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Planar Cell Polarity Pathway Genes and Risk for Spina Bifida

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Abstract

Spina bifida, a neural tube closure defect (NTD) involving the posterior portion of what will ultimately give rise to the spinal cord, is one of the most common and serious birth defects. The etiology of spina bifida is thought to be multi-factorial and involve multiple interacting genes and environmental factors. The causes of this congenital malformation remain largely unknown. However, several candidate genes for spina bifida have been identified in lower vertebrates, including the planar cell polarity (PCP) genes. We used data from a case-control study conducted in California to evaluate the association between variation within several key PCP genes and the risk of spina bifida. The PCP genes included in this study were the human homologues of the *Xenopus* genes *Flamingo*, *Strabismus*, *Prickle*, *Dishevelled* and *Scrib*, two of the homologues of *Xenopus Wnt* genes, *WNT5A* and *WNT11*, and two of the homologues of *Xenopus Frizzled*, *FZD3* and *FZD6*. None of the 172 SNPs that were evaluated were significantly associated with spina bifida in any racial/ethnic group after correction for multiple testing. However, several SNPs in the *PRICKLE2* gene had unadjusted p value < 0.01. In conclusion our results, though largely negative, suggest that the *PRICKLE2* gene may potentially modify the risk of spina bifida and deserves further investigation.

Keywords

Neural Tube Defects (NTDs); Spina Bifida; Planar Cell Polarity (PCP); candidate gene; association study

INTRODUCTION

Neural tube defects result from a failure of neural tube closure during early embryonic development. While the molecular mechanisms that govern neural tube closure are poorly understood [MRC 1991], it is known that evolutionarily conserved common molecular mechanisms are employed to regulate morphogenesis of multi-cellular organisms. For

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example, the planar cell polarity (PCP) pathway is important in the embryonic development of a range of species including human, and recent studies indicate that this pathway is important in neural tube development [Copp et al., 2003; Curtin et al., 2003; Doudney and Stanier 2005; Kibar et al., 2007; Kibar et al., 2001; Murdoch et al., 2001; Murdoch et al., 2003; Ueno and Greene 2003; Wallingford 2006; Wallingford and Harland 2002].

Determination of cell polarity is a fundamental developmental process [Green et al., 2008]. The PCP pathway was first discovered in *Drosophila*, where it is involved in determining the position and direction of wing hairs [Adler and Lee 2001; Adler et al., 2000; Keller 2002; Mlodzik 2002]. In vertebrates, the PCP signaling pathway is essential for neural tube closure [Doudney and Stanier 2005; Ueno and Greene 2003]; any significant perturbation of this pathway results in a high prevalence of NTD-affected embryos in both *Xenopus* and mouse models [Curtin et al., 2003; Hamblet et al., 2002; Jenny et al., 2003; Kibar et al., 2007; Kibar et al., 2001; Lu et al., 2004; Murdoch et al., 2003; Qian et al., 2007; Tada and Smith 2000; Wallingford and Harland 2002; Wallingford et al., 2000; Wallingford et al., 2001; Wang et al., 2006]. In *Xenopus laevis*, disruption of PCP signaling results in an abnormally wide neural plate, which prevents the convergence of the neural folds at the midline and therefore proper closure of the neural tube [Copp et al., 2003; Wallingford 2006; Wallingford and Harland 2001]. Among genetically modified mouse models of NTDs, four involve PCP pathway genes: *Vangl2*, *Scrib1*, *Celsr1* and *Dsh1/Dsh2* [Doudney and Stanier 2005]. In addition, studies in humans have identified several non-synonymous mutations in another PCP pathway gene, *VANGLI1*, in individuals with spina bifida and other types of NTDs [Kibar et al., 2009b; Kibar et al., 2007].

