotype and spina bifida in a case-control analysis in the Californian population in the USA. Infants that were homozygous for both the A55V (V/V) and 3' untranslated region (UTR) 45 bp deletion/insertion polymorphisms (D/D) in exon 8 had more than a three-fold higher risk of spina bifida occurrence. However, in a study of the Irish population, UCP2 polymorphism did not influence the risk of NTDs^[31].

Considering the previous studies and other results that implicate UCP2 variants in obesity, type 2 diabetes and gene expression, we hypothesize that maternal UCP2 polymorphism is a risk factor for NTDs, which can also be affected by environmental factors including the nutrition of mothers in Shanxi province. We investigate two polymorphisms, A55V and the 3' UTR 45 bp D/I of UCP2 and other factors including environmental and nutritional factors that contribute toward the development of NTDs in children born in Shanxi province in northern China using 1: 1 age-matched case-control analyses. Moreover, we evaluate the interactions between UCP2 polymorphisms and other NTD risk factors to elucidate whether the UCP2 gene and environmental factors have combined effects on NTD occurrences in Shanxi province. In addition, we provide epidemiological evidence for identifying genetic markers of NTDs to further our understanding of the etiology of NTDs.

RESULTS

Baseline analysis of participants

A total of 156 women with a history of pregnancy affected by NTDs as diagnosed with ultrasound and fetal autopsy reports were selected as the case group. The control group consisted of 156 women with similar economic conditions, identical nationality, similar education level, a gestational age difference of ± 4 weeks, an age difference of ≤ 3 years and no history of pregnancies affected by NTDs. All subjects were of Han origin and had lived in Lüliang and Changzhi districts in northern China for more than 20 years. There were no significant differences between the control and case groups in terms of age, periconceptual folic acid use, education level and place of residence ($P > 0.05$). Data collected from all subjects were included into the final analysis, and all subjects participated in the conclusion of the study (Table 1).

UCP2 gene polymorphism detection and Hardy-Weinberg equilibrium matching results

PCR was used to test the polymorphism of UCP2 3'UTR and A55V. Chi-square test was used to prove the Hardy-Weinberg equilibrium in the control group. The observed values for the 3' UTR locus in the control group ($\chi^2 = 3.641$, $P = 0.056$) matched

expected values for Hardy-Weinberg equilibrium, as did the A55V locus in the control group ($\chi^2 = 3.733$, $P = 0.053$). These data indicate that there were no obvious effects of natural selection and migration on genetic equilibrium.

Table 1 Comparison of baseline data between case and control groups

Item	Case group	Control group	P
n	156	156	
Age (mean \pm SD, year)	26.9 \pm 3.8	24.8 \pm 4.3	0.066
Education level [n (%)]			0.433
< high school graduation	129(82.6)	135(86.5)	
\geq high school graduation	27(17.4)	21(13.5)	
Periconceptual folic acid use [n (%)]			0.503
Yes	133(85.2)	138(88.5)	
No	23(14.8)	18(11.5)	

UCP2 genotype distribution and allele frequency

The genotype/allele frequency difference for the UCP2 gene between case and control groups was evaluated by a chi-square test. The odds ratio (OR) with a 95% confidence interval (CI) was calculated to estimate the risk of NTDs in relation to UCP2 gene polymorphism. The results are presented in Table 2. Genotype distribution analyses between case and control groups showed significant differences for the 3' UTR D/I polymorphism and no significant differences for the A55V polymorphism. The genotype frequency of UCP2 3' UTR D/D was significantly higher in the case group than that of the control group (OR: 3.233; 95% CI: 1.103–9.476; $P < 0.001$) and the frequency of the D allele was also significantly higher in the case group (OR: 3.484; 95% CI: 2.109–5.753; $P < 0.05$). There were no differences in the A55V genotype and allele frequency between case and control groups ($P > 0.05$).

Conditional logistic regression analysis of NTD risk factors

Sixteen meaningful factors were concluded *via* conditional logistic regression univariate analysis of the risks for NTD occurrence in offspring (Table 3). The risk factors were further analyzed by a conditional logistic regression multivariate analysis model. Four risk factors including maternal pregnancy time (OR = 3.168, 95% CI = 1.518–6.610), germinated potato consumption (OR = 7.280, 95% CI = 2.356–22.495), body mass index (BMI) (OR = 1.420, 95% CI = 1.102–1.829) and UCP2 3' UTR D/D polymorphism (OR = 2.815, 95% CI = 1.395–5.641) were concluded to be risk factors for NTDs in offspring based on the results shown in Table 4.

