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Digenic variants of planar cell polarity genes in human neural tube defect patients

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ABSTRACT

Neural tube defects (NTDs) are considered to be a complex genetic disorder, although the identity of the genetic factors remains largely unknown. Mouse model studies suggest a multifactorial oligogenic pattern of inheritance for NTDs, yet evidence from published human studies is surprisingly absent. In the present study, targeted next-generation sequencing was performed to screen for DNA variants in the entire coding regions and intron-exon boundaries of targeted genes using DNA samples from 510 NTD cases. These candidate genes were PCP genes, including *VANGL1, VANGL2, CELSR1, SCRIB, DVL2, DVL3* and *PTK7*. Candidate variants were validated using Sanger sequencing. A total of 397 single nucleotide variants(SNVs) were identified with a mean depth of approximately 570×. Of these identified SNVs, 74 were predicted to affect protein function and had a minor allele frequency of < 0.01 or unknown. Among these 74 missense SNVs, 10 were identified from six NTD cases that carried two mutated genes. Of the six NTD cases, three spina bifida cases and one anencephaly case carried digenic variants in the *CELSR1* and *SCRIB* gene; one anencephaly case carried variants in the *CELSR1* and *DVL3* gene; and one spina bifida case carried variants in the *PTK7* and *SCRIB* genes. Three cases that parental samples were available were confirmed to be compound heterozygous. None of the digenic variants were found in the 1000 genome database. The findings imply that genetic variation might interact in a digenic fashion to generate the visible NTD phenotypes and emphasize the importance of these genetic interactions in the development of NTDs in humans.

1. Introduction

Neural tube defects (NTDs), such as anencephaly and spina bifida, are congenital malformations of the central nervous system caused by a partial or complete failure of the neural tube to close during embryogenesis [1]. Although the etiology of human NTDs has been intensively studied for over 40 years [1–5], the causative genetic mechanism of NTD development is largely unknown. It is estimated that up to 70% of the risk of NTDs is attributed to genetic factors [6]; yet the main predisposing genetic factors for human NTDs remain unknown. Given that NTDs present a mainly sporadic pattern and exhibit a relatively high prevalence across the world, it is thought that the etiology of these disorders represents a multifactorial oligogenic or polygenic pattern of inheritance, together with an important role for non-genetic factors such as the environment [2].

It is now well established that over 300 genes were causally linked to the expression of a NTD phenotype in mice [7–10], indicating the complex genetic requirements for neurulation during the period of neural tube closure. Interestingly, a disproportionately large number of these genes are localized in the planar cell polarity (PCP) pathway. The PCP pathway, also called the non-canonical Frizzled/Dishevelled pathway, controls the process by which cells become polarized within the plane of an epithelium in numerous tissues in both *Drosophila* and vertebrates [11,12]. Genetic studies in *Drosophila* have initially identified a group of "core" PCP genes including: *frizzled (fz), dishevelled (dvl), vang gogh/strabismus (vang/stbm), flamingo (fmi), prickle (pk),* and *diego (dg)* [12]. These genes encode proteins that are highly conserved in vertebrates, where they mediate a complex morphogenetic process called convergent extension during gastrulation and neurulation. Convergent extension is a polarized cellular rearrangement that leads to the narrowing of the mediolateral axis and lengthening of the anteroposterior axis for gastrulation and neural tube formation. Mouse

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Table 1
Mouse mutants of key PCP Genes in a digenic fashion and NTD phenotypes.

Gene and mutant	NTD Type	Penetrance	References
Vangl2$^{Lp/+}$, Dvl2$^{-/-}$	CRN	100%	Wang et al. (2006) [25]
Vangl2$^{Lp/+}$, Dvl3$^{+/-}$	CRN, EX	30%	Etheridge et al. (2008) [26]
Vangl2$^{Lp/+}$,Scrib$^{Crc/+}$	CRN or SB	55% CRN, 5% SB	Murdoch et al. (2001) [27]
Vangl2$^{Lp/+}$,Scrib$^{Crc/+}$	CRN	54%	Murdoch et al. (2014) [2]
Vangl2$^{Lp/+}$,Celsr1$^{Crsh/+}$	CRN	not stated	Copp et al. (2003) [41]
Vangl2$^{Lp/+}$,Celsr1$^{Crsh/+}$	CRN	54%	Murdoch et al. (2014) [2]
Vangl2$^{Lp/+}$, Ptk7$^{+/-}$	SB	95%	Lu et al. (2004) [47]
Vangl2$^{Lp/+}$,Vangl1$^{+/-}$	CRN	60%	Torban et al. (2008) [28]
Dvl2$^{-/-}$,Dvl3$^{+/-}$	CRN	~100%	Etheridge et al. (2008) [26]
Dvl2$^{+/-}$,Dvl3$^{-/-}$	CRN	some	Etheridge et al. (2008) [26]
Scrib$^{Crc/+}$,Celsr1$^{Crsh/+}$	CRN	8%	Murdoch et al. (2014) [2]

PCP, planar cell polarity; NTD, neural tube defect; CRN, craniorachischisis; SB, spina bifida; EX, exencephaly.

model studies show that mutants that disrupt core components of the PCP pathway, including *Vangl1, Vangl2, Celsr1, Fzd3, Fzd6, Dvl1, Dvl2* and *Dvl3,* can cause NTDs [7,13–16].

