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Screening for Novel *PAX3* Polymorphisms and Risks of Spina Bifida

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Abstract

Background—*PAX3* plays an important role in mammalian embryonic development. Known mutations in *PAX3* are etiologically associated with Waardenburg syndrome and syndromic neural tube defects (NTDs). Mutations in the murine homologue, *Pax3*, are responsible for the phenotype of Splootch mice, in which nullizygotes are 100% penetrant for NTDs.

Methods—The study sample included 74 infants with spina bifida (cases) and 87 non-malformed infant controls. The conserved paired-box domain, as well as upstream genomic region of *PAX3* was subjected to re-sequencing and those identified SNPs were evaluated as haplotypes. The associations of haplotypes for selected gene regions and the risks of spina bifida were further studied.

Results—Nineteen SNPs were observed; 15 observed in controls had been submitted to National Center for Biotechnology Information (NCBI) database with allele frequencies. The *PAX3* gene variant T-1186C (rs16863657) and its related haplotype, T -C-T-C-C-G-C-C of 9 SNPs, were found to be associated with an increased risk of spina bifida, with an odds ratio of 3.5 (95% confidence interval, 1.2–10.0) among Hispanic whites.

Conclusions—Our analyses indicated that *PAX3* SNPs were not strong risk factors for human spina bifida. However, additional follow-up of the *PAX3* gene variant T-1186C (rs16863657) and its related haplotype, T-C-T-C-C-G-C-C may be important in other populations.

Keywords

PAX3; polymorphisms; spina bifida; haplotype; association study

Introduction

Neural tube defects (NTDs) have complex etiologies that include both genetic and environmental factors. Some environmental factors such as folic acid intake have been well described in terms of their importance to NTD risk (Berry et al., 1995). However, potential genetic contributions to risk have been more resistant to identification.

The Paired-box (*PAX*) gene family encodes specific DNA binding transcription factors that play important roles in embryonic development. Of the 9 *PAX* genes, *PAX* 2, 3, 5, 6, 7 and 8 are expressed during the formation and organization of the neural tube along its entire axis (Hol et al. 1996; Helwig et al. 1995; Tremblay and Gruss 1994; Balling et al. 1988). *PAX* genes contain a paired-box DNA binding domain, with or without an octapeptide and homeo domain (Dahl et al., 1997). The paired box domain is a highly conserved DNA-binding domain of 128 amino acids located at the amino terminus of the protein. Analysis of mouse models and human syndromes has revealed the importance of *PAX* genes in their role as regulators of normal development (Mansouri et al., 1999; Pani et al., 2002). Heterozygous *splootch* mice display pigmentary abnormalities while homozygotes die during gestation with spina bifida or exencephaly (Vogan et al. 1993; Auerbach 1954). Mutations within the human *PAX3* gene have been associated with Waardenburg syndrome, a condition occasionally associated with NTDs (Baldwin et al. 1992; Hoth et al. 1993; Hol et al. 1995). In 1995, Hol and colleagues identified a 5bp deletion in exon 5 of the *PAX3* gene in a patient with spina bifida and mild manifestations of Waardenburg syndrome. A subsequent study identified 2 spina bifida patients who also had small interstitial chromosomal deletions involving *PAX3* (Nye et al., 1998).

In the current study, we investigated a California population to determine whether polymorphisms in *PAX3* were associated with risk of human spina bifida.

Methods

Study Subjects

The study design is a population-based case-control study. Cases and controls were ascertained by the California Birth Defects Monitoring Program, a population-based active surveillance system for collecting information on infants and fetuses with congenital malformations. Diagnostic and demographic information was collected by program staff from multiple sources of medical records for all liveborn and stillborn fetuses. Nearly all major structural anomalies diagnosed within 1 year of delivery were ascertained. Overall ascertainment for major malformations has been estimated as 97% complete (Schulman J, et al., 1993). Eligible for this study were live born infants only, owing to the fact that the source of DNA was from newborn screening bloodspot.

