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Original Article

Association of *SMO* polymorphisms and neural tube defects in the Chinese population from Shanxi Province

Zhen Wang*, Shaofang Shangguan*, Xiaolin Lu, Shaoyan Chang, Rui Li, Lihua Wu, Yihua Bao, Bo Niu, Li Wang, Ting Zhang

*Beijing Municipal Key Laboratory of Child Development and Nutriomics, Capital Institute of Pediatrics, Beijing 100020, China. *Equal contributors.*

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Abstract: Objective: This study aimed to investigate the single nucleotide polymorphisms (SNPs) of *SMO* and neural tube defects (NTDs) in Chinese population. Method: A total of 113 NTDs cases and 138 healthy controls were used in this study. 10 selected single nucleotide polymorphism (SNP) sites in the *SMO* gene were analyzed with MassArray high-throughput DNA analyzer with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. A series of statistical methods were carried out to investigate the correlation between the SNPs and the patient susceptibility to NTDs. Results: The C allele of rs3824 increased the risk of spina bifida (OR=2.52; 95% CI: 1.18, 5.38; p=0.026) but not the risk of anencephaly or encephalocele. Significant differences were found between spina bifida and controls when we compared the GG group with the CC+CG group (OR=2.66; 95% CI: 1.26, 5.61; p=0.011). CC+CG genotype was a risk factor for spina bifida. Conclusions: The gene polymorphism loci rs3824 of SMO was closely related to spina bifida in Chinese population from Shanxi. The haplotype GA in rs3824 and rs9706 increased the risk of NTDs particularly spina bifida in women.

Keywords: Neural tube defects (NTDs), single nucleotide polymorphisms (SNPs), *SMO*, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

Introduction

Neural tube defects (NTDs) are severe birth defects (congenital anomalies), which are the leading cause of death in babies under 1 year of age. NTDs are the second most common cause of birth defects worldwide and include anencephaly, spina bifida and encephalocele etc. They are associated with a failure of neural tube closure during early embryogenesis. NTDs can result in miscarriages, infant death, or serious lifetime disabilities, which can be a major burden for surviving children and their families [1-4]. The incidence of NTDs in Shanxi Province is very high. It is more than 10-fold higher than the national Chinese average, with an observed frequency of 199.38 NTDs per 10,000 pregnancies from 2002-2004. Insufficient intake of vegetables due to geographical reasons has been evoked as one environmental factor potentially leading to the high risk of NTDs [5].

Indeed, for the past 30 years, NTDs in humans have been considered to be multifactorial in origin. They are caused by the interplay of multiple genes, along with the interaction of genes with their environment [1, 6, 7]. Several single nucleotide polymorphisms (SNPs) in different signaling pathways have been shown to be associated with human NTDs, including rs1801133 in the *MTHFR* gene, rs3733890 in the *BHMT* gene, rs2851391 and rs234713 in the *CBS* gene, rs202676 in the *GCPII* gene and rs357564 in the PTCH1 gene [8-16].

The protein SMO encoded by proto-oncogene *smoothened* is a receptor for *PTCH1* and acts as a positive regulator of the signaling pathway. PTCH1 can inhibit the activity of SMO and control proliferation, differentiation and patterning in almost every tissue and organ during embryogenesis [17-19]. Genetic inactivation of *Smo* cells autonomously blocks the ability of cells to transduce the Hedgehog (Hh) signal. Hh signaling organizes pattern formation in the embryonic limb, neural tube, and other structures and Hh pathway dysfunction can lead to birth defects and tumors. It has been confirmed that

the abnormal expression of SMO was implicated in a multitude of tumour types such as basal cell carcinoma, small cell lung cancer, pancreatic cancer, oral squamous cell carcinoma, prostate cancer, bladder cancer tumor and so on. SMO was homologous with G protein-coupled receptor and was a receptor required in Sonic hedgehog (SHH) signal transmission. SHH signaling plays a crucial role during neurulation. Several researchers have suggested that it is involved in regulating the folding of the neural plate. Activated SMO can lead to the activation of Hedgehog target genes in the absence of SHH and PTCH [19-22].

