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Genes Encoding Catalytic Subunits of Protein Kinase A and Risk of Spina Bifida

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Abstract

BACKGROUND—*PRKACA* and *PRKACB* are genes encoding the cAMP-dependent protein kinase A (PKA) catalytic subunits alpha and beta, respectively. PKA is known to be involved in embryonic development, as it downregulates the Hedgehog (Hh) signaling pathway, which is critical to normal pattern formation and morphogenesis. The PKA-deficient mouse model, which has only a single catalytic subunit, provided intriguing evidence demonstrating a relationship between decreased PKA activity and risk for posterior neural tube defects (NTDs) in the thoracic to sacral regions of gene-knockout mice. Unlike most other mutant mouse models of NTDs, the PKA-deficient mice develop spina bifida with 100% penetrance. We hypothesized that sequence variations in human genes encoding the catalytic subunits may alter the PKA activity and similarly increase the risk of spina bifida.

METHODS—We sequenced the coding regions and the exon/intron boundaries of *PRKACA* and *PRKACB*. We also examined 3 common single-nucleotide polymorphisms (SNPs) of these 2 genes by allele discrimination.

RESULTS—Five sequence variants in coding region and 2 intronic sequence variants proximal to exons were detected. None of the 3 SNPs examined in the association study appeared to be associated with substantially increased risk for spina bifida.

CONCLUSIONS—Our results did not reveal a strong association between these PKA SNPs and spina bifida risk. Nonetheless, it is important to examine the possible gene-gene interactions between *PRKACA* and *PRKACB* when evaluating the risk for NTDs, as well as genes encoding regulatory subunits of PKA. In addition, interactions with other genes such as Sonic Hedgehog (*SHH*) should also be considered for future investigations.

Keywords

PRKACA; *PRKACB*; case-control study; spina bifida

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INTRODUCTION

The cAMP-dependent protein kinase A (PKA) is a serine/threonine kinase composed of 2 catalytic (C) subunits and 2 regulatory (R) subunits. Previous studies have demonstrated that PKA is a ubiquitous signaling molecule that mediates multiple extracellular signals in eukaryotic cells (Griffeoen and Thevelein, 2002; Poueymirou and Schultz, 1989; Skalhegg et al., 2002). PKA's role in embryonic development primarily is mediated through down-regulating the Hedgehog (Hh) signaling pathway (Noveen et al., 1996; Ungar and Moon, 1996). *PRKACA* and *PRCAKB* are the genes encoding the 2 catalytic subunits α and β, respectively. PKA-deficient mice were generated by intercrossing double heterozygotes of Cα and Cβ ($C\alpha^{+/-}C\beta^{+/-}$) by Huang et al. (2002). Mice with only 1 allele of each catalytic subunit ($C\alpha^{+/-}C\beta^{-/-}$ or $C\alpha^{-/-}C\beta^{+/-}$) developed spinal neural tube defects (NTDs) with 100% penetrance. Studies on these mice provided intriguing evidence that decreased PKA activity induced posterior NTDs in both the thoracic and sacral regions of the affected fetuses. In these mutant animals, the sonic hedgehog (SHH) signal response domain was expanded into the regions of the observed morphologic abnormalities. Decreased PKA activity also resulted in an increase in apoptosis in these animals (Huang et al., 2002).

The SHH signaling pathway is highly conserved throughout evolution and has been implicated in diverse processes in vertebrate development, including cartilage differentiation, myotome and sclerotome specification, limb morphogenesis, and the specification of different neuronal cell types along the dorsoventral axis of the neural tube (Ingham, 1998). During the early development of human embryos, *SHH* induces cell proliferation in a tissue-specific manner. It is expressed in the notochord, the floor plate of the neural tube, and, eventually, in the brain, in the zone of polarizing activity in the developing limbs, and in the gut (Odent et al., 1999). Abnormal expression of the *SHH* gene was observed in the floor plate of embryos with craniorachischisis and spina bifida (Kirillova et al., 2000). PKA is known to be a negative regulator of *SHH* in vertebrates (Hammerschmidt et al., 1996; Epstein et al., 1996); however, whether PKA is involved in all of these processes and just how PKA functions as a negative regulator (directly in the Hh pathway or in a parallel pathway) remains unclear (Huang et al., 2002).