Included for study were 74 infants with spina bifida and 87 non-malformed control infants born during the period 1983–1986 in selected counties in California. Among cases, 47.3 % were non-Hispanic Whites, 41.9 % were Hispanic Whites including U.S. born and foreign born, 4.0 % were African American and 6.8 % were other ethnic groups. Among controls, 52.9 % were non-Hispanic Whites, 34.5 % were Hispanic Whites including U.S. born and

foreign born, 4.6 % were African American and 8.0 % were other ethnic groups. All newborn blood 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 spot on filter paper using Puregene DNA Purification kit (Gentra, Minneapolis, MN) according to the manufacturer's instructions and was amplified using PCR (Schwartz EI, et al, 1990).

PAX3 Gene Re-Sequencing

The genomic region of *PAX3* gene, including the paired-box domain, was re-sequenced with VariantSEQR re-sequencing system (Applied Biosystems, Foster city, CA). The primer set (RSS000013589 _02) for *PAX3* included nine pairs of primers and covered a 6-kb genomic region containing 5 exons and flanking intronic sequences. PCRs (30s at 94 °C, 45s at 60 °C, 60 s at 72 °C for 40 cycles) were performed in a final volume of 10 µl containing 60 ng genomic DNA, 2.0 µl VariantSEQR primer mix, 250 µM of each dNTP, in 2.0 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 8% Glycerol, and 1.5 U of *Taq* DNA polymerase on PE9700 thermocycler. Then, PCR products were cleaned up by digestion with ExoSAP-IT enzymes (USB Corporation, Cleveland, Ohio) and 3 µl of final products were applied to sequencing reaction (10 s at 96 °C, 5 s at 50 °C, 4 m at 60 °C for 25 cycles) with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA). The fragments were precipitated with isopropanol, denatured with 12 µl HiDi formamide solution at 95 °C for 5 min, and loaded onto 3730 DNA Analyzer. Sequencing results were analyzed with SeqScape v2.5 software (Applied Biosystems, Foster City, CA).

(CA) n Repeats Genotyping

Primers (forward: 5'-CCCAGATGCCTTCTTA and reverse: 5' FAM-CAGGGAG-ATGGCAGTT-3') were used to amplify fragments. The fragment is located ~300 bp upstream of transcription start site and contains the (CA) n dinucleotide repeats. PCR (35 s at 94 °C, 30s at 55.7 °C, 60 s at 72 °C for 35 cycles) was performed in a final volume of 25 µl containing 60 ng of genomic DNA, 6.25 pmol of each primer, 200 µM of each dNTP, in 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1 µg / ml bovine serum albumin and 0.4 U *Taq* DNA polymerase. Data were collected by 3730 DNA Analyzer (ABI, Foster city, CA) and analyzed by GeneMapper v3.5 (ABI, Foster city, CA).

Statistical analyses

Deviations from Hardy-Weinberg Equilibrium among control infants were evaluated using a χ^2 test. Genotyping frequencies for detected variants within the *PAX3* gene were compared between cases and controls and odds ratios and 95% confidence intervals (CIs) were estimated utilizing SAS software (version 9.1). Haplotype analysis was performed with Haploview software (version 3.2) (Barrett JC, et al, 2005) and evaluated with χ^2 tests.

Results

Nineteen SNPs were observed by re-sequencing in upstream genomic region and exons 1–4 of *PAX3* gene containing the paired-box domain (table 1, figure 1). Two SNPs in coding regions did not result in an amino acid substitution. The flanking sequences and SNPs are

shown in Table 2. Fifteen novel SNPs observed in controls had been submitted to National Center for Biotechnology Information (NCBI) database with allele frequencies. These were rs28945085, rs16863657, rs28945086, rs28945087, rs28945088, rs28945668, rs28945089, rs28945090, rs28945091, rs12623857, rs28945092, rs28945093, rs28945094, rs28945095, rs28945096,.