At the molecular level, the process of cell polarization is controlled by a membrane-associated signaling complex composed of several distinct proteins. The vertebrate PCP pathway consists of secreted ligands (Wnts), membrane proteins (Frizzled, Flamingo and strabismus/Van Gogh) and cytoplasmic proteins (Dishevelled, Prickle, Diego, small G, Rho/Rac and Rho kinase). Based on previous findings, we hypothesized that the variants in PCP pathway genes are risk factors for human neural tube defects. This study focuses on screening PCP genes (other than *VANGLI1*) for their potential associations with NTD.

MATERIALS AND METHODS

Study Population

The data for this study were obtained from a case-control study conducted by the California Birth Defects Monitoring Program (CBDMP). The CBDMP is an active, population-based surveillance system for collecting information on infants and fetuses with congenital malformations, which has been described elsewhere [Croen et al., 1991]. Included for study were 338 singleton infants with spina bifida (cases) and 338 non-malformed infants (controls). These included all live born cases and a random sample of non-malformed control infants ascertained by the CBDMP corresponding to birth years 1994–1998.

The case and control infants were linked to their newborn bloodspot. DNA was extracted from the blood spots using the Gentra Puregene DNA Extraction Kit (Qiagen, Valencia, CA) following the standard protocol. All samples were obtained with approval from the State of California Health and Welfare Agency Committee for the Protection of Human Subjects.

Candidate genes and SNPs

The 14 candidate genes, their *Xenopus* homologue and the number of SNPs genotyped are listed in Table I. SNPs were selected for each candidate gene using the SNPbrowser™ Software (ABI, Foster City, CA). tagSNPs were selected from the four HapMap populations (CEU, YRI, JPT and CHB) [De La Vega et al., 2006]. For the nine small genes (*WNT5A*,

WNT11, *DSH*, *DVL2*, *DVL3*, *VANGL2*, *CELSR2*, *CELSR3* and *SCRIB*), all the validated HapMap SNPs with a minor allele frequency (MAF) no less than 5% were selected for genotyping. For the five large genes (*PRICKLE1*, *PRICKLE2*, *CELSR1*, *FZD3* and *FZD6*), we used a more stringent criteria to select tagSNPs in order to reduce the number of SNPs to be genotyped (MAF 10%). In addition, all non-synonymous SNPs in these genes were genotyped.

Genotyping Approach

The SNPs selected for analyses were submitted to Applied Biosystems to create a unique SNPlex assay. The SNPlex[™] genotyping system (Applied Biosystems, Foster City, CA) is based on a universal multiplexed oligonucleotide ligation assay (OLA)/PCR and drag chute mobility modifier technology. With this method, up to 48 SNPs can be tested in a single reaction. The resulting genotype data was analyzed using GeneMapper[™] v4.0 (Applied Biosystems, Foster City, CA).

Analytic procedures

Associations between spina bifida and infant sex, maternal education, maternal age and maternal ethnicity were assessed by Chi-square analyses using the full data set. All other analyses were performed separately within each ethnic/race group. The subgroup of Hispanic infants was further subdivided into those whose mother was born in the United States, or native-born (Hisp-NB) and those who were foreign-born (Hisp-FB), as it has been shown that these two groups are different with respect to ethnic composition and social economic status [Baluja et al., 2003; Curry et al., 2005]. Allele frequencies were estimated and departure from Hardy-Weinberg Equilibrium (HWE) was assessed by Chi-square analysis in each subgroup using data from controls only. The Odds Ratios (OR) for each SNP genotype was calculated using logistic regression analyses. Only genotype data was included in the logistic regression model. For each SNP, potential association with NTD risk were evaluated assuming dominant, recessive and additive models of inheritance. Chi-square tests were used to assess the significance of dominant and recessive models. Cochran-Armitage trend tests were used to assess the significance of additive models. Individual tests with a p value < 0.01 were considered significant. Haplotype associations for a subset of SNPs were evaluated by Chi-square tests. These analyses were performed using SVS v7.1.1 (Goldenhelix, Bozeman, MT) software. The false discovery rate (FDR) [Benjamini and Hochberg, 1995] or the Bonferroni procedure was used to account for multiple testing.