The human *PRKACA* gene maps to chromosome 19p13.1. The human mRNA displays 90% homology to its mouse counterpart. The human *PRKACB* gene maps to chromosome 1p36.1. The mRNA sequence shows 85% homology to the mouse *PRKACB* gene. There is a 77% homology between the *PRKACA* and *PRKACB* mRNA sequences. We hypothesized that sequence variations in human genes encoding the catalytic subunits may decrease the PKA activity and increase the risk of spina bifida in humans. In the current study, we searched for sequence variants in coding regions of *PRKACA* and *PRKACB* genes and investigated whether polymorphic variants of the *PRKACA*/*PRKACB* genes influenced risk for spina bifida in a cohort of California infants.

MATERIALS AND METHODS

Subjects

Pregnancy and outcome data were obtained from the California Birth Defects Monitoring Program, a population-based active surveillance system for collecting information on infants and fetuses with congenital malformations (Croen et al., 1991). Program staff collected diagnostic and demographic information from multiple sources of medical records for all live-born infants, stillborn fetuses (defined as >20 weeks gestation), and pregnancies electively or spontaneously terminated. Nearly all structural anomalies diagnosed within 1 year of delivery were ascertained. Overall ascertainment has been estimated as 97% complete (Schulman and Hahn, 1993).

Included for study were 207 infants with spina bifida (cases) and 209 nonmalformed infants (controls). Among the 207 case infants, 71 were born in 1983–1986 and 136 were born in 1994–1995. The 209 controls were randomly selected among all live-born infants from the same time period and geographic regions from which the cases were ascertained. That is, 70 control infants were selected at random from among all eligible births in the 1983–1986 birth period and 139 controls were selected at random from among all eligible births in the 1994–1995 birth period. All samples were obtained with approval from the State of California Health and Welfare Agency Committee for the Protection of Human Subjects. Genomic DNA was extracted from dried newborn screening blood spots on filter paper using the Puregene DNA Extraction Kit (Gentra, Minneapolis, MN) according to the manufacturer's instructions and was amplified using PCR (Schwartz et al., 1990).

DNA Resequencing

In order to identify possible functional variations in *PRKACA* and *PRKACB* genes, we sequenced the coding regions of the *PRKACA* and *PRKACB* genes and exon/intron junctions. We randomly sequenced 48 samples from the 207 cases and 48 samples from the 209 controls. Sequencing was performed using the BigDye Terminator (version 3.1) Kit from Applied Biosystems on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). The primers used for the PCR and sequencing reactions are listed in Table 1. Sequencing data were aligned and compared with GenBank sequences using Sequencher software version 4.2 (Gene Codes, Ann Arbor, MI).

Selection of Single-Nucleotide Polymorphism (SNP) Markers

For candidate gene association studies, it is preferable to examine polymorphisms known or suggested to affect the function of the gene product. Nonsynonymous SNPs that change amino acid coding, or SNPs at exon/intron junction that may affect the splicing site or SNPs in regulatory regions all fall into this category. However, based on our resequencing effort for these 2 genes, *PRKACA* and *PRKACB*, the 4 SNPs in coding region list in NCBI dbSNP were not found in our study population. We found 4 nonsynonymous SNPs, but the frequencies of the minor alleles are <10%. Therefore, we selected intronic SNPs based on the assumption that these SNPs are in linkage disequilibrium with the potential disease-causing variation(s). Minor allele frequency has also been considered for SNP selection. SNP markers selected have a minor allele frequency of no less than 0.10.