Deviations from Hardy-Weinberg equilibrium were evaluated in controls for each SNP. Statistical evidence ($P < .05$) for deviations was observed for rs28945089 and rs28945094. Stratified by maternal race/ethnicity, deviations of those 2 SNPs remained significant in non-Hispanic Whites.

Analyses focused on (CA) n repeats, single SNPs, and haplotypes. We did not observe significant associations between (CA) n repeats polymorphism and risk of spina bifida (data not shown). For SNP analyses, Table 3 shows allele frequencies by case and control status and by maternal race/ethnic background. Overall, substantial differences between cases and controls in allele frequencies were not observed. One exception, however, was rs16863657. The major allele (T) for this SNP in Hispanic white case infants was substantially less frequent than in control infants.

The risk associated with rs16863657 for spina bifida was further explored (table 4). The odds ratio associated with rs16863657 was 3.5 (95% CI: 1.2–10.0), indicating that SNP, rs16863657, was associated with a substantial increased risk of spina bifida in Hispanic Whites. Haplotype analyses were performed for various race/ethnic groups. Since all observed SNPs were in linkage disequilibrium, these SNPs were defined as whole block for these analyses. Results of these analyses are shown in Table 5. For African Americans, 5 haplotypes were investigated, including C-C-G-C, G-C-G-C, C-T-G-C, C-T-C-C, and C-T-G-G, which were represented by four SNPs, rs28945092 (C/G), rs12623857 (T/C), PAX3_G-396C, and rs28945085 (C/G). For Hispanic whites, 7 haplotypes were investigated, T-C-T-C-C-G -G-T-C, T-C-T-C-C-G- C-C-C, T-C-T-C-C-G-C -T-C, T-C-T-T-C-G-C- T-G, T-C-T-C-A-G-C-C -C, T-C-T-T-C-G-C-T- C, and C-C-T-C-C-G-C-T-C, corresponding to rs28945096 (T/C), rs28945095 (C/T), rs28945094 (T/C), rs12623857 (T/C), rs28945091 (C/A), rs28945090 (G/A), rs28945089 (C/G), rs16863657 (T/C) and rs28945085 (C/G). For non-Hispanic white, 5 haplotypes were investigated, T-T-C-A-C-G-C-C-C-T-T-C, T-T-C-A -C-G-C-C-C-T-C-C, T-T-C-A-T-G-C-C-C-T-T-G, T- T-C-A-T-G-C-C-C-T-T-C, and T-C-C-A-C-G-C-C-C-T-T-C, corresponding to rs28945096 (T/C), rs28945094 (T/C), PAX3_C701A, rs28945093 (A/C), rs12623857 (T/C), PAX3_G-569C, rs28945668 (C/T), rs28945088 (C/G), rs28945087 (C/T), rs28945086 (T/C), rs16863657 (T/C), and rs28945085 (C/G). For the other race/ethnic groups, 6 haplotypes were investigated, T-C-G-T, T-C-C-T, T-C-C-C, C-C-G-T, T-T-G-C, and T-T-C-C, corresponding to rs28945094 (T/C), rs12623857 (T/C), rs28945089 (C/G), and rs28945085 (T/C). For these various comparisons, only 1 haplotype appeared to be more frequent among infants with spina bifida than control infants. That is, the haplotype frequency of T-C-T-C-C-G-C-C- C in Hispanic Whites was 0.35 and 0.15 in cases and controls respectively, including the SNP, rs16863657. Because of the numbers of analytic comparisons made in this study, we cannot exclude the possibility that this latter result is also consistent with expected random variation.