In this study, single gene polymorphisms (SNPs) of SMO in NTD cases and healthy controls were investigated to evaluate the genetic risk factors for NTDs.

Materials and methods

Ethics statement

This study was reviewed and approved by the Research Ethics Committee of the Capital Institute of Pediatrics (IRB00008963) and written informed consent was obtained from each of the participants or their parents. The objective and procedures of this study were explained to all of the subjects and the patient's parents. All of the subjects and the patient's parents signed the informed consent forms. All potential participants who declined to participate or otherwise did not participate were eligible for treatment and were not disadvantaged in any other way by not participating in the study.

Study design

A case-control study was designed for testing the possible association between *SMO* SNPs and NTD susceptibility. NTD and control samples were obtained from the Lvliang area of Shanxi Province in northern China. All subjects were Han Chinese. Stillborn and aborted NTD cases were obtained from nine county hospitals in the same area from 2004-2009. NTDs were diagnosed by B-mode ultrasound and pathology, with sex, gestational age and general development of the embryo recorded in details. Diagnosis of NTDs was performed by experienced pathologists according to the International Classification of Disease, Tenth Revision codes Q00 anencephaly, Q05 spina bifida, and Q01 encephalocele (http://apps.who.int/classifications/). Subjects (*n*=113) chosen for analysis included 55 spina bifida, 66 anencephaly and 17 encephaloceles. Control subjects (*n*=138), aborted for nonmedical reasons, were selected from the same region and selected according to sex and gestational week. Fetuses displaying any pathologic malformation were excluded from the control group.

DNA extraction

All samples for DNA extraction were stored at -20°C in local hospitals before being shipped on ice to the laboratory for study. The separation of different tissues was performed by pathologists. Brain tissue was collected. Genomic DNA was extracted from frozen samples using a Maxwell 16 system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and purity of DNA samples were determined by measuring their absorbance at 260 and 280 nm.

Genotyping

SNPs (*n*=10) spanning the *SMO* gene were selected for investigation. These included seven tag SNPs, which were selected using the Haploview software (version 3.32; http://www.broad.mit.edu/mpg/haploview/). Additional informative SNPs (n=3) were selected from the National Center for Biotechnology Information (NCBI) SNP database (www.ncbi.nlm.nih.gov/SNP). rs3824, rs9607, rs1061280, rs2228617, rs13231139, rs2566871, rs4731562, rs4731563, rs6962740 and rs12674259 were analyzed in our study. Genotyping was conducted with a MassArray high-throughput DNA analyzer with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, CA, USA). It was repeated and 10% samples were sequenced to validate the consistency. Amplifications were conducted according to the manufacturer's protocol.

Statistical analysis

Data were analyzed with SPSS16.0 (McGraw-Hill Inc., New York, NY, USA). All *p*-values were two-sided. A *p*-value less than 0.05 was considered statistically significant. The Hardy-Weinberg equilibrium was assessed using a chi-square test. Clinical data regarding continuous

Table 1. Characteristics of samples in the studied case and control groups

Characteristic	case	control	P^1	spina bifida	anencephalus	encephalocele
N	113	138		55	66	17
Gestational week[2]	20±3.9	21±3.9	0.96	22±4.1	20±3.1	22±5.2
Sex [n (%)]						
M	56 (50.0)	74 (53.6)	0.52	30 (54.5)	39 (59.1)	5 (29.4)
F	57 (50.0)	62 (46.4)		23 (45.5)	27 (40.9)	12 (70.6)

[1]x^2 test for categorical variables and Mann-Whitney U test for continuous variables. [2]Mean ± SD.

variables are expressed as mean ± SD, and differences between groups were assessed using a Mann-Whitney U test. When data did not meet normality, a Wilcoxon signed-rank test was used. Our analysis concerned the whole study group stratified into three major types: spina bifida, anencephaly and encephalocele. In each stratum, cases were compared with the corresponding control groups. Genotype and allele frequencies for each SNP were compared between cases and controls using a chi-square test. Adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for gestation week and sex were established using multiple logistic regressions. Linkage disequilibrium for rs3824 and rs9706 was estimated using Haploview. Power calculations were carried out using Quanto software (http://hydra.usc.edu/GxE). Based on the smallest cohort size 113 patients and 138 controls), our study had 80% power to detect an OR of 2.0 at a significance level of 5%.