Genotyping

We examined SNPs within these 2 candidate genes for NTD risk by TaqMan allele discrimination analysis (Assay-on-Demand) using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), following the manufacturer's protocols. The intronic SNP rs729372 was used as a marker for the *PRKACA* gene, whereas rs1565823 was used as a marker for *PRKACB*. An SNP in the 3′ untranslated region (3′-UTR) of *PRKACB*, rs600674, located 124 kb downstream of rs1565823 on chromosome 1, was also interrogated. Genotyping analyses were performed 2 times for all samples. Only samples with consistent results from both tests were considered successfully genotyped. Experiments were performed by laboratory personnel who did not know the case/control status of samples.

Statistical Analyses

Odds ratios and 95% confidence intervals (CIs) were used to estimate risks. These measures were calculated using SAS software (version 9.1). Individuals with wild-type genotypes were considered referents for risk estimation associated with heterozygous and homozygous mutant genotypes. Deviation from Hardy-Weinberg equilibrium among control infants was evaluated

by the χ^2 test. Information on maternal race/ethnicity was obtained for case and control infants from California birth certificates.

RESULTS

Coding Region Resequencing of *PRKACA* and *PRKACB*

Sequence variants found in this study are listed in Table 2. Forty-four cases (91.7%) and 46 control individuals (95.8%) sequenced provided data for all exons sequenced for *PRKACA*. Three sequence variations were identified in the *PRKACA* coding region. One case was found to be homozygous for 288A>G in exon 2, which changes the amino acid from lysine to glutamic acid. Both lysine and glutamic acid are polar (hydrophilic) amino acids. One case infant and 1 control infant were determined to be heterozygotes for 654G>C (GI, 46909581), which changes the amino acid from valine to leucine. Both valine and leucine are nonpolar (hydrophobic) amino acids with similar structures. One case infant and 2 control infants were heterozygotes for 658T>G, which changes the amino acid from leucine to arginine. This mutation changes the non-polar leucine to a positively charged hydrophilic arginine. Furthermore, we found a common intronic SNP located 76 bp downstream of exon 6 (749 +76G>A). No subject had a variant allele for the SNPs rs3729858 and rs11541563, which have been listed in the dbSNP database.

Forty-one cases (85.4%) and 40 control individuals (83.3%) sequenced provided data for all exons sequenced for *PRKACB*. Two sequence variants were found in the *PRKACB* coding region. One control was found to be a heterozygote for 700G>A (GI, 46909585), which changes glutamic acid to leucine. These 2 amino acids have different base/acid nature, as mentioned previously. Another control was found to be a heterozygote for a synonymous change 690G>A. One control infant was found to be a heterozygote for 649-25G>C. No subject had either of 2 synonymous SNPs, rs11552595 and rs3729866, listed in the dbSNP database.

Spina Bifida Association Study

Genotyping data were successfully obtained for SNP rs729372 in 200 cases (96.6%) and 201 controls (96.2%). For SNP rs1565823, 195 cases (94.2%) and 197 controls (94.3%) were successfully genotyped. For SNP rs600674, 205 cases (99.0%) and 201 controls (96.2%) were successfully genotyped. Genotype frequencies for cases and controls are presented in Tables 3–5. None of the 3 SNPs appeared to reveal substantially increased risk for spina bifida in the overall population or in the 2 major ethnic groups (Hispanic white vs. non-Hispanic white) comprising the study population.

DISCUSSION

To our knowledge, this is the first epidemiologic study investigating possible associations between genes encoding catalytic subunits of PKA, which is a signaling molecule, and the risk of spina bifida. There was no apparent increase in spina bifida risk for infants who carried uncommon alleles for any of the 3 *PRKACA* and *PRKACB* SNPs we genotyped.