Discussion

PAX3 belongs to the family of paired domain proteins that bind DNA and regulate gene expression. Previous studies in mouse and chick embryos demonstrate that PAX3 play an important role in neural tube development. Expression of PAX3 is detected in gestation day (GD) 8.5 embryos, with peaks of expression from day 9 to 12 followed by declining levels until no expression is evident in GD 17 embryos. The time window of PAX3 high expression is coincident with the critical time for neural tube closure. Homozygotes (*splotch* mice) have 100% penetrance of neural tube defects. Our study focused on conserved, paired-box domain of PAX3 gene. Nineteen SNPs were found and 16 novel ones were identified and submitted to the NCBI database. Haplotypes including these SNPs were investigated in a study population of California infants. Overall, our analyses indicated that PAX3 SNPs were not strong risk factors for human spina bifida. Despite the fact that our study is strengthened by its population-based design, our lack of observing a modest role for PAX3 SNPs and spina bifida risk may be explicable to limited sample sizes and the exclusion of spina bifida-affected pregnancies that were terminated. Nevertheless, additional follow-up of PAX3 gene variant T-1186C (rs16863657) and its related haplotype, T-C-T-C-C-G-C-C-C may be important in other populations.

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Figure 1.

Table 1Primer Sequences for Resequencing *PAX3* Gene

Primer ID	Sequences (5' → 3')	Fragment length (bp)
RSA000021427	Fw: TGTAAAACGACGOCAGTGGTGCCAGCACICTAAGAACCCA	595
	Re: CAGGAAACAGCIATGACCGGTGATCTGACGGCAGCCAA	
RSA000021430	Fw: TGTAAAACGACGGCCAGTAAGAAGTGTCCAGGGCGCGT	571
	Re: CAGGAAACAGCIAIGACCGGTCIGGGTCIGGGAGTCCG	
RSA000021432	Fw: TGTAAAACGACGGCCAGTTATCCGGAGCGTGGAGAGCC	578
	Re: CAGGAAACAGCTATGACCTTCTCCGCCCTCAGCAACTG	
RSA000021433	Fw: TGTAAAACGACGGCCAGTCGATTGGCCGACGGGTAGAC	554
	Re: CAGGAAACAGCTATGACCGGGCACTGACCACAGGAAGG	
RSA000579700	Fw: TGTAAAACGACGGCCAGTAATGGCAACAGAGTGAGAGCTTCC	587
	Re: CAGGAAACAGCTATGACCAGGAGACACCCGCGAGCAGT	
RSA000579702	Fw: TGTAAAACGACGGCCAGTTAAACGCTCTGCCTCCGCCT	600
	Re: CAGGAAACAGCTATGACCGGGATGTGTTCTGGTCTGCCC	
RSA001307840	Fw: TGTAAAACGACGGCCAGTCAGTTGCTGAGGGCGGAGAA	596
	Re: CAGGAAACAGCTATGACCAGAACCGCAGCTTGCCAGAG	
RSA001307844	Fw: TGTAAAACGACGGCCAGTAGAGCAGCGCGCTCCATTTG	596
	Re: CAGGAAACAGCTATGACCGCTCGCCGTGGCTCTCTGA	
RSA001307846	Fw: AACGACGGCCAGTCTGGGAGGAGTCCAGGGTGC	461
	Re: AAACAGCTATGACCAGGCGAGTTCGTGGCACTT	

The 5' end was flanked with 18 bp of universal M13 sequence.

Table 2SNPs Observed in Genomic Region of *PAX3* Gene Containing Paired-Box Domain