RESULTS

The distribution of infant sex and maternal age were not significantly different between cases and controls (Table II). The distributions of maternal education level and maternal race/ethnicity were significantly different between cases and controls, with mothers of cases being less educated and more likely to be Hispanic (primarily foreign born) and less likely to be Asian as compared to the mothers of control infants.

Genotyping of 211 SNPs was performed in 676 samples. The numbers of subjects from each ethnic/race groups were listed in table II. Seven samples were excluded because of poor genomic DNA yield. In addition, SNPs with genotyping call rates lower than 95% (n=17) and with overall MAFs lower than 2% (n=22) were excluded from further analysis. After making these exclusions, genotype data for 172 SNPs from 669 samples were left for further study. Evidence of departure from HWE (i.e. p<0.01) resulted in the exclusion of one additional SNP in Asians, five additional SNPs in Hisp-NB, three additional SNPs in Hisp-

FB and three additional in Blacks. 9 cases and 13 controls with missing maternal ethnicity data were also excluded from further analysis.

The association between each SNP and spina bifida was assessed separately in the four largest ethnic groups (White, Asian, Hisp-NB, Hisp-FB). Data from the black subgroup were not analyzed due to small numbers (19 cases and 19 controls). A total of 2037 tests of association were performed. Table III lists the SNPs and association tests with un-adjusted P -value ≤ 0.01 . Although none of these associations remained significant when the FDR for the 2037 comparisons was controlled at 0.05, it is of interest to note that among the 13 SNPs whose association tests have un-adjusted P -values ≤ 0.01 , 6 were in the *PRICKLE2* gene. For the SNPs that showed $p < 0.01$ in either Hisp-NB or Hisp-FB, we subsequently analyzed the data using the combined data from these two groups. The results with $p < 0.01$ were also listed in table III. The association analyses using the whole data set did not yield additional positive findings.

We then performed haplotype association analysis for the five *PRICKLE1* SNPs that showed $p < 0.01$ in the Asian group (rs149105, rs695936, rs153717, rs153726 and rs888405). LD among these five SNPs in Asian group was measured and the D' value ranges from 0.85 to 1. Haplotype frequencies were estimated based on the EM algorithm. The estimated frequency of TCGCG haplotype appeared to be enriched in cases compared to other haplotypes (0.94 in cases, 0.43 in controls, Chi-square test $P = 0.0003$).

Haplotype analyses using moving windows of two-, three-, and four-SNPs were also performed for all other genes in each of the four ethnic groups. None of the haplotypes was significantly associated with spina bifida risk after adjusted for multiple comparisons using the Bonferroni approach at 0.05 level (data not shown).

DISCUSSION

In this study, we analyzed the associations between SNPs in 14 PCP pathway genes and risk of spina bifida in multiple racial/ethnic groups. Although no association achieved statistical significance after accounting for the 5% false discovery among multiple SNP testing, 6 of the 13 SNPs (46%) with the smallest P -value are variants of *PRICKLE2*, while only 15% of all genotyped SNPs were among this gene. Furthermore, variants in *PRICKLE2* gene have the smallest P -value in both Asian and Hisp-FB groups, and have the second smallest P -value in Hisp-NB group. The haplotype formed by the five positive *PRICKLE2* SNPs in Asian group also showed association with spina bifida. While the limited sample size of our study compromised the power to detect associations with SNPs that are only weakly or moderately associated with spina bifida, the observation of small P -values in *PRICKLE2* gene in three out of the four ethnic groups could not rule out the potential association between this gene and spina bifida risk. Other than the low power, it is also of note that for the five large genes (including *PRICKLE2*), we only investigated SNPs with MAF equal or larger than 10%; thus some less common variants were ignored even if they have potential impact on the risk for spina bifida.