Genetic and environmental interaction

From the directly estimated interaction index, we analyzed the interaction between UCP2 3' UTR D/I polymorphism, BMI, germinated potato consumption as well as fruit and vegetable consumption.

Table 2 Comparisons of genotype distribution [*n* (%)] and allele frequency [*n* (%)] between control and case groups and their association with NTDs

Marker	Genotype/allele	Controls (<i>n</i> = 156)	Cases (<i>n</i> = 156)	χ^2	OR	95%CI	<i>P</i>
3'UTR D/I	I/I	12(7.7)	5(3.8)		1		
	D/I	46 (29.4)	18(12.2)	17.971 ^a	0.826	0.271–2.522	0.776
	D/D	98(62.8)	133(83.9)		3.233	1.103–9.476	0.040 ^a
	I	70(22.4)	23(7.6)		1		
A55V	D	242(77.6)	277(92.3)	25.888 ^a	3.484	2.109–5.753	< 0.001 ^b
	V/V	17(10.9)	10(6.4)		1		
	A/V	85(54.5)	86(55.1)		1.720	0.745–3.971	0.220
	A/A	54(34.6)	60(38.5)	2.136	1.889	0.797–4.478	0.199
	V	119(38.1)	77(33.2)		1		
	A	193(61.9)	155(66.8)	1.415	1.241	0.869–1.772	0.242

Genotype/allele frequency difference for the UCP2 gene between case and control groups was evaluated by a chi-square test. An odds ratio (OR) with a 95% confidence interval (CI) was calculated to estimate the risk of NTDs in relation to UCP2 gene polymorphism. ^a*P* < 0.05, vs. I/I genotype; ^b*P* < 0.001, vs. I allele. UTR D/I: Untranslated region deletion/insertion; NTDs: neural tube defects; UCP2: uncoupling protein 2.

Table 3 Conditional logistic regression univariate analysis of NTDs susceptible risk factors

Variable	β	SE	Wald χ^2	<i>P</i>	OR	95%CI
Weight before pregnancy	0.103	0.028	13.144	0.000 ^b	1.108	1.048–1.171
Gravidity	1.267	0.285	19.759	0.000 ^b	3.550	2.031–6.206
History of NTD-affected-pregnancy	3.978	1.856	4.591	0.032 ^a	53.393	1.404–2031.0
History of abortion	1.649	0.488	11.398	0.001 ^b	5.200	1.977–13.541
Family history of diabetes	1.012	0.584	3.002	0.083	2.750	0.876–8.636
Gestational fever	2.708	1.033	6.875	0.009 ^b	15.000	1.981–113.556
Gestational vomit	0.511	0.327	2.446	0.118	1.667	0.879–3.161
Threatened abortion	1.335	0.503	7.055	0.008 ^b	3.800	1.419–10.177
Gestational anemia	0.693	0.548	1.602	0.206	2.000	0.684–5.851
Pesticide exposure	2.197	1.054	4.345	0.037 ^a	9.000	1.140–71.038
Fertilizer exposure	1.609	0.775	4.317	0.038 ^a	5.000	1.096–22.820
Antimicrobial exposure	1.179	0.572	4.249	0.039 ^a	3.250	1.060–9.967
Vegetable intake	-0.539	0.336	2.569	0.109	0.583	0.302–1.128
Fruit intake	-0.758	0.313	5.863	0.015 ^a	0.469	0.254–0.866
Germinated potato intake	1.040	0.375	7.687	0.006 ^b	2.828	1.356–5.899
Passive smoking	0.695	0.294	10.791	0.001 ^b	2.625	1.476–4.669
Body mass index	0.312	0.079	15.722	0.000 ^b	1.365	1.171–1.594

Sixteen meaningful factors were concluded through conditional logistic regression univariate analysis on the risk of NTDs infants. ^a*P* < 0.05, vs. control group; ^b*P* < 0.01, vs. control group. All the variables refer to the mother just before or during pregnancy. SE: Standard error; OR: odds ratio; CI: confidence interval; NTD: neural tube defect.