It has been suggested that NTDs could be the result of a failure of neural tube epithelial cell differentiation, resulting in too few cells reaching terminal differentiation and continuing to proliferate. Cellular differentiation of neural plate cells is coordinately regulated through the signaling mechanisms that either activate or repress transcription factors appropriate to the cells' position and fate (Robertson et al., 2001). These behaviors can be affected by subtle alterations in the availability of cyclic nucleotides. The cellular cyclic nucleotide composition can also determine the cells' responses to exogenous signals. For example, cGMP enhances the SHH signals to a neural plate cell, whereas cAMP will inhibit these signals. This is

important, as SHH signaling is involved in the differentiation of the ventral cell types of the fusing neural tube (Roelink et al., 1994, 1995). Therefore, a change in the nucleotide composition of neural tube cells at critical time points during neural fold fusion can have a major impact during neural tube closure.

We know that folic acid, a major modifier of NTD risk, regulates nucleotide composition in cells. We also know that folate concentrations regulate SHH expression. In the absence of sufficient folates, there is an upregulation of SHH signaling (Tang and Finnell, 2003). This serves to ventralize cells in the neural tube, while repressing PAX3, 6 and 7 signaling in the medial floor plate of the neural tube. cAMP is a second messenger that acts through PKA to modulate the SHH response. Thus, it is possible that polymorphisms in the PKA genes would result in ectopic SHH signaling and excessive ventralization of the neural tube, resulting in NTDs. The rationale for exploring genetic variation within the *PRKACA* and *PRKACB* genes as possible risk factors governing abnormal neural tube closure was based on the knowledge that cAMP-dependent PKA plays an important role in embryonic development via its negative regulation of the Hh signaling pathway. Decreased PKA activity during development has been shown to result in the dorsal expansion of the SHH signal from the thoracic to sacral regions of the developing neural tube (Huang et al., 2002).

In this study, we searched sequence variants in coding regions but not all the noncoding regions, such as the promoter and regulatory elements upstream of the gene. This could limit our findings on the role of these 2 genes in spina bifida risk. A more robust DNA resequencing study of these genes is warranted to identify and genotype more SNP markers, not only in coding regions but also in non-coding regions. We investigated SNPs in these 2 genes using PCR-based methods. This method, however, cannot exclude the possibility of certain types of gene variations, such as microdeletions. Although this is an unlikely situation, if any subject were hemizygous for a microdeletion, the remaining allele would give a result that would appear to be homozygous. PKA protein consists of 2 catalytic subunits and 4 regulatory subunits. Considering the importance of downregulating *SHH*, it will be of interest to determine if gene variations in the genes encoding regulatory subunits (*PRKAR1A*, *PRKAR1B*, *PRKAR2A*, *PRKAR2B*) are NTD risk factors. In addition, interaction with other genes, such as *SHH* and *SMO* (Ohlmeyer and Kalderon, 1997), should also be considered for future evaluations.

Our study used a population-based ascertainment of both case and control infants and incorporated the 2 major types of catalytic subunits of PKA, *PRKACA* and *PRKACB*. The study population consists of multiple ethnic groups; therefore, population admixture may bias the association between genetic polymorphism and risk for spina bifida. In order to estimate possible confounding due to ethnic difference in allele frequencies, we performed stratified analysis (Tables 3–5), and none of the 3 SNPs appeared to reveal substantial change in risk for spina bifida in the 2 major ethnic groups (Hispanic white vs. non-Hispanic white). Due to the lack of functional variations with high frequency, we used nonfunctional SNPs as markers in the case-control study, which is an indirect method and likely to underestimate gene–disease association. We assumed that these SNPs may be in linkage disequilibrium with disease, causing variations, and this is only true when recombination is infrequent in the gene locus under study. Our investigation may also have been limited by the lack of information on every case fetus with spina bifida. Fetuses with spina bifida that do not survive to term, due to either elective or spontaneous termination of pregnancy, may have higher or lower frequencies of the variant *PRKACA* and *PRKACB* genotypes. Therefore, these cases may have a different risk relation. Further investigations are warranted to answer these questions.

The lack of a strong association between *PRKACA* and *PRKACB* polymorphisms and spina bifida risk does not rule out potential contributions of other genetic variations of PKA. More

profound search for sequence variants in the locus regions, as well as genes encoding the regulatory subunits of PKA protein, is necessary. It is also important to study gene–gene interactions among functionally related genes such as *SHH* and *SMO* and PKA genes.