Location	AA position	Submitter ID*	Submitted SNP (NCBI)	Reference SNP (NCBI)	Relevant sequence
Genomic region		<i>PAX3_C-1219G</i>	ss38343224	rs28945085	GCTTGCCAGAG (C/G) GGTTTTAC
Genomic region		<i>PAX3_T-1186C</i>	ss38343225	rs16863657	GCCAGGTTTT (T/C) TYCTGCACCCCTCC
Genomic region		<i>PAX3_T-1184C</i>	ss38343226	rs28945086	GCCAGGTTTTYT (T/C) CTGCACCCCTCC
Genomic region		<i>PAX3_C-1166T</i>	ss38343227	rs28945087	CCCTCCCCCATA (C/T) CCCCAGCCT
Genomic region		<i>PAX3_C-1072G</i>	ss38343228	rs28945088	CAAAAGAACAT (C/G) AGCGCACCCCT
Genomic region		<i>PAX3_G-900A</i>	N/A	N/A	GCCAAGAGAAATGAGAGC (G/A) AGACCTACAG
Genomic region		<i>PAX3_C-749T</i>	ss38343229	rs28945668	GAGAAAAGACACACA (C/T) ACACACACACACACA
Genomic region		<i>PAX3_C-693G</i>	ss38343230	rs10193524 rs28945089	CACACACACACAGAGTGACA (C/G) AGACAGAGACAGACACAGAGA
Genomic region		<i>PAX3_G-576A</i>	ss38343231	rs28945090	CCTGGGCAAGGGGC (G/A) CAGCGCGGGT
Genomic region		<i>PAX3_G-569C</i>	N/A	N/A	AGGGGCGCAGCGC (G/C) GGTCCCCCTCGGGGCCAGC
Genomic region		<i>PAX3_C-565A</i>	ss38343232	rs28945091	CAGCGCGGT (C/A) CCCCCTCGGGGCCAGCA
Genomic region		<i>PAX3_G-396C</i>	N/A	N/A	AAGAACTAATAAATGCTCCC (G/C) AGCCCGGATCCCCGCACTCG
Exon 3	43	<i>PAX3_T129C</i>	ss38343233	rs12623857	CGTCAACCAAGCTGGCGG (T/C) GTTTTATCAACGGCAGG
Exon 3	52	<i>PAX3_C156G</i>	ss38343234	rs28945092	GGCAGGCCGCTGCC (C/G) AACACATCCGCC
3'-UTR		<i>PAX3_A672C</i>	ss38343235	rs28945093	ATCTGACGGCAGCCA (A/C) GCCCAGCTCGGATCAAGG
3'-UTR		<i>PAX3_C701A</i>	N/A	N/A	CTCGGATCAAGGTCCCTTCATG (C/A) GCGGTGTCTCTGCGCCTGA
3'-UTR		<i>PAX3_T722C</i>	ss38343236	rs28945094	GTGTCTCTGCGCTGAG (T/C) AACGACATGTCC
3'-UTR		<i>PAX3_C1020T</i>	ss38343237	rs28945095	GGTCTCCGGAGTTT (C/T) CTCGCATTAAAGGAG
3'-UTR		<i>PAX3_T1087C</i>	ss38343238	rs28945096	CAAAATTGCCCCACACA (T/C) TGGCTGCCTTA

* Nucleotide +1 is the A of the ATG-translation initiation codon; the nucleotide 5' to +1 is numbered -1.

N/A: not applicable.

Table 3

Single SNP Associations

SNP	Major allele	Allele frequencies: case, control (X^2 , P value)			
		Hispanic Whites	Non-Hispanic Whites	African-American	Others
rs28945096	T	0.98, 0.98 (0.00, .98)	1.00, 0.99 (0.78, .38)	0	0
rs28945095	C	1.00, 0.98 (1.01, .32)	0	0	0
rs28945094	T	1.00, 0.98 (0.98, .32)	1.00, 0.95 (3.17, .07)	0	0.90, 1.00 (1.05, .30)
PAX3_C701A	C	0	0.99, 1.00 (1.24, .26)	0	0
rs28945093	A	0	0.99, 0.99 (0.02, .88)	0	0
rs28945092	C	0	0	1.00, 0.88 (0.81, 0.37)	0
rs12623857	T	0.97, 0.90 (2.07, .15)	0.85, 0.91 (1.04, .31)	0.67, 0.88 (0.88, 0.35)	1.00, 0.88 (1.32, .25)
PAX3_G-396C	G	0	0	0.83, 1.00 (1.44, 0.23)	0
rs28945091	C	0.95, 0.98 (1.03, .31)	0	0	0
PAX3_G-569C	G	0	0.99, 1.00 (1.07, .30)	0	0
rs28945090	G	1.00, 0.98 (1.01, .32)	0	0	0
rs28945089	C	0.59, 0.58 (2.67, .10)	0	0	0.80, 0.50 (1.8, .18)
rs28945668	C	0	1.00, 0.99 (0.80, .37)	0	0
rs28945088	C	0	1.00, 0.99 (0.69, .41)	0	0
rs28945087	C	0	1.00, 0.99 (0.74, .39)	0	0
rs28945086	T	0	1.00, 0.99 (0.82, .37)	0	0
rs16863657	T	0.60, 0.82 (6.82, .01 [*])	0.81, 0.82 (0.02, .90)	0	0.80, 0.80 (0.39, .53)
rs28945085	C	0.98, 0.93 (2.11, .15)	0.92, 0.94 (0.31, .58)	0.83, 1.00 (1.44, 0.23)	0