PRICKLE2 is one of the two known human homologues for the *Prickle* gene [Katoh, 2003]. It is expressed in the neural tube tissue during early mammalian development [Bekman and Henrique, 2002] and is a critical component of the PCP pathway. Prickle physically interacts with Vangl to maintain the asymmetric distribution of PCP core components in polarized cells during development [Jenny et al., 2005]. Inactivation and over-expression of Prickle causes spina bifida in *Xenopus* embryos [Takeuchi et al., 2003], as well as defects in convergent extension, which is important in gastrulation and neural tube closure, in other vertebrates, such as zebrafish [Veeman et al., 2003]. The SNPs in the human *PRICKLE2*

gene that were most strongly associated with spina bifida are intronic tagSNPs covering the region around the first 4 exons, which codes the PET domain and LIM domain. According to a previous study [Tree et al., 2002], loss-of-function for either PET domain or LIM domain of *Drosophila Prickle* is sufficient to abolish the binding between *Prickle* and *Dishevelled*, which is critical in maintaining the polar cell polarity and normal embryonic development [Jenny et al., 2005; Tree et al., 2002]. Taken together, this information supports the hypothesis that variation in the human *PRICKLE2* gene contributes to development of spina bifida.

In summary, of the 172 SNPs examined among 14 PCP genes, none were significantly associated with risk of spina bifida after correction for multiple testing. However, our analysis of these data suggests that the potential association between *PRICKLE2* gene variants and risk for spina bifida deserves further investigation. The strengths of our study include its population-based ascertainment of cases and controls. A larger and more robust study of the polymorphisms in PCP genes, especially the *PRICKLE2* gene, is warranted.

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TABLE I

14 Planar Cell Polarity Genes Investigated

Gene name	Xenopus homologue	# of SNPs genotyped
<i>WNT5A</i>	<i>Wnt</i>	5
<i>WNT11</i>	<i>Wnt</i>	7
<i>FZD3</i>	<i>Frizzled</i>	11
<i>FZD6</i>	<i>Frizzled</i>	20
<i>DSH</i>	<i>Dishevelled</i>	22
<i>DVL2</i>	<i>Dishevelled</i>	2
<i>DVL3</i>	<i>Dishevelled</i>	4
<i>PRICKLE1</i>	<i>Prickle</i>	29
<i>PRICKLE2</i>	<i>Prickle</i>	36
<i>VANGL2</i>	<i>Strabismus</i>	8
<i>CELSR1</i>	<i>Flamingo</i>	44
<i>CELSR2</i>	<i>Flamingo</i>	11
<i>CELSR3</i>	<i>Flamingo</i>	5
<i>SCRIB</i>	<i>Scrib</i>	7
Total		211

TABLE II

Characteristics of Spina Bifida Cases and Controls

Variable		Control (%)	Case (%)	P-value
Gender	Female	166(49.1%)	169(50.0%)	0.88
	Male	172(50.9%)	169(50.0%)	
Maternal Age	Less than 20	49(14.5%)	43(12.7%)	0.20
	20–24	75(22.2%)	98(29.0%)	
	25–29	89(26.3%)	84(24.9%)	
	30–34	78(23.1%)	69(20.4%)	
	35–55	47(13.9%)	42(12.4%)	
Maternal Education	Less than high school	125(37.0%)	181(53.6%)	0.000012
	High school	94(27.8%)	81(24.0%)	
	College	116(34.3%)	71(21.0%)	
Maternal Ethnicity	White	79(23.4%)	61(18.0%)	0.00000031
	Hisp-NB	52(15.4%)	61(18.0%)	
	Hisp-FB	132(39.1%)	178(52.7%)	
	Black	19(5.6%)	19(5.6%)	
	Asian	43(12.7%)	8(2.4%)	
	Missing	13(3.8%)	9(2.7%)	
Total		338	338	