Table 4 Conditional logistic regression multivariate analysis of NTD risk factors

Variable	β	SE	Wald χ^2	<i>P</i>	OR	95%CI
Gravidity	1.153	0.375	9.439	0.002	3.168	1.518–6.610
Germinated potato intake	po-1.985	0.576	11.897	0.001	7.280	2.356–22.495
BMI	0.350	0.129	7.340	0.007	1.420	1.102–1.829
UCP2 3'UTR D/I	0.316	0.121	6.855	0.009	1.372	1.082–1.738
UCP2 3'UTR D/D	1.035	0.358	8.345	0.003	2.815	1.395–5.641

Data via conditional logistic regression univariate analysis of the risk of NTD infants. UCP2: Uncoupling protein 2; BMI: body mass index; SE: standard error; OR: odds ratio; CI: confidence interval; NTDs: neural tube defects; UTR: untranslated region.

Analysis of mothers with either a I/I, D/I genotype or low germinated potato consumption (< three times per week) representing the reference group and marked as 0, and mothers with a D/D genotype or frequent germinated

potato consumption (\geq three times per week) marked as 1, then interactions between the maternal UCP2 3' UTR D/D polymorphism and frequent germinated potato consumption were observed because the combined effects on the risk for NTD occurrence were stronger compared with those of the individual effects. Using an interaction additive model, the relative excess risk of interaction (RERI), synergy index (S) and attributable proportion of infection (API) showed that there was synergy between the maternal UCP2 3' UTR D/D polymorphism and frequent germinated potato consumption (RERI > 0, S > 1) and the synergy attributed 43% toward NTD-affected infants (API = 0.43). Results are listed in Table 5.

Analysis of mothers with either I/I, D/I genotypes or a BMI \leq 22 kg/m² representing the reference group and marked as 0, and mother with the D/D genotype or BMI > 22 kg/m² marked as 1, then interactions were observed between the maternal UCP2 3' UTR D/D genotype and

BMI > 22 kg/m². The results of RERI, S and API showed that there was synergy between the maternal UCP2 3' UTR D/D polymorphism and BMI > 22 kg/m² (RERI > 0, S > 1) and the synergy attributed 63% toward NTD-affected infants (API = 0.63). Results are listed in Table 6.

Table 5 Interaction between UCP2 3' UTR I/D polymorphism and germinated potato consumption and the odds ratio for NTDs

D allele	Germinated potato intake	Cases	Controls	OR
0	0	14	39	1 (OR ₀₀)
1	1	38	20	5.29 (OR ₁₁)
1	0	75	78	2.68 (OR ₁₀)
0	1	9	19	1.32 (OR ₀₁)

$$OR_{11} - 1.00 > (OR_{01} - 1.00) + (OR_{10} - 1.00)$$

$$RERI = OR_{11} - (OR_{01} + OR_{10}) + 1 = 5.29 - (2.68 + 1.32) + 1 = 2.29 > 0$$

$$S = (OR_{11} - 1) / ((OR_{01} - 1) + (OR_{10} - 1)) = 11.400 / 3.804 = 2.15 > 1$$

$$API = \{OR_{11} - (OR_{01} + OR_{10}) + 1\} / OR_{11} = 2.29 / 5.29 = 0.43$$

0: Mothers with either I/I or D/I genotypes or low germinated potato consumption (1–2 times/week) are marked as 0 and represent the reference group. 1: Mothers with the D/D genotype or frequent germinated potato consumption (≥ three times per week) are marked as 1.

UCP2: Uncoupling protein 2; NTDs: neural tube defects; OR: odds ratio; RERI: relative excess risk of interaction; S: synergy index; API: attributable proportions of interaction; UTR: untranslated region.