Results

Subjects

Clinical and demographic characteristics of subjects are shown in **Table 1**. The average week of gestation was about 21 weeks in controls and cases. The two groups contained both male and female fetuses. There did not appear to be any apparent difference with respect to sex or gestational week between the two study groups.

Distribution of SMO polymorphisms

Associations were discovered between the rs3824 polymorphism in the *SMO* gene and different types of NTD (**Table 2**). For genotypes and alleles, significant differences were not found between cases and controls. The C allele of rs3824 increased the risk of spina bifida but not the risk of anencephaly or encephalocele. Significant differences were found between spina bifida and controls when we compared the GG group with the CC+CG group. We found that the CC+CG genotype was a risk factor for spina bifida. The *p* value in the table has not been adjusted by multiple corrections. When the data were analyzed on the basis of gender, it showed that there was no significant difference between the case and control group (**Table 3**).

Distribution of SMO haplotypes

Each SNP site involved with multiple associations was investigated for linkage disequilibrium. Evidence for linkage disequilibrium was observed for rs3824 and rs9607. Two are located in the SMO gene 3'UTR region. The two SNPs generated four common haplotypes and in female the GA haplotype is associated with a slightly elevated risk for NTDs, especially spina bifida (**Table 4**).

Discussion

In this study, the association between SMO polymorphisms and risk of NTDs was analyzed for the Lvliang region, which has the highest prevalence of NTDs in China [5]. The results showed that infants with the CC genotype of the SMO rs3824 polymorphism and the GA haplotype have an increased risk of spina bifida. The result analyzed by the Haploview program showed that the GA genotype comprises two SNPs in 3'UTR. We found this GA genotype was associated with a slightly increased risk of NTD in female.

The Hh signaling pathway is composed of patched (PTCH) and SMO, two transmembrane proteins, and downstream Gli transcription factors. SMO protein, a 7-pass transmembrane protein and key transcriber in Hh signaling pathways, plays a crucial role in regulating the

Table 2. Genotype and allele counts for rs3824 variants in control and NTD subtypes

	control	Case			spina bifida			anencephalus			Encephalocele		
		N	P	OR (95% *CI*)	N	P	OR (95% *CI*)	N	P	OR (95% *CI*)	N	P	OR (95% *CI*)
GG	59	56	0.252	1	15	0.026	1	31	0.83	1	10	0.205	1
CG	1	4		4.21 (0.46-38.86)	2		7.87 (0.67-92.67)	1		1.90 (0.16-31.48)	1		5.90 (0.34-102.17)
CC	39	52		1.41 (0.81-2.44)	25		2.52 (1.18-5.38)	24		1.17 (0.60-2.89)	3		0.45 (0.12-1.75)
G	119	113	0.066	1	32	0.001	1	63	0.508	1	21	0.134	1
C	79	108		1.44 (0.98-2.12)	52		2.45 (1.45-4.14)	49		1.17 (0.73-1.87)	7		0.502 (0.20-1.24)
GG	59	56	0.163	1	15	0.011	1	31	0.608	1	10	0.399	1
CC+CG	40	56		1.47 (0.85-2.55)	27		2.66 (1.26-5.61)	25		1.19 (0.61-2.31)	4		0.59 (0.17-2.01)

x^2 tests were used to calculate *P*-values. Fisher's exact test was used when the sample size was less than 5.

Table 3. Genotype comparison of rs3824 between cases and controls in males and females

SEX	Genotype	Control	case	p	OR (95% CI)	spina bifida	P	OR (95% CI)
F	GG	27	29	0.72	1	17	0.67	1
	CG	0	2		--	0		--
	CC	21	31		1.37 (0.64-2.95)	16		1.21 (0.50-2.95)
M	GG	32	26	0.62	1	12	0.64	1
	CG	1	2		2.46 (0.21-28.69)	1		2.67 (0.15-46.11)
	CC	18	20		1.37 (0.60-3.11)	10		1.48 (0.53-4.10)

x^2 tests were used to calculate *P*-values. Fisher's exact test was used when the sample size was less than 5.