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Table 1

Human *PRKACA* and *PRKACB* Resequencing Primers (Coding Regions)

Amplicons	Primer sequence	Fragment size (bp)
PRKACA		
Exon1	Fw: 5′-GAGGATGAGGGGCCCCGTAG-3′ Rev: 5′-CGAGCGAGTGAATGGCCGAG-3′	513
Exon2	Fw: 5′-CCTAGGGAGCAAATGGGGAG-3′ Rev: 5′-AAGGTCAGGCTCTGGGTTGG-3′	316
Exon3	Fw: 5′-AGCAAGTGAGTGAACGGGTGG-3′ Rev: 5′-CCTGTCCTCACCCCGCTGAC-3′	361
Exon4	Fw: 5′-CTGATTGTCCTCAGCTCCGACC-3′ Rev: 5′-ATCAGCTAAGGGCCCGACAG-3′	341
Exon5	Fw: 5′-GGACTCCTCTGCATTCCCAGG-3′ Rev: 5′-CCTACAGGGAGCCAGGATCG-3′	298
Exon6	Fw: 5′-CAAGAACAGGCAGTTGGCAGG-3′ Rev: 5′-GCTATCTAAGAATCAGGTGCCTTG-3′	347
Exon7	Fw: 5′-ACCAGTCCACGGCCTTGTTG-3′ Rev: 5′-ACATTCAGGTGCCCACCAGG-3′	313
Exon8	Fw: 5′-CAACAAGCAGGGCTCCCCAG-3′ Rev: 5′-GCAAAGTAGGAGCCTCCCCAG-3′	311
Exon9	Fw: 5′-TTCTCTATTTCTCTATTGGCTGTCC-3′ Rev: 5′-GAAACTGTAGGGTAGGCTCAAGG-3′	409
Exon10 (coding region)	Fw: 5′-TAACCCTGGAGGGTGGGGTG-3′ Rev: 5′-GCTACATTAAGAGTTTCGGGCTG-3′	432
PRKACB		
Exon1	Fw: 5′-GGTGCTAAGGAGTTCGCTGGA-3′ Rev: 5′-CTAGGGACTTCTTTCCTCGGACC-3′	262
Exon2	Fw: 5′-TTTTAAGTGAGAAAACCATATGCAA-3′ Rev: 5′-GGCAAAATTCCCATTTTAAGC-3′	320
Exon3	Fw: 5′-TTCAAAGGGAAAAATAGAACCAA-3′ Rev: 5′-TTGAAGCTGTCAATGGTAAGTCA-3′	434
Exon4	Fw: 5′-CCAACCTAAAAGTTGGTAATTGTT-3′ Rev: 5′-TCTTCAGGCTTATCATCTGGTTT-3′	375
Exon5	Fw: 5′-GCATGTTCCATTTATTCTTTATTTCTC-3′ Rev: 5′-CCACCTAATGCTTCATATGGC-3′	377
Exon6	Fw: 5′-TGTTCCCAAAGTACCTCTACTTCA-3′ Rev: 5′-TGTAATAGCAGCAAAACTCTGAGC-3′	409
Exon7	Fw: 5′-AAACTTTCAACGTAGGTGCAAT-3′ Rev: 5′-CCCAGAGTTAAAATTGACAGAGG-3′	410