Table 6 Interaction between UCP2 3' UTR I/D, BMI and the odds ratio for NTDs

D allele	BMI	Cases	Controls	OR
0	0	9	31	1 (OR ₀₀)
1	1	50	18	9.57 (OR ₁₁)
1	0	63	81	2.67 (OR ₁₀)
0	1	14	27	1.78 (OR ₀₁)

$$OR_{11} - 1.00 > (OR_{01} - 1.00) + (OR_{10} - 1.00);$$

$$RERI = OR_{11} - (OR_{01} + OR_{10}) + 1 = 6.12 > 0;$$

$$S = (OR_{11} - 1) / ((OR_{10} - 1) + (OR_{01} - 1)) = 3.50 > 1;$$

$$API = \{OR_{11} - (OR_{01} + OR_{10}) + 1\} / OR_{11} = 0.63$$

0: Mothers with either I/I or D/I genotypes or BMI ≤ 22 kg/m² are marked as 0 and represent the reference group. 1: Mothers with the D/D genotype or BMI > 22 kg/m² are marked as 1.

OR: Odds ratio; RERI: relative excess risk of interaction; S: synergy index; API: attributable proportions of interaction; NTDs: neural tube defects; BMI: body mass index; UTR: untranslated region.

Results of the interaction between the 3' UTR D/I polymorphism and fresh fruit and vegetable consumption are listed in Table 7. Analysis of mothers either with I/I, D/I genotypes or low fruit and vegetable consumption (< three times per week) representing the reference group and marked as 0, and mothers with the D/D genotype or frequent fruit and vegetable consumption (≥ three times per week) marked as 1, then the combined effects of the maternal UCP2 3' UTR D/D genotype and frequent fruit and vegetable consumption (≥ three times per week) on the risk for NTD occurrence were weaker

compared with those of the individual effects. Moreover, there was antagonism between frequent fruit and vegetable consumption and D/D susceptibility alleles (RERI < 0, S < 1).

Table 7 Interaction between UCP2 3' UTR I/D polymorphism and fresh fruit and vegetable consumption and the odds ratio for NTDs

D allele	Fruit and vegetable intake	Cases	Controls	OR
0	0	11	21	1
1	1	48	86	1.066
1	0	65	12	10.34
0	1	12	27	0.85

$$OR_{11} - 1.00 < (OR_{01} - 1.00) + (OR_{10} - 1.00)$$

$$RERI = OR_{11} - (OR_{01} + OR_{10}) + 1 = -10.12 < 0$$

$$S = (OR_{11} - 1) / ((OR_{10} - 1) + (OR_{01} - 1)) = 0.007 < 1$$

$$API = \{OR_{11} - (OR_{01} + OR_{10}) + 1\} / OR_{11} = -9.49$$

0: Mothers with either I/I or D/I genotypes or low fresh fruit and vegetable consumption (1–2 times/week) are marked as 0 and represent the reference group. 1: Mothers with the D/D genotype or frequent fresh fruit and vegetable consumption (≥ three times per week) are marked as 1.

OR: Odds ratio; RERI: relative excess risk of interaction; S: synergy index; API: attributable proportions of interaction; NTDs: neural tube defects; UTR: untranslated region.

DISCUSSION

In this study, we analyzed the genotype and various characteristics of the mother because several studies have found that the maternal genotype is responsible for the environment in which the embryo develops^[32-34]. From our results, we found that the maternal UCP2 3' UTR D/I polymorphism, BMI, pregnancy time and germinated potato consumption may be major risk factors for NTDs in offspring, while frequent consumption of fruit and vegetables is a protective factor. In addition, there are probable interactions between UCP2 polymorphism and other environmental factors in Changzhi and Lüliang districts in Shanxi province in northern China.

To the best of our knowledge, the association between UCP2 polymorphism and the risk of NTDs in offspring, which was previously investigated in Californian and Irish populations, is inconsistent^[28, 31]. Our results show that there is an association between the maternal UCP2 3' UTR D/I genotype or allele frequencies, but not the A55V polymorphism (Table 1), and NTDs in offspring in the northern Chinese population. Reasons for the inconsistencies include ethnic variations and the heterogeneity of environmental factors^[35-36]. These environmental factors and ethnic variations may affect the role of the UCP2 gene in NTDs, which is referred to as a gene-environment interaction and will be discussed later. Another reason may be our relatively small sample size. All of these explanations require further research. Various reports have shown that obese women have a 2–5-fold higher risk of having a child with NTDs compared

