Gene-gene interaction between *VANGL1*, *FZD3*, and *FZD6* correlated with neural tube defects in Han population of Northern China

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ABSTRACT. We evaluated the influence of gene-gene interactions between *VANGL1*, *FZD3*, and *FZD6* on the risk of neural tube defects (NTDs) in Han population in the north of China. Two single nucleotide polymorphisms (SNPs) (rs4839469 and rs34059106) within *VANGL1*, two SNPs (rs2241802 and rs28639533) within *FZD3*, and three SNPs (rs827528, rs3808553, and rs12549394) within *FZD6* were genotyped in 135 NTD patients and 135 controls. The gene-gene

interactions between *VANGL1*, *FZD3*, and *FZD6* were analyzed using multifactor dimensionality reduction (MDR) software. The distribution of genotypes of rs4 839 469 within *VANGL1* and rs3 808 553 within *FZD6* differed significantly difference between patients and controls ($P < 0.05$). MDR revealed significance in models with 2 SNPs (rs4839469 and rs3808553) (OR = 3.18, 95%CI = 1.85-5.44; $\chi^2 = 18.39$, $P < 0.0001$), 3 SNPs (rs4839469, rs2241802, and rs3808553) (OR = 4.17, 95%CI = 2.43-7.14; $\chi^2 = 28.5$, $P < 0.0001$), and 4 SNPs (rs4839469, rs2241802, rs827528, and rs3808553) (OR = 7.34, 95%CI = 3.98-13.54; $\chi^2 = 45.3$, $P < 0.0001$). Gene-gene interaction between *VANGL1*, *FZD3*, and *FZD6* may exist, which may increase the risk of NTDs. This work provides insight into the understanding of the etiology of NTDs.

Key words: *VANGL1* gene; *FZD3* gene; *FZD6* gene; Gene-gene interaction; Neural tube defects

INTRODUCTION

Neural tube defects (NTDs) are a group of birth defects of the brain, spine, or spinal cord due to failure of neural tube fusion during embryo development, including anencephaly, encephalocele, spina bifida, and myeloschisis (Greene et al., 2009). Studies have confirmed that the etiology of NTDs is associated with both genetic and environmental factors. Additionally, the planar cell polarity (PCP) signaling pathway, which is indispensable for cellular oriented and coordinated movement in the period of embryonic information, is an attractive candidate for investigations of human NTDs. The findings demonstrate rare variations in the PCP pathway make significant differences in the occurrence of NTDs and are involved in mutant genes, such as *VANGL*, *FZD*, *CELSR1*, and *DVL*, among others (Cai and Shi, 2014). Recent investigations both in China and other countries have demonstrated close relationships between *VANGL1* and NTDs (Kibar et al., 2007; Kibar et al., 2009; Xu et al., 2011). There are both Ala116Pro variations in *VANGL1* and an obviously short structure of the α-helix fragment after variation in Han population of Northern China (Cai et al., 2013). Recent studies have shown that *FZD3* and *FZD6* are associated with NTDs (Wang et al., 2006; De Marco et al., 2012). Because they are core genes of the PCP pathway, *VANGL1 FZD3/6* may influence NTDs. In this study, the association of NTDs with *VANGL1* and *FZD3/6* was evaluated by analyzing gene-gene interactions among single nucleotide polymorphisms (SNPs) in order to weaken generative risks in preconception and increase the probability of a healthy birth outcome.

MATERIAL AND METHODS

Subjects

The case group included 135 NTDs patients who were chosen from hospitalized patients and diagnosed by two experienced doctors depending on clinical manifestations, images, and surgery findings in the Department of Neurosurgery of Tianjin Children's Hospital in China from November 2003 to August 2010. Additionally, 135 patients who were not infected with NTDs were considered as controls, which had age and gender composition similar to that of

the NTD patient group. All cases of maternal periconceptional supplementation of folate were included. All patients were from Chinese Han population in the north, northwest, and northeast of China. Blood drawn from peripheral venous was added to sodium citrate anticoagulant. Samples were stored at -80°C until genomic DNA was extracted. This work was approved by the Tianjin Children's Hospital Ethics Committee and written informed consent was signed by the guardians of each subject.

Extraction and identification of genomic DNA

Genomic DNA was extracted from blood using the DNA Extraction Kit (Tiangen, China) according to the manufacturer instruction. DNA samples were stored at -80°C until use.

Selection of SNPs

We straightly searched the dbSNP database (build 130) and applied the SNPper software (Riva and Kohane, 2002) to investigate the search results, selecting two common missense SNPs (rs4839469 and rs34059106) in the coding region of *VANGL1*, three missense SNPs (rs827528, rs3808553, and rs12549394) in *FZD6*, and two sense SNPs (rs2241802 and rs28639533) in *FZD3* because there were no missense SNPs within *FZD3*.

