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Identification and characterization of novel rare mutations in the planar cell polarity gene *PRICKLE1* in human neural tube defects

Ciprian M. Bosoi[1], **Valeria Capra**[2], **Redouane Allache**[1], **Vincent Quoc-Huy Trinh**[1], **Patrizia De Marco**[2], **Elisa Merello**[2], **Pierre Drapeau**[3], **Alexander G. Bassuk**[4], and **Zoha Kibar**[1,*]

[1]Department of Obstetrics and Gynecology, CHU Sainte Justine Research Center and University of Montréal

[2]Laboratorio del Servizio di Neurochirurgia, Instituto G. Gaslini, Genova, Italy

[3]Department of Pathology and Cell Biology, University of Montreal, Montreal, Canada

[4]Department of Pediatrics, University of Iowa, Iowa City, Iowa, USA

Abstract

The planar cell polarity (PCP) pathway controls the process of convergent extension (CE) during gastrulation and neural tube closure and has been implicated in the pathogenesis of neural tube defects (NTDs) in animal models and human cohorts. In this study, we analyzed the role of one core PCP gene *PRICKLE1* in these malformations. We screened this gene in 810 unrelated NTD patients and identified 7 rare missense heterozygous mutations that were absent in all controls analyzed and predicted to be functionally deleterious using bioinformatics. Functional validation of 5 *PRICKLE1* variants in a zebrafish model demonstrated that one variant, p.Arg682Cys, antagonized the CE phenotype induced by the wild-type zebrafish *prickle1a* in a dominant fashion. Our study demonstrates that *PRICKLE1* could act as a predisposing factor to human NTDs and further expands our knowledge of the role of PCP genes in the pathogenesis of these malformations.

Keywords

PRICKLE1; Planar cell polarity; PCP; neural tube defects; NTD; rare mutations

Planar cell polarity (PCP) is the process by which epithelial cells become polarized along the plane of the epithelium. It was first described and studied in details in *Drosophila* where it controls the orientation of the hair cells in the developing wing and abdomen and of the sensory organ precursor cells of the notum and the ommatidial arrangement in the compound eye. Genetic studies of a wide range of mutants affecting these highly organized PCP structures led to the identification of a group of “core” PCP genes that include: *frizzled (fz), dishevelled (dsh), prickle-spiny legs (pk), strabismus/ van gogh (stbm/vang), flamingo/ starry night (fmi/stan)* and *diego (dgo)* (Simons and Mlodzik, 2008). The PCP or non-canonical Fz/Dsh pathway is highly conserved in vertebrates where all these core PCP genes have corresponding orthologs and homologs (Simons and Mlodzik, 2008). In vertebrates, PCP mediates a complex morphogenetic process called convergent extension (CE) during gastrulation and neural tube formation. During this process, cells elongate, move mediolaterally and intercalate between neighboring cells, leading to convergence towards

*To whom correspondence should be addressed: Zoha Kibar, CHU Sainte Justine Research Center, 3175 Cote-Ste-Catherine, Room A711, Montreal, QC H3T 1C5 Canada; Tel: (514) 345 4931 X 3984; zoha.kibar@recherche-ste-justine.qc.ca.

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the mediolateral axis and extension along the anteroposterior axis (Bassuk and Kibar, 2009). PCP is also implicated in other developmental processes including directional migration of facial motor neurons, oriented cell division, regulation of neuronal and nonneuronal cellular morphology and shape changes, axon guidance, orientation of sensory hair cells in the organ of Corti and ciliogenesis (Simons and Mlodzik, 2008). Defects in PCP signaling in mouse result in a wide range of developmental anomalies including neural tube defects (NTDs), polycystic kidneys, conotruncal heart defects and deafness (Simons and Mlodzik, 2008). In humans, mutations in two PCP genes, *VANGL2* and *VANGL1*, homologs of *van gogh*, were associated with NTDs, providing the first lines of evidence for the involvement of PCP in the pathogenesis of these malformations (Kibar et al., 2007; 2009; 2011).

NTDs, including anencephaly and spina bifida, are the most common and severe malformations of the central nervous system, affecting 1–2/1000 births. They result from embryonic failure of neural tube closure that can occur at any level of the embryonic axis. Despite the protective effect of periconceptional supplementation with folic acid against a large fraction of NTDs (~50–70%), thousands of families are still affected with these conditions urging the need for better understanding of the underlying pathogenesis. NTDs have a complex etiology involving interactions among environmental and genetic factors whose identities remain largely unknown (Bassuk and Kibar, 2009).