with that of women with normal weights^[21, 24-25, 37-38]. This study supports the role of BMI in NTD-affected pregnancies. However, the standard of obesity differs between various races. Therefore, the obesity standard in this study is a BMI of > 28 kg/m² based on Chinese investigation. Moreover, this study shows no association between NTDs and maternal age, which is inconsistent with previous studies^[39-40]. We found an increased risk for NTDs associated with maternal pregnancy time, which has been seldom reported. The reason may be that with the time of pregnancy increasing, germ cells do not efficiently develop and contribute toward fertilized ovum dysplasia, while maintaining the burden of the endometrium will lead to decidual cell dysplasia. These results suggest that professional advice should be given to NTD high-risk women with regard to pregnancy. Nutritional factors have been implicated in the etiology of NTDs^[39-41] such as protein, vitamin and other micronutrient deficiencies, most notably folic acid^[42]. During winter and spring, the main vegetables in northern China are stored potatoes and cabbage, which lack rich vitamins, folic acid and other minerals. Moreover, food stored for long periods, in particular germinated potatoes, contain more alkaloids, which has been reported to cause strong embryonic toxicity^[43]. Our study reveals a significant association between germinated potato consumption and an increased risk of NTD-affected pregnancy, while frequent consumption of fruit and vegetables is associated with a decreased risk of NTDs. This observation may be due to the abundance of vitamins, micronutrients and folic acid in fresh fruit and vegetables. The study of gene-environment interactions may lead to a better understanding of the biological mechanisms and pathological processes that contribute toward the development of birth defects. In this study, there was an interaction between the maternal UCP2 D allele and germinated potato consumption ($S = 2.15$), fresh fruit and vegetable consumption ($S = 0.007$) and BMI ($S = 3.50$) in a population from Shanxi province in China. Gene-environment interactions play an important role in the occurrence of NTDs in offspring. These data may provide some useful clues to identify the risk factors for NTDs and better understand the inconsistent results between various regions and ethnicities, although the mechanism still requires investigation. Finally, some limitations that commonly occur in case-control studies require discussion such as misclassification, recall bias and method issues. We conducted our study using a population with very high NTD prevalence, thereby increasing our likelihood of identifying important risk factors. The risk of misclassification is almost zero because NTDs are major external congenital anomalies, which are easily identified by existing birth defect diagnostics. Local women are too poorly educated to know the existence of environmental risk factors for NTDs, thus, a recall bias can be ignored. For quality control purposes, 10% of the samples were

randomly selected and genotyped by a second investigator. The second round of genotyping was compared with that of the first, and demonstrated 100% concordance. Moreover, because very little is known about NTD risk factors, the gene markers, risk factors and gene-environment interactions identified in this study may assist other researchers to conduct additional etiological studies and devise more comprehensive and efficient strategies for the prevention of NTDs.

SUBJECTS AND METHODS

Design

A 1: 1 age-matched case-control study.

Time and setting

Samples were collected at Lüliang and Changzhi hospitals. This experiment was performed in Shanxi Medical University, China from December 2006 to December 2009.

Subjects

A total of 156 NTD cases and 156 controls completed an interview and donated blood for inclusion in this study. The following Chinese affiliations were involved in this study: Zhongyang People's Hospital, Zhongyang Maternal and Child Health Hospital, Liulin People's Hospital, Liulin Red Cross Hospital, Linxian County Hospital, Linxian County Maternal and Child Health Hospital.

Case group

A total of 156 mothers with a history of pregnancy with NTD-affected offspring who had selectively terminated an NTD-affected fetus or had a live or stillbirth with an NTD represented NTD cases from Shanxi province in northern China between 2006 and 2009. Among the 156 NTD cases, 78 and 73 infants were male and female, respectively, and the remaining five infants were of unknown gender. Altogether, 76 infants suffered from anencephaly, 42 infants had spina bifida, 20 infants exhibited encephalocele and 18 infants had combined defects.

Inclusion criteria: NTDs were diagnosed by ultrasound and fetal autopsy reports using the International Classification of Diseases, Tenth Revision codes Q00.0, Q05.9, Q01.9.

Exclusion criteria: Infants that were diagnosed or strongly suspected of chromosomal abnormalities and/or nervous system diseases were excluded from the study. Mothers with nervous system diseases or a family history of nervous system diseases were also excluded. In addition, mothers that provided incomplete information on the questionnaire were excluded.