Polymerase chain reaction (PCR)

Primers (Table 1) were manufactured by Liuhehuada Company (Beijing, China). The PCR system was composed of 2.5 μL 10X Ex Taq buffer, 2 μL dNTPs, 0.3 μL sense primer, 0.3 μL antisense primer, 2 μL template DNA, 0.2 μL Ex Taq, and 17.7 μL water. The reaction conditions were as follows: pre-denaturation at 94°C for 5 min; 35 cycles at 94°C for 40 s, 56°C for 40 s, and 72°C for 40 s; 72°C for 10 min, and 12°C for storage (Arya et al., 2005; Maheaswari et al., 2016).

Table 1. Primer sequences of loci for *VANGL1*, *FZD3*, and *FZD6*.

Gene	Dbsnp rs	Primer (5' to 3')	Length (bp)
VANGL1	rs4839469	F: cagccaaacagacagaaag	549
		R: aggtccctatgagcagaat	
VANGL1	rs34059106	F: aagtgacctttaggattt	473
		R: gtgatcgcagctattt	
FZD3	rs2241802 rs28639533	F: tctccaggaataaataag	559
		R: gtgtcactgtggaagc	
FZD6	rs827528	F: gagtgattcatccaagc	388
		R: aacataagtcagtagaggg	
FZD6	rs3808553	F: tgggatatggtactgac	584
		R: agctagaacttggtgac	
FZD6	rs12549394	F: gataaaggtggacactggtt	300
		R: aatttgcttctgagtaacga	

Sequencing reaction

The conditions of sequencing reaction were as follows: 95°C denaturation for 2 min; 35 PCR cycles (95°C for 15 s, 50°C for 15 s, 60°C for 90 s); and storage at 12°C after amplification. The sequencing map was analyzed by the Liuhehuada Company of Beijing (Beijing, China).

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was used to test whether the samples were consistent with Mendel's laws of inheritance. Online HWE software was used (http://www.biology.ualberta.ca/jbrzusto/hwenj.html) (Huang and Yang, 2004). Data were analyzed using SPSS17.0. Differences in the genotype frequency between case and control groups were tested by Chi-square test or Fisher's exact test. Multifactor dimensionality reduction (MDR) 2.0 software was utilized to evaluate gene-gene interactions.

RESULTS

Hardy-Weinberg equilibrium text

The genotype frequencies of 7 polymorphisms conformed to HWE between NTDs and controls ($P > 0.05$).

Genotype frequencies of SNP loci between cases and controls

Because the polymorphism of two SNPs (*VANGL1* rs34 059 106 and *FZD3* rs28 639 533) were not found, these two loci were not further statistically analyzed. The genotype distribution of *VANGL1* rs4 839 469 and *FZD6* rs3 808 553 showed significant differences between cases and controls ($P < 0.05$) (Table 2). The number of cases and controls was unequal in different loci, as shown in Table 2, resulting from the low quality of the sequencing test.

Table 2. Genotype frequencies of loci for *VANGL1*, *FZD3*, and *FZD6.*

Genotype	Genotype frequencies N *(%)*	
	Control	Case
VANGL1 rs4839469	N = 131	N = 129
GG	99 (75.6)	87 (67.4)*
GA	28 (21.4)	33 (25.6)
GC	1 (0.8)	9 (7.0)
AA	3 (2.2)	0 (0)
FZD3 rs2241802	N = 123	N = 129
AA	12 (9.8)	15 (11.6)
AG	68 (55.3)	64 (49.6)
GG	43 (35.0)	50 (38.8)
FZD6 rs827528	N = 109	N = 113
AA	106 (97.2)	111 (98.2)
AG	3 (2.8)	2 (1.8)
FZD6 rs3808553	N = 129	N = 130
GG	42 (32.5)	29 (22.3)*
TG	70 (54.3)	68 (52.3)
TT	17 (13.2)	33 (25.4)
FZD6 rs12549394	N = 128	N = 131
CC	123 (96.1)	120 (91.6)
CA	5 (3.9)	11 (8.4)

*P value < 0.05 indicates that the genotype distribution of *VANGL1* rs4839469 and *FZD6* rs3808553 showed significant differences between case and control groups.

Association between MDR optimal model in *VANGL1*, *FZD3*, *FZD6*, and NTDs

MDR optimal models to determine gene-gene interaction were obtained from MDR software with the following results: *VANGL1* rs4839469 - *FZD6* rs3808553, *VANGL1* rs4839469 - *FZD3* rs2241802 - *FZD6* rs3808553, *VANGL1* rs4839469 - *FZD3* rs2241802 - *FZD6* rs827528 - *FZD6* rs3808553 (Table 3). The cross-validation consistency showed a maximum (10/10) and testing balance accuracy was relatively high in the best models. Two important loci, *VANGL1* rs4839469 and *FZD6* rs3808553, were included in all of the best models. Significant difference was found between optimal models and NTDs ($P < 0.001$), revealing that gene-gene interactions between *VANGL1*, *FZD3*, and *FZD6* significantly increased the risk of NTD occurrence.