In this study, we focused on investigating the role of one core PCP gene, *PRICKLE1* (MIM# 608500), homolog 1 of *pk* in Drosophila, in human NTDs. *PRICKLE1* encodes a cytoplasmic protein with an N-terminal PET (Pk, Espinas, Testin) domain, three LIM domains and a C-terminal PKH (prickle homologous) domain (Katoh and Katoh, 2003). The LIM domain is a cysteine-rich sequence with two zinc-finger motifs that mediates protein-protein interactions. The N-terminal PET domain combines with the three LIM domains during interactions with other proteins. The C-terminal domain contains a CaaX-motif prenylation site that determines protein–protein and protein–membrane interactions (Veeman et al., 2003). Pk physically interacts with other PCP components to modulate signaling through this pathway. Upon Fz activation, Stbm recruits Pk to the cell membrane, and through this interaction, Pk affects Stbm membrane localization and can cause clustering of Stbm. Pk competes with Dgo for Dsh binding and is thought to antagonize Dsh by affecting its membrane localization (Jenny et al., 2003; Das et al., 2004). Both gain-of-function and loss-of-function of *PRICKLE1* in *Xenopus* and zebrafish lead to defective convergent extension movements, manifested mainly by a shortened body axis (in both organisms) and spina bifida (in frog embryos) (Carreira-Barbosa et al, 2003; Takeuchi et al., 2003; Veeman et al., 2003). In mouse, knocking out *prickle1* leads to early embryonic lethality that is associated with failure of distal visceral endoderm migration and primitive streak formation resulting mainly from loss of apico-basal (AB) polarity in the epiblast tissue, suggesting a cross-talk between AB and PCP (Tao et al., 2009).

The well-established role of PCP signaling in the pathogenesis of NTDs in mouse models and humans as well as the effect of Prickle1 on CE movements in lower vertebrates prompted us to analyze this core PCP gene in human NTDs. We sequenced the open reading frame and exon-intron junctions of *PRICKLE1* in a large and multi-ethnic cohort of open and closed forms of NTDs. The cohort consisted of 810 NTD patients affected with non-syndromic or isolated NTDs, where 66% of patients were affected with myelomeningocele or open spina bifida and 32% were affected with various forms of closed spinal NTDs. Of all 810 patients, 42% were male and 84% were of Caucasian White non-Hispanic origin. The two other major ethnic groups present in this cohort consisted of Hispanics (9%) and African Americans (4%) (Supp. Table S1) (Supp. Materials and Methods).

Our screening strategy of *PRICKLE1* in human NTDs focused on the coding exons where many of the disease-associated mutations are found (Stenson et al., 2009) (Supp. Materials and Methods). We have identified 9 rare missense mutations that were all absent in either 346 ethnically-matched Italian control subjects (for the Italian cohort) or 1050 HGDP individuals (for the American multi-ethnic cohort) (Table 1). All these mutations were also absent from the dbSNP and 1000 genome databases. Of these, 7 mutations were predicted to be damaging to protein function using bioinformatics tools (PolyPhen and SIFT) (Table 1). These 7 "potentially pathogenic" variants are described in the next paragraphs in the order that they occur in the PRICKLE1 protein. The DNA mutation and amino acid numbering systems are based on *PRICKLE1* cDNA sequence with the accession NM_001144881.1 and PRICKLE1 protein sequence with the accession NP_001138353.1 respectively. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Two variants, p. Ile69Thr (c.206T>C) and p.Asn81His (c.241A>C), were identified in the PET domain of PRICKLE1 (Supp. Figure S1). The p. Ile69Thr variant was identified in a 22 year-old Italian female affected by diastematomyelia type II which represents a complex dysraphic state caused by the failure of the midline integration of the notochord leading to a variably elongated separation of the spinal cord in two hemicords (Rossi et al., 2004). At birth, the proband showed a tuft of hair in the lumbar-sacral region. MRI showed schisis of the lumbar vertebrae (L2-L3) associated with a low-lying spinal cord and diastematomyelia characterized by the presence of two hemicords at level D12-L1. This mutation was transmitted by her unaffected mother. Isoleucine is invariant across all species analyzed except in *Drosophila* where it is replaced by valine (Supp. Figure S1). While isoleucine and valine are both highly similar in their hydrophobic non polar nature, a substitution by threonine is not conservative as it reduces the hydrophobicity at this position. The p.Asn81His variant was detected in an American male patient of a mixed Caucasian and African American origin and affected with lumbosacral myelomeningocele, a severe form of open NTDs characterized by exposure of the placode (a segment of flattened, non-neurulated embryonic neural tissue) through a midline skin defect in the back. The mutation was transmitted by his mother. Remarkably, Asn81 forms part of a 12 amino acids block that is absolutely conserved to Drosophila (Supp. Figure S1). This change is not conservative as it introduces a positively charged amino acid at a normally neutral position.