Control group

The control group included age-matched women of an identical ethnic background and did not suffer from an NTD or gave birth to NTD-affected offspring. The control group women gave birth at the same time as the case group. Mothers with a family history of nervous system diseases were excluded.

All included subjects were of Han origin and voluntarily participated in this study. Written informed consent was obtained from all participants. The protocols were performed in accordance with the *Administrative Regulations on Medical Institutions* formulated by the State Council of China^[44].

Methods

Data collection

Upon participant recruitment, an in-person interview that used a questionnaire was conducted and blood samples (2 mL) were collected from each subject. To avoid bias, interviewers underwent professional training for conducting the interview. In addition, interviewers were blinded to the interviewees' status and forbidden to ask inducing questions. There were more objective indices than subjective indices. A general state of affairs including name, age, career, income, residence, literacy, height and weight were ascertained before pregnancy had occurred. Risk factors including past medical history, medical history during early pregnancy, exposure to toxic substances, medication history, diet and living habits, as well as UCP2 genotype were assessed.

Genotyping

Whole blood was collected into ethylenediamine tetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA) coated tubes and centrifuged at $150 \times g$ for 15 minutes, followed by isolation of the buffy coat layer. Genomic DNA was extracted from 200 mL of buffy coat using standard techniques^[45]. All subjects were uniformly questioned about risk factors for NTDs and were genotyped for UCP2 3' UTR D/I and UCP2 A55V polymorphisms. Agarose gels, and polymorphisms were assessed by technologists blinded to the study groups.

The UCP2 A55V polymorphism was detected by PCR amplification and digested with BbvI (Shanghai Sangon Biotech Co., Ltd., China). The homozygous V/V genotype showed a single 126 bp fragment, the heterozygous A/V genotype showed three fragments of 126, 98 and 28 bp, and the homozygous A/A showed two fragments of 98 and 28 bp. 30 μ L PCRs system contained 50–100 ng genomic DNA, 7.5×10^{-6} μ mol each primer, 1.5 U Taq polymerase, 3 μ L Tris-HCl (pH 8.8), 6 M dNTPs and 25 M MgCl₂. PCR conditions were as follows: 94°C for 5 minutes, 30 cycles of 94°C for 60 seconds, 66°C for 120 seconds, followed by 72°C for 10 minutes.

The UCP2 3' UTR D/I gene polymorphism was genotyped using a PCR method^[45]. A similar PCR assay using the appropriate primers was used. PCR conditions were as follows: 94°C for 5 minutes, 35 cycles of 94°C for 60 seconds, 70°C for 60 seconds, 72°C for 60 seconds, followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis in 2%. The heterozygous D/I genotype showed two fragments of 412 and 457 bp, and the homozygous D/D or I/I showed one fragment of 457 or 412 bp, respectively.

Primers were synthesized by Shanghai Sangon Biology Co., Ltd., China. The sequences are as follows:

For UCP2 3'UTR locus detection:

P₁: 5'-CAG TGA GGG AAG TGG GAG G -3'

P₂: 5'-GGG GCA GGA CGA AGA TTC -3'

For UCP2 A55V locus detection:

P₁: 5'-GGG CCA GTG CGC GCT ACA G-3'

P₂: 5'-CAT TTG GCG CTG CAG GCC GG-3'

To quality control the genotyping results, 10% of samples were randomly selected and genotyped by a second investigator, and resulted in 100% concordance. Samples that failed PCR amplification or digestion as well as uncertain results were repeated to ensure correct identification of variants.

Statistical analysis

Allele and genotype frequencies in NTD-affected subjects and controls were determined by counting alleles and calculating proportions. The probability of the Hardy-Weinberg equilibrium and the frequencies of both genotypes and alleles were used to compare each group to the control by a chi-square analysis. The risk factors for NTDs were analyzed by conditional logistic regression, and an odds ratio with a 95% CI was used to measure the association. Multivariable logistic regression analysis was performed to decrease the confounding bias. Interactions were also examined to elucidate whether the risk factors demonstrated combined effects on NTDs. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for all analyses, and $P < 0.05$ was considered statistically significant. The results were evaluated using the blind method.

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