TABLE III

Planar Cell Polarity SNPs with Smallest Un-adjusted *P*-values

Marker	dbSNP_ID	Gene	Ethnicity	Test Statistics	<i>P</i> -value	Association Test Model	Odds Ratio [*]	95% CI
hCV11329570	rs153717	<i>PRICKLE2</i>	Asian	Armitage Trend Test	0.0068	additive (Dd/dd)	9.17	0.998–83.33
				Chi-square	0.0061	dominant	14.54	1.62–130.86
hCV618532	rs149105	<i>PRICKLE2</i>	Asian	Armitage Trend Test	0.0078	additive (Dd/dd)	6.67	1.05–83.33
				Chi-square	0.0067	dominant	14	15.56–125.26
hCV618602	rs153726	<i>PRICKLE2</i>	Asian	Armitage Trend Test	0.006	additive (Dd/dd)	8.07	0.87–76.92
				Chi-square	0.0061	dominant	14.54	1.62–130.86
hCV8802585	rs695936	<i>PRICKLE2</i>	Asian	Armitage Trend Test	0.0086	additive (Dd/dd)	7.41	0.79–71.43
				Chi-square	0.01	dominant	13.38	1.46–122.72
hCV8802720	rs888405	<i>PRICKLE2</i>	Asian	Armitage Trend Test	0.0019	additive (Dd/dd)	14.71	1.59–142.86
		Asian		Chi-square	0.0014	dominant	21.7	2.37–198.46
		Hisp-NB		Chi-square	0.0074	dominant	0.33	0.15–0.73
hCV11329629	rs2306380	<i>PRICKLE2</i>	Hisp-FB	Armitage Trend Test	0.00096	additive (Dd/dd)	2.42	1.43–4.09
						(DD/Dd)	1.02	0.19–5.60
		Hisp-FB		Chi-square	0.00081	dominant	2.44	1.45–4.00
		Hisp ^{**}		Chi-square	0.0095	dominant	1.72	1.14–2.61
hCV11879006	rs3788723	<i>CELSRI</i>	Hisp ^{**}	Armitage Trend Test	0.0071	additive (Dd/dd)	0.66	0.44–1.01
						(DD/Dd)	0.66	0.33–1.32
hCV26334885	rs4823831	<i>CELSRI</i>	Hisp-FB	Armitage Trend Test	0.0037	additive (Dd/dd)	0.72	0.48–1.09
						(DD/Dd)	0.41	0.17–0.96

Marker	dbSNP_ID	Gene	Ethnicity	Test Statistics	P-value	Association Test Model	Odds Ratio*	95% CI
hCV27516514	rs3788706	<i>CELSR1</i>	Hisp**	Armitage Trend Test	0.0008	additive (Dd/dd)	2.08	1.37–3.17
						(DD/Dd)	1.00	0.32–3.08
			Hisp-FB	Chi-square	0.0067	dominant	1.92	1.20–3.13
			Hisp**	Chi-square	0.0004	dominant	2.08	1.38–3.13
hCV29072668	rs5768773	<i>CELSR1</i>	Hisp**	Armitage Trend Test	0.0007	additive (Dd/dd)	1.35	0.87–2.10
						(DD/Dd)	2.17	1.22–3.85
hCV12059877	rs3895894	<i>PRICKLE1</i>	Hisp-NB	Armitage Trend Test	0.0061	additive (Dd/dd)	1.87	0.77–4.56
						(DD/Dd)	2.79	0.89–8.73
hCV16076911	rs2131860	<i>FZD6</i>	white	Armitage Trend Test	0.0093	additive (Dd/dd)	0.52	0.22–1.22
hCV27504171	rs3808558	<i>FZD6</i>	Hisp-FB	Armitage Trend Test	0.0049	additive (Dd/dd)	6.54	1.48–28.97

* D: Minor allele; d: major allele

** Hisp: combined data of Hisp-FB and Hisp-NB