One variant, p.Thr275Met (c.824C>T), was identified in the 3rd LIM domain of PRICKLE1 (Supp. Figure S1). This mutation was detected in a 22 year-old Italian patient affected with lumbosacral myelomeningocele, hydrocephalus, Chiari type II malformation, and tethered cord. Thr275 forms part of an absolutely conserved "HWHAT" motif. The substitution of a threonine residue (neutral polar) by a methionine (neutral non polar) is not conservative as it reduces hydrophilicity.

Four variants were identified in the last 284 amino acids at the carboxy terminus of PRICKLE1 (Supp. Figure S1). One variant, p.Val550Met (c.1648G>A), was identified in a white Caucasian male affected with myelomeningocele. Val550 is highly conserved and a valine-to- methionine substitution is non-conservative; although both residues are apolar, methionine is less lipophylic due to its thiogroup. One variant, p.Arg682Cys (c.2044C>T), was identified in a white Caucasian American male affected with myelomeningocele. This mutation was transmitted from his mother. This arginine residue is conserved in all vertebrates analyzed and is replaced by serine in Drosophila (Supp. Figure S1). An arginine (basic)-to-cysteine (neutral, polar) substitution is not conservative as it removes the positive charge at this residue. One variant, p.Ser739Phe (c.2216C>T), was detected in a white Caucasian American male affected with myelomeningocele and that represents a familial NTD case with an affected paternal first degree cousin (not available for this study). This

mutation was transmitted from the mother. However, the family NTD history was reported on the paternal side of the family; this reflects the complexity of the etiology of NTDs. One could hypothesize the presence of a cluster of gene variants (that remain largely unknown) on both paternal and maternal sides of the family that may interact with each other and/or with environmental factors to modulate NTD incidence; or alternatively, the NTD in the affected case and the NTD in his paternal cousin are caused by different risk factors as expected for a heterogeneous disease such as NTDs. Ser739 is highly conserved in human, mouse, dog, cow, chicken and frog (Supp. Figure S1). A serine to phenylalanine substitution is not conservative as it reduces the hydrophilicity at this position. One variant, p.Asp771Asn (c. 2311G>A), was detected in a white Caucasian American male affected with caudal agenesis, which represents a heterogeneous constellation of anomalies comprising total or partial agenesis of the spinal column, anal imperforation, genital anomalies, bilateral renal dysplasia or aplasia, pulmonary hypoplasia, and lower limb abnormalities. The mutation was transmitted from the unaffected mother. Asp771 is invariant across all species analyzed except in zebrafish and Drosophila where it is replaced by glutamate and serine respectively (Supp. Figure S1). A substitution of aspartate (acidic) to asparagine (neutral) is not conservative as it removes the negative charge at this position.

Our genetic data implicate *PRICKLE1* in the pathogenesis of a fraction of human NTDs (7 in 810 or 0.8%) where we identified 7 rare missense mutations that were absent in all controls analyzed and that were predicted to be pathogenic *in silico*. All these mutations were heterozygous and private. In each case, when parents were available for DNA analysis, *PRICKLE1* mutations were detected in one of the parents indicating incomplete penetrance; or alternatively this could be caused by phenotypic variability since the parent could be affected by a mild undetected form of closed NTDs. None of these mutations map close to the intron-exon boundaries and hence most likely do not lead to splicing defects.