As suggested from the mouse NTD literature, PCP genes were subsequently determined to have potentially a causal role in humans with NTDs [7]. Single-nucleotide variants (SNVs) in the core PCP genes *CELSR1, FZD6, PRICKLE1, DVL2, VANGL1* and *VANGL2*, and the PCP associated genes *SEC24B, DACT1, FUZ* and *SCRIB* [7,17–23] have been proposed as human NTD risk factors. In contrast to the association of mouse PCP gene mutations with craniorachischisis in homozygotes, most of the variants identified in human NTDs are heterozygous variants, and the PCP-mutation-associated NTD phenotypes vary widely ranging from the “open NTD” of craniorachischisis, myelomeningocele, and anencephaly, to the “closed NTD” of lipomyelomeningocele, lipomyelocele, and lipoma [2,7]. Unlike mouse models, variants in single genes may not explain the genetic mechanism underlying the development of human NTDs.

Recently, a digenic or polygenic pattern of inheritance was suggested to contribute to the cause of NTDs. Actually, mouse studies showed that several digenic combinations involving the core PCP gene *Vangl2* and other genes (*Sec24b, Sfrp1/Sfrp2/Sfrp5, Dvl3, Scrib, Celsr1, Ptk7, Vangl1*), most of which were double heterozygotes, could cause either open spina bifida or exencephaly or craniorachischisis, in contrast to only the craniorachischisis phenotype when homozygous mutants were detected in some of these PCP genes [2,24–28]. Additionally, double mutants of *Scrib$^{Crc/+}$*and *Celsr1$^{Crsh/+}$, Dvl2$^{-/-}$* and *Dvl3$^{+/-}$, Dvl2$^{+/-}$* and *Dvl3$^{-/-}$* induced a phenotype of craniorachischisis in mice [2,26]. Although these findings raise the possibility of similar genetic combinatorial mechanisms in human NTDs, little evidence was available in support of this hypothesis in human studies except a small chohort study including 90 patients with cranial NTDs in England [29]. Therefore, the present study aimed to find double or multiple heterozygous variant combinations of critical PCP genes in a large number of human NTD cases, which may provide novel insight into the comprehensive genetic mechanisms in humans.

2. Materials and methods

2.1. Study subjects

The subjects were recruited from five rural counties (Xiyang, Shouyang, Taigu, Pingding, and Zezhou) of Shanxi Province in northern China, utilizing a population-based birth defect surveillance program, which has been previously described elsewhere [30]. Birthing hospitals report to the system newborns with major external structural birth defects or fetuses that are terminated due to prenatal diagnosis of such defects. Maternal venous blood samples, cord blood samples and umbilical cord tissues were collected at delivery or at the time of termination of NTD-affected pregnancies. Venous blood samples were collected from NTD case fathers. Dried blood spots were made using cord blood and paternal blood samples. The tissue samples were stored at −80 °C and the dried blood spots were stored at −20 °C until analysis. The study protocol was approved by the institutional review board of Peking University, and written informed consent was obtained from the mothers prior to the investigation.

2.2. DNA extraction

Fetal DNA from umbilical cord tissues or cord blood samples was extracted with QIAamp DNA Mini Kit Tissue kit (Qiagen, Germany). The concentration of DNA was measured by NanoDrop2000 Ultramicro spectrophotometer (Thermo Fisher Scientific, USA). All the DNA samples were stored at −80 °C until utilized for analysis.

2.3. Target gene selection

We selected candidate PCP genes for sequencing according to the following criteria: heterozygous mutants that have been reported as NTD-causing factors when they interact with other variants in a digenic or polygenic fashion. The NTD-causing digenic mutant combinations of non-homologous PCP genes in mice are shown in Table 1. These candidate genes sequenced are: *VANGL1, VANGL2, CELSR1, SCRIB, DVL2, DVL3,* and *PTK7*.

2.4. Multiplex PCR amplification and next-generation sequencing

Multiplex PCR amplification and next-generation sequencing was used to screen for DNA variants along the entire coding regions and intron-exon boundaries of targeted genes. Primers were designed using primer3. One hundred oligonucleotide pairs were constructed to cover all of the coding sequences and intron-exon boundaries of the targeted genes. After the first round of primer design, under the most stringent conditions (no SNPs in primer annealing region, amplicon length between 200 and 270 bp, GC content between 30 and 80%), the 100 oligonucleotide pairs were put into 6 multiplex PCR panels that amplified all of the target regions. The amplification reactions were carried out on an AB 2720 Thermal Cycler (Life Technologies Corporation, USA). The cycling program was 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 63 °C–0.5 °C per cycle for 40 s, 72 °C for 1 min; 24 cycles of 94 °C for 20 s, 65 °C for 30 s, 72 °C for 1 min; 72 °C for 2 min.

The PCR product of each sample was labeled with 8 bp barcode; all the libraries of each sample were pooled. After cluster generation and hybridization of sequencing primer, base incorporation was carried out on a MiSeq Benchtop Sequencer (Illumina, Inc., San Diego, CA) in one single lane following the manufacturer's standard cluster generation and sequencing protocols. The sequencing reactions ran for 300 cycles per read to generate paired-end reads including 300 bp at each end and 8 bp of the index tag.