* $P < .01$.

Table 4*PAX3*_T-1186C (rs28945085) Genotype in Hispanic White Population

	Control (%)	Case (%)	OR	95% CI
Overall				
TT	19 (63.3)	10 (33.3)	Reference	
CC, TC	11 (36.7)	20 (66.7)	3.5	1.2–10.0

Table 5

Haplotype Association

Haplotype	Frequency	Case, control frequencies	X ²	P
Hispanic Whites: rs28945096 (T/C), rs28945095 (C/T), rs28945094 (T/C), rs12623857 (T/C), rs28945091 (C/A), rs28945090 (G/A), rs28945089 (C/G), rs16863657 (T/C), and rs28945085 (C/G)				
TCTCCGGTC	0.52	0.46, 0.58	1.78	0.18
TCTCCGCCC	0.25	0.35, 0.15	6.37	0.01 *
TCTCCGCTC	0.09	0.10, 0.10	0.00	0.98
TCTTCGCTG	0.03	0.02, 0.05	1.03	0.31
TCTCAGCCC	0.03	0.05, 0.02	1.03	0.31
TCTTCGCTC	0.03	0.02, 0.05	0.86	0.35
CCTCCGCTC	0.02	0.02, 0.02	0.00	1.00
Non-Hispanic whites: rs28945096 (T/C), rs28945094 (T/C), <i>PAX3</i> _C701A, rs28945093 (A/C), rs12623857 (T/C), <i>PAX3</i> _G-569C, rs28945668 (C/T), rs28945088 (C/G), rs28945087 (C/T), rs28945086 (T/C), rs16863657 (T/C), and rs28945085 (C/G)				
TTCACGCCCTTC	0.65	0.64, 0.66	0.10	0.76
TTCACGCCCTCC	0.17	0.20, 0.16	0.23	0.63
TTATGCCCCTTG	0.06	0.06, 0.06	0.00	0.95
TTATGCCCCTTC	0.04	0.06, 0.20	2.16	0.14
TCCACGCCCTTC	0.02	0.00, 0.04	2.48	0.12
African Americans: rs28945092 (C/G), rs12623857 (T/C), <i>PAX3</i> _G-396C, and rs28945035 (C/G)				
CCGC	0.71	0.67, 0.75	0.12	0.73
GCGC	0.07	0.00, 0.13	0.81	0.37
CTGC	0.07	0.00, 0.13	0.81	0.37
CTCC	0.07	0.17, 0.00	1.44	0.23
CTGG	0.07	0.17, 0.00	1.44	0.23
Others: rs28945094 (T/C), rs12623857 (T/C), rs28945089 (C/G), and rs16863657 (T/C)				
TCGT	0.59	0.70, 0.47	1.06	0.30
TCCT	0.21	0.10, 0.33	1.53	0.22
TCCC	0.10	0.10, 0.10	0.00	1.00
CCGT	0.05	0.10, 0.00	1.05	0.30
TTGC	0.03	0.00, 0.05	0.51	0.47
TTCC	0.03	0.00, 0.05	0.51	0.47

* $P < .05$.