In order to test the potential impact of these *PRICKLE1* variants on protein function, we used a zebrafish model where overexpression of the wild-type *pk1a* leads to a defective CE manifested mainly by shortened body axis (Veeman et al., 2003) (Supp. Materials and Methods). We introduced the human mutations in the zebrafish *prickle1a* (*zpk1a*) open reading frame using site-directed mutagenesis. With this assay, we were only able to validate 5 variants (p.Ile69T, p.Asn81His, p.Thr275Met, p. Val550Met and p.Arg682Cys), since the two other variants (p.Ser739Phe and p.Asp771Asn) were not conserved in zebrafish. We first injected *zpk1a* RNA at 100, 200, 300 pg and 400 pg doses to determine the optimal dose that causes a significant perturbation in CE movements in injected embryos (Supp. Figure S2). Injection of 200–400 pg wild-type z*pk1a* alone produced a range of phenotypes that were clustered into three groups based on the phenotype severity (grade 1: wild-type like, grade 2: intermediate and grade 3: severe) (Fig. 1A). We examined the distributions of these three clusters at a dose of 400 pg among all 6 groups described above. The anticipated results of this overexpression assay are as follows: if the *zpk1a* variant has no effect on protein function, it will behave like the wild-type *zpk1a* and produce similar phenotypes and a similar distribution of the 3 groups defined above; if the *zpk1a* variant is a hypermorph, it will produce more severe phenotypes and a larger number of grade 2 and/or grade 3 embryos; and lastly if the *zpk1a* variant is a loss-of-function mutation, then it will not lead to CE defects and will behave like the uninjected embryos. The distribution of the 3 clusters in uninjected wild-type fish was similar to that observed in wild-type fish injected with the vital dye fast-green (Fig. 1B). As expected, the distribution of the clusters obtained with wild-type *zpk1a* was significantly different from uninjected wild-type fish ($P<0.05$) and from wild-type fish injected with the vital dye fast-green ($P<0.05$) (Fig. 1B). When comparing each of the 5 *zpk1a* variants to wild-type *zpk1a*, overexpression of each of four variants, $zpk1a^{I75T}$, $zpk1a^{N87H}$, $zpk1a^{T282M}$ and $zpk1a^{R682C}$, significantly perturbed CE movements with larger numbers of intermediate and severely- affected embryos (grades 2

and 3 respectively) (P<0.01 for *zpk1a*^{I75T}, P<0.001 for *zpk1a*^{N87H} and *zpk1a*^{R682C}, and P<0.0001 for z*pk1a*^{T282M} as compared to wild-type z*pk1a*) (Fig. 1B), suggesting that these variants could act as hypermorphs. For the variant, *zpk1a*^{V545M}, a high level of mortality of ~50% was observed at a dose of 400 pg and hence its overexpression was carried out at a lower dose of 300 pg. At this lower dose, this variant behaved like the wild-type *zpk1*, causing a reduction in axial length that was equal to control (Fig. 1B).

To test whether any of the *pk1a* variants could antagonize the effects of overexpressed wild-type *zpk1a* and hence could act in a dominant negative fashion, we co-injected 200 pg of each of these variants and the wild-type *zpk1a* RNA and studied the resulting phenotype (Fig. 1C–G). If a variant antagonizes the effect of overexpressed wild-type *zpk1a*, a reduction in phenotype severity would be observed. On the contrary, if there is no antagonizing effect, an increase in phenotype severity would be expected as both the variant and wild-type *zpk1a* would be acting jointly in the same pathway to cause CE defects. Only one variant, *zpk1a*^{R682C}, seemed to antagonize the wild-type *zpk1a* in a dominant fashion because its co-injection with the wild-type *zpk1* rescued the phenotype produced when either the wild-type or variant was injected alone (P was significant at <0.05 only when compared to the variant alone) (Fig. 1G). In contrast, the phenotype of wild-type *zpk1a* was not rescued but significantly enhanced with each of the two variants, *zpka1*^{I75T} , and *zpka1*^{N87H}, suggesting that they do not antagonize the wild-type *zpk1a* at the injected dose (P<0.0001 and P< 0.025 respectively) (Fig. 1C,D). Interestingly, two variants, z*pk1a*^{T282M} and *zpk1a*^{V545M}, seem to be antagonized by the wild-type, since their co-injection with the wild-type *zpk1a* rescued the phenotype produced with the variant alone (P<0.001 and P<0.01 respectively) (Fig. 1E,F).