Table 4. Associations between haplotypes (rs3824 and rs9706) and risk of NTDs

SEX	haplotype	Control fre	case fre	OR (95% CI)	spina bifida fre	OR (95% CI)
M	CG	0.088	0.098	1	0.049	1
F		0.101	0.154	1.52 (0.66-3.51)	0.076	1.62 (0.69-3.82)
M	GA	0.081	0.064	1	0.033	1
F		0.061	0.078	3.75 (1.01-13.94)	0.045	4.86 (1.12-21.11)
M	CA	--	0.005	1	0.003	1
F		0.002	0.004	--	0.0.3	--
M	GG	0.067	0.065	1	0.032	1
F		0.067	0.065	0.43 (0.12-1.63)	0.042	0.42 (0.09-1.89)

x^2 tests were used to calculate *P*-values. Fisher's exact test was used when the sample size was less than 5.

folding of the neural plate. It can transport Hh signals to a cytoplasmic transduction cascade that ultimately regulates the Gli family of zinc finger transcription. Therefore, it activated nuclear gene transcription and the Hh signaling pathway. Hh pathway dysfunction can lead to birth defects or proliferative disorders [19-22].

According to the international classification standard, NTDs can be divided into three subtypes. There were 55 patients with spina bifida, 66 with anencephaly and 17 with encephalocele in our study. It is worth noting that a significant association was observed between spina bifida and the rs3824 polymorphisms in the *SMO* gene in our work, it is the C allele of rs3824 that is related to spina bifida. In our previous study we also found SNPs of PTCH1 gene was related to spina bifida [23]. These suggested that the polymorphisms of these key genes affected the activity of the SHH pathway and resulted in neural tube closure obstacles, and then leaded to spina bifida.

The process of neural tube closure occurs with a characteristic sequence of events along the embryonic axis. The human embryo appears to have two closure sites, at the hindbrain/cervical boundary (closure 1) and the forebrain (closure 2) [2, 24]. Fusion at closure 1 spreads bidirectionally into the anterior neuropore and along the spinal region into the posterior neuropore. Spina bifida most commonly results from failure to complete closure at the posterior neuropore. Anencephaly is caused by failure of neural tube closure in the cranial region, thereby development of the brain is disrupted [24-26]. Our findings indicate that the SMO gene may preferentially affect the progress of closure from closure 1 to the posterior neuropore. In the caudal spinal region, closing of the neural tube is characterized by dorsolateral hinge points (DLHPs), with no median hinge point (MHP) formed [27]. DLHPs are formed by a combination of BMP2 and Noggin in the presence of low levels of SHH. Previous studies have found that an increase in SHH signalling can prevent neural tube closure by repressing the formation of DLHPs [28, 29]. The rs3824 polymorphisms have a possible role in activating the SHH signaling pathway, thereby resulting in less effective formation of DLHPs.

There was gender difference in the incidence of NTDs [30]. There were no statistically significant differences between the case and control groups when they were stratified by gender in this study. This may be due to small sample

size. But in the haplotype analysis, when the samples were stratified by gender, we found that the haplotype GA in rs3824 and rs9706 increased the risk of NTDs particularly spina bifida in women.

In conclusion, rs3824 single nucleotide polymorphism of SMO gene may be associated with spina bifida. The haplotype GA in rs3824 and rs9706 increased the risk of NTDs particularly spina bifida in women. These may be due to the SNP site located at the 3'UTR region and lead to the change of Hh pathway activity.

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Disclosure of conflict of interest

None.

Address correspondence to: Li Wang, Beijing Municipal Key Laboratory of Child Development and Nutriomics, Capital Institute of Pediatrics, Beijing 100020, China. Tel: +86-10-85695585; Fax: +86-10-85631504; E-mail: liwangl1@163.com; lily_wang@yeah.net

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