Table 3. MDR models of *VANGL1*, *FZD3*, and *FZD6*.

Models	Training balance accuracy	Testing balance accuracy	Cross-validation consistency	χ^2 (P)	OR (95%CI)
X1-X4	0.6238	0.6000	10/10	18.39 (P < 0.0001)	3.1771 (1.8540-5.4344)
X1-X2-X4	0.6630	0.5331	10/10	28.50 (P < 0.0001)	4.1667 (2.4325-7.1371)
X1-X2-X3-X4	0.7053	0.5441	10/10	45.30 (P < 0.0001)	7.3373 (3.9777-13.5366)

X1-X4 are used to represent rs4839469, rs2241802, rs827528, and rs3808553 respectively.

DISCUSSION

In recent years, studies have indicated that disease occurrence is correlated with the joint effects of multiple genetic loci polymorphisms, particularly gene-gene interactions. MDR (Ritchie et al., 2001) is a nonparametric and multifactor method for analyzing interactions according to the susceptibility of diseases. Using this method, several factors are considered multifactor combinations, such that the high-dimensional structure is degraded to a unidimensional structure with two levels (high risk and low risk), which may reveal dimensionality reduction. MDR is superior to traditional statistical methods in case-control studies for analyzing gene-gene interactions. This method is convenient for users to assess interactions because MDR is model-free, where no genetic pattern is supposed, and uses known data about the disease for which inheritance-model is unknown or are extreme complex (Li et al., 2016). This software can be freely obtained from the Internet (http://sourceforge.net/projects/mdr/).

Maternal folic acid deficiency in pregnancy plays an important role in NTD occurrence (Li et al., 2000). Periconceptional folate supplementation may reduce the incidence of NTDs by 50-70%, and thus related genetic investigations have focused on the folic acid metabolic pathway. For example, methylene tetrahydrofolate reductase (*MTHFR*) C677T polymorphism significantly increased the risk of NTDs (Fang et al., 2015). However, folic acid supplementation could not completely prevent NTD occurrence, and the offspring of mothers without folic acid deficiency also developed NTDs (van der Linden et al., 2006). Therefore, other pathways may be related to NTDs. However, studies have verified that formation of the neural tube is directly correlated with the orientation of cell division and reduction of migration, in which one of the key factors is the regulation of the PCP pathway (Wang and Nathans, 2007). The impact of *VANGL1* and *FZD* on neural tube formation is well-known in the PCP pathway (Wang et al., 2006; Kibar et al., 2007, 2009; Xu et al., 2011; De Marco et al., 2012; Cai et al., 2013).

In humans, *VANGL1* is located on chromosome 1p11-p13.1 and encodes 524 amino acids and the size of *VANGL1* is 56 kb; *VANGL2* is located on chromosome 1q22-q23 and encodes 521 amino acids, and its size is 28 kb.

In humans, *FZD* contains ten members, *FZD1* to *FZD10*. *FZD3* and *FZD6* influence neural tube formation (Wang et al., 2006; De Marco et al., 2012). The *FZD3* gene is located on chromosome 8p21, encoding protein composed of 666 amino acids, and its DNA sequence spans 70 kb; The *FZD6* gene is located on chromosome 8q22.3-q23.1, encoding protein composed of 706 amino acids, and its DNA sequence spans 34 kb.

FZD and VANGL, as transmembrane proteins, play critical roles in transducing cellular signals. They recruit each other to the junction side of the adjacent cell (Strutt and Strutt, 2007); this recruitment is induced by a weakly unbalanced effect. When either *FZD* or *VANGL* is absent from cloned cells, unbalanced interactions cause abnormal signals in adjacent cells. In this study, we confirmed a clearly positive interactive risk of *VANGL1*, *FZD3*, and *FZD6* on NTDs by analyzing gene-gene interactions. Therefore, an interaction between *VANGL* and *FZD* was supported at the gene level.

In conclusion, based on MDR analysis, gene-gene interactions between *VANGL1*, *FZD3*, and *FZD6* may exist and increase the risk of NTD occurrence in the Han population of northern China. However, this study only involved primary research of the gene-gene interaction, and further analysis is required. Furthermore, there were additional limitations to this study. First, non-genetic factors related to NTDs, such as environment and nutrient in periconception, should be evaluated in detail. Second, the number of enrolled subjects, from the Han population of northern China, was not high, and further studies should include larger populations. There are other genes in the PCP pathway other than the genes discussed in this study, and thus the relationship between other genes and NTD occurrence should be evaluated in further analysis.

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