In PCP signaling, knockdown of PCP core genes by antisense morpholino oligos as well as their overexpression leads to similar CE defects, indicating a finely-tuned and dosage sensitive pathway. We consequently did dose-response experiments to assess the effect of higher doses (300 and 400 pg) of each of the 5 variants when co-injected with 200 pg of wild-type *zpk1a* respectively. A significant reduction in phenotype severity was observed only for the variant *zpk1a*^{I75T} when co-injected with the wild-type *zpk1a* at 300 pg and 400 pg respectively (P<0.0001) (Fig. 1C,H), suggesting that this variant could antagonize the effect of the wild-type *zpk1a* at higher doses . A reduction in phenotype severity was observed for the variant *zpka1*^{N87H} but was not significant (Fig. 1D,H). For each of the two variants, z*pk1a*^{T282M}, *zpk1a*^{V545M}, co-injection of higher doses of either 300 pg or 400 pg with 200 pg of wild-type *zpk1a* did not change significantly the phenotypes obtained with co-injection of 200 pg (Fig. 1E,F,H). The variant *zpk1a*^{R682C} manifests a complex dose response when co-injected with wild-type *zpk1a* at higher doses. At 300pg, this variant continues to antagonize the wild-type as manifested by a smaller percentage of affected phenotypes (P<0.05); this antagonistic activity seems to peak at 300 pg and then levels off at a higher dose of 400 pg where we observe an increase in CE phenotype severity(Fig. 1H). This could be interpreted as an excess of the variant (at a dose of 400 pg) that is exerting its hypermorphic activity. In conclusion, our basic validation data in zebrafish suggest that 4 variants, *zpka1*^{I75T}, *zpk1a*^{T282M}, *zpk1a*^{V545M}, and *zpk1a*^{R682C}, act antagonistically with the wild-type *zpk1a*, at different doses. Of these, only one variant, *zpk1a*^{R682C}, seems to antagonize wild-type *zpk1a* in a dominant negative fashion in this model. No significant antagonistic effect was observed between the variant *zpka1*^{N87H} and the wild-type *zpk1a* at any dose of co-injection.

Additional experiments in cellular or animal models are needed to further validate the “hypothesized” pathogenicity of *PRICKLE1* variants detected in our study. In Drosophila, it was shown that upon binding of an unknown ligand to the Fz receptor, several PCP proteins become localized asymmetrically in the plane of the epithelium in a mutually interdependent

manner, forming different subsets of PCP proteins at the proximal (Vang/Pk) and distal (Fz/Dsh) sides of adjacent cells (Simons and Mlodzik, 2008). Fmi interacts with both Dsh/Fz and Vang/Pk complexes for stabilizing and propagating the polarity signals across adjacent cells. Dgo competes with Pk for Dsh binding ensuring tight control over PCP signaling. In particular, Pk was shown to interact with three other PCP core proteins: Dsh through its PET/LIM domains, Vang and Dgo through a stretch of 131 amino acids close to, but not including, its very C terminus (Supp. Figure S1) (Jenny et al., 2003).The Vang/Dgo binding domain of Pk overlaps with 6 amino acids of its PKH domain. Three variants (p.Ile69Thr, p.Asn81His and p.Thr275Met) map to the PET/LIM Dsh-binding domains and three variants (p.Arg682C, p. Ser739Asn, p.Asp771Asn) map to or close to the Vang/Dgo binding domain (Supp. Figure S1). Any of these mutations could affect the interaction of PRICKLE1 with its binding partner(s) and interfere with the polarized protein distribution required for PCP signaling. One other variant, p.Val550Met, maps close to a Destruction-box (D-box motif) implicated in Dvl3 ubiquitination/degradation in canonical Wnt/Fz β-catenin signaling in human hepatocellular carcinoma (Chan et al., 2006) (Supp. Figure S1). In zebrafish, it was shown that pk1a blocks the Fz7-dependent membrane localization of Dsh by down-regulating levels of Dsh protein; however the mechanisms of such degradation are not determined yet. One could hypothesize that the variant p.Val550Met could affect the ability of PRICKLE1 to down-regulate DVL levels and negatively regulate the PCP pathway.

In humans, a homozygous missense mutation in *PRICKLE1* was identified in an autosomal recessive form of progressive myoclonus epilepsy-ataxia syndrome. This mutation disrupts the interaction of PRICKLE1 with REST *in vitro* and the function of Pk1 in convergent extension in a zebrafish model (Bassuk et al., 2008). Mouse Prickle 1 was discovered independently in a yeast two-hybrid screen based on its ability to functionally interact with a transcriptional repressor called REST/NRSF (RE-1 silencing transcription factor/neuron-restrictive silencer factor) that is an essential regulator of neural genes. It was hypothesized that the disruption of this interaction caused by the epilepsy mutation in *PRICKLE1* would lead to constitutive activation of REST and consequently inappropriate silencing of REST target genes in neuronal and nonneuronal cells. A recent study showed that mutations in *prickle* genes are associated with seizures in humans, mice, and flies (Tao et al., 2011). Our results led us to hypothesize that either interactions of PRICKLE1 with various interacting proteins (PCP members or REST) could be differently affected by PRICKLE1 variations, thus resulting in distinct alterations of downstream pathways or processes and therefore explaining the phenotypic diversity of the PRICKLE1-related spectrum. Transgenic animal models bearing variations in *PRICKLE1* will be necessary in future to dissect the multifaceted role of this gene in development and maintenance of the central nervous system.