Table 2

Sequence Variations Found in *PRKACA* and *PRKACB*

Gene	Location in gene	Variant type	SNP detail	Alternate amino acids	MAF[a] in controls
PRKACA (GI:46909581)					
288A>G	Exon 2	Nonsynonymous	AAGAAGATTT TCTTAAA [A/G] AA TGGGAAAGTCCCGCTC	K30E	0.000[b]
749+76G>A	76 bp downstream of exon 6	Intronic	gtggccctgcagagcctacc [c/t] Cagaggaagacacctccagtc	—	0.354
654 G>C	Exon 6	Nonsynonymous	TTCTACGCGGCCCAGATC [G/C] TCCTGACCTTTGAGTATCT	V152L	0.011
658 T>G	Exon 6	Nonsynonymous	ACGCGGCCCAGATCGTCC [T/G] GACCTTTGAGTATCTGCA	L153R	0.022
PRKACB (GI:46909585)					
649-25G>C	25 bp upstream of exon 6	Intronic	gaatgtattaactcatttttaca [g/c] aaaatcaatggttttatttctttg	—	0.013
690G>A	Exon 6	Synonymous	GTTCTATGCAGCTCAGATAGT [G/A] CTAACATTCGAGTACCTCCAT	V199V	0.013
700G>A	Exon 6	Nonsynonymous	GATAGTGCTAACATTC [G/A] AGTACCTCCATTCACTAGACC	E203K	0.013

[a] Minor allele frequency (MAF): number of minor alleles detected/number of individuals successfully sequenced × 2.

[b] Minor allele found in spina bifida case.

Table 3

PRKACA (rs729372) Genotyping Frequencies among NTD Cases and Controls

	Case (%)	Control (%)	OR	95% CI
Overall				
CC	135 (67.5)	141 (70.1)	Reference	
CA	56 (28.0)	53 (26.4)	1.1*	0.7–1.7[a]
AA	9 (4.5)	7 (3.5)	1.3*	0.5–3.7[a]
Non-Hispanic white				
CC	54 (79.4)	60 (75.9)	Reference	
CA	13 (19.1)	17 (21.5)	0.8	0.4–1.9
AA	1 (1.5)	2 (2.5)		—
Hispanic white				
CC	58 (50.9)	43 (53.8)	Reference	
CA	46 (40.4)	32 (40.0)	1.1	0.6–1.9
AA	10 (8.8)	5 (6.3)	1.5	0.5–4.7

[a] Adjusted for ethnicity group.

OR, odds ratio; CI, confidence interval.

Table 4

PRKACB (rs1565823) Genotyping Frequencies among NTD Cases and Controls

	Case (%)	Control (%)	OR	95% CI
Overall				
CC	73 (37.4)	68 (34.5)	Reference	
CT	88 (45.1)	99 (50.3)	0.8[a]	0.5–1.3[a]
TT	34 (17.4)	30 (15.2)	1.0[a]	0.6–1.9[a]
Non-Hispanic white				
CC	14 (21.2)	19 (24.7)	Reference	
CT	30 (45.5)	39 (50.6)	1.1	0.5–2.4
TT	22 (33.3)	19 (24.7)	1.6	0.6–4.0
Hispanic white				
CC	46 (44.2)	34 (43.0)	Reference	
CT	48 (46.2)	39 (49.4)	0.9	0.5–1.7
TT	10 (9.6)	6 (7.6)	1.2	0.4–3.7

[a] Adjusted for ethnicity group.

OR, odds ratio; CI, confidence interval.

Table 5

PRKACB 3′-UTR (rs600674) Genotyping Frequencies among NTD Cases and Controls

	Case (%)	Control (%)	OR	95% CI
Overall				
CC	136 (66.3)	137 (67.8)	Reference	
CT	56 (27.3)	52 (25.7)	1.1[a]	0.7–1.7[a]
TT	13 (6.3)	13 (6.4)	1.0[a]	0.5–2.3[a]
Non-Hispanic white				
CC	49 (74.2)	55 (68.8)	Reference	
CT	15 (22.7)	21 (26.3)	0.8	0.4–1.7
TT	2 (3.0)	4 (5.0)	—	—
Hispanic white				
CC	69 (62.7)	58 (72.5)	Reference	
CT	33 (30.0)	19 (23.8)	1.5	0.8–2.8
TT	8 (7.3)	3 (3.8)	—	—

[a] Adjusted for ethnicity group.

OR, odds ratio; CI, confidence interval.