The cohort used in this study was previously screened for mutations in two other PCP genes, *VANGL1* and *VANGL2*. To date, all known variations of the PCP genes are missense; however, the presence of pathogenic copy number variants in these genes in NTDs cannot be excluded by our screening strategies. All mutations are heterozygous, partially penetrant and present in sporadic and familial cases of NTDs. The majority of these mutations are private. The phenotype of *VANGL1* and *VANGL2*, and *PRICKLE1* mutations is variable including open and closed forms of NTDs. No patient carried potentially pathogenic mutations in more than one of these 3 PCP genes. It will be interesting to screen other PCP genes in NTDs and examine digenic combinations of mutations since variants at PCP loci might act in combination to confer a high risk for developing NTDs, as may be the case with a multifactorial threshold model for NTDs in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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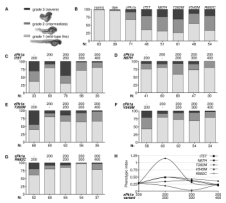

Figure 1.

(A) Lateral views of 2 days post fertilization zebrafish illustrating the 3 clusters identified based on phenotype severity. (B) The distribution of the 3 clusters in each of the experimental groups: uninjected, injected with vital dye, and injected with each of wild-type *zpk1a* and of the 5 *zpk1a* variants. The variants p.Ile69Thr, p.Asn81His, p.Thr275Met, p.Val550Met and p.Arg682Cys in human PRICKLE1 are orthologous to p.Ile75Thr, p.Asn87His, p.Thr282Met, p.Val545Met and p.Arg682Cys in zpk1a respectively. All injections were done at 400 pg except for the variant p.Val545Met that was injected at 300 pg. (C–G) The distribution of the 3 clusters in each of the experimental groups injected with 200pg of each of the *zpk1a, p.Ile75Thr, p. Asn87His, p.Thr282Met, p.Val545Met* and *p.Arg682Cys* mRNA as well as in the experimental groups where 200 pg, 300pg and 400pg of the mutant *zpk1a* was co-injected respectively with 200 pg of the wild-type *zpk1a*. The Y-axis represents the percentages of the 3 clusters. (H) A dose-response curve for the co-injection assay of 200 pg wild-type *zpk1a* with 200 pg, 300 pg and 400 pg of each of the 5 *zpk1a* variants respectively. The Y-axis represents the phenotypic index that measures the distribution of phenotypic classes described in panel A and that is determined by multiplying the number of embryos in each grade by an assigned value (grade 1 = 0, grade 2 = 1, grade 3 = 2) and dividing the sum by the total number of embryos (see Supp. Materials and Methods).

Table 1

Rare missense variants (<1%) identified in *PRICKLE1* in human neural tube defects[a]

Amino acid change	Nucleotide change	Freq. in patients[b]	Freq. in controls[b]	Exon	Domain	PolyPhen prediction	SIFT prediction
Potentially damaging							
p.Ile69Thr	c.206T>C	1	0	2	PET	Probably damaging	Intolerant
p.Asn81His	c.241A>C	1	0	2	PET	Possibly damaging	Intolerant
p.Thr275Met	c.824C>T	1	0	6	LIM3	Probably damaging	Intolerant
p.Val550Met	c.1648G>A	1	0	7	Close to D-Box1	Benign	Intolerant
p.Arg682Cys	c.2044C>T	1	0	7	Vangl/Dgo binding	Benign	Intolerant
p.Ser739Phe	c.2216C>T	1	0	7	Vangl/Dgo binding	Probably damaging	Intolerant
p.Asp771Asn	c.2311G>A	1	0	7	PKH	Benign	Intolerant
Benign							
p.Val121Ile	c.361G>A	1	0	3	Close to LIM1	Benign	Tolerant
p.Ala124Thr	c.370G>A[c]	6	40	3	LIM1	Benign	Tolerant
p.Ser799Cys	c.2396C>G	1	0	7		Benign	Tolerant

[a] The position of the mutations is given with reference to sequence accession NM_001144881 for the *PK1* cDNA and NP_001138353.1 for the protein.

[b] The patients' group included 421 Italian patients and 389 American patients; the control group included 346 Italian controls and 1050 HGDP individuals (www.cephb.fr/)

[c] The variant c.370G>A is reported in the dbSNP as rs79087668 and was not considered for further evaluation