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Exon Sequencing of PAX3 and T (Brachyury) in Cases with Spina Bifida

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Abstract

BACKGROUND—Based on studies in animals and humans, *PAX3* and *T (brachyury)* are candidate genes for spina bifida. However, neither gene has been definitively identified as a risk factor for this condition.

METHODS—Sanger sequencing was used to identify variants in all *PAX3* and *T* exons and promoter regions in 114 spina bifida cases. For known variants, allele frequencies in cases were compared to those from public databases using unadjusted odds ratios. Novel variants were genotyped in parents and assessed for predicted functional impact.

RESULTS—We identified common variants in *PAX3* (n=2) and *T* (n=3) for which the allele frequencies in cases were significantly different from those reported in at least one public database. We also identified novel variants in both *PAX3* (n=11) and *T* (n=1) in spina bifida cases. Several of the novel *PAX3* variants are predicted to be highly conserved and/or impact gene function or expression.

CONCLUSION—These studies provide some evidence that common variants of *PAX3* and *T* are associated with spina bifida. Rare and novel variants in these genes were also identified in affected individuals. However, additional studies will be required to determine whether these variants influence the risk of spina bifida.

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URLs
Exome Sequencing Project: http://evs.gs.washington.edu/EVS/
HapMap: http://hapmap.ncbi.nlm.nih.gov
Human Splicing Finder 2.4.1: http://www.umd.be/HSF
GERP++: http://mendel.stanford.edu/SidowLab/downloads/gerp/
Polyphen 2: http://genetics.bwh.harvard.edu/pph2
Sequenom MassARRAY iPLEX Gold genotyping assay: http://www.sequenom.com/files/genetic-analysis-files/snp-genotyping-pdfs/iplex-gold-application-guide)
Study questionnaire used September 2006 through 2010: https://sph.uth.tmc.edu/sbrr/questionnaire.htm.
Targetscan: http://www.targetscan.org
TESS: http://www.cbil.upenn.edu/cgi-bin/tess/tess
UCSC Genome Browser: http://genome.ucsc.edu/
1,000 Genomes: http://www.1000genomes.org

Keywords

spina bifida; myelomeningocele; genetic epidemiology; sequencing; *PAX3*; *T* locus

BACKGROUND

Spina bifida is a serious congenital malformation that results from abnormal development of the caudal neural tube. The etiology of spina bifida is complex, involving both genetic and non-genetic factors. While there is a long list of candidate genes for spina bifida (e.g. (Au et al., 2010; Harris and Juriloff, 2010)), few of these genes have been thoroughly investigated as potential causes of spina bifida in humans.

In the mouse, mutations in *Pax3* give rise to the *Splotch* (*Sp*) phenotype, which includes spina bifida and other neural crest abnormalities in homozygotes (Greene and Copp, 2005; Harris and Juriloff, 2007; Kubic et al., 2008). In humans, mutations in *PAX3* (MIM: 606587) cause Waardenburg syndrome, an autosomal dominant condition that affects neural crest derived structures and includes spina bifida as part of its phenotypic spectrum (Pingault et al., 2010). Moreover, in a study including 406 children with spina bifida, 8 (2%) were identified as having probable WS, including two with confirmed deletions encompassing *PAX3* (Nye et al., 1999).

The human *T (brachyury) gene* (MIM: 601397) was suggested as a candidate for spina bifida because mice lacking T protein (i.e. *Brachyury* mutants) have defective mesoderm formation and abnormal axial development (Stott et al., 1993), and several studies have identified an association between a common variant in *T* (i.e. *TIVS7-2*, rs3127334) and the risk of neural tube defects in humans (Carter et al., 2011; Jensen et al., 2004; Morrison et al., 1996; Morrison et al., 1998; Shields et al., 2000).

To further our understanding of the relation between spina bifida and these genes, we sequenced all *PAX3* and *T* exons and promoter regions in 114 spina bifida cases and evaluated the identified variants relative to publically available genomic data. As mutations in *PAX3* cause Waardenburg Syndrome (WS), analyses of *PAX3* variants considered the presence of WS features in cases and their relatives.

METHODS

STUDY SUBJECTS

This study is based on data from a family-based study of spina bifida (i.e. myelomeningocele) that recruited cases and their parents through support organizations, clinics and websites in the United States, 1997–2010 (Mitchell, 2008). Study participants (usually the case's mother) completed a telephone interview covering parental race and ethnicity, pregnancy and medical histories, family history of birth defects and genetic disorders and (since September 2006) the presence of WS features (i.e., patches of white hair, eyebrows or lashes; heterochromia; hypopigmented skin, premature graying, and deafness) in the case and other family members. Blood or saliva samples were also collected from cases and their parents. The present study is based on data from cases enrolled after September 2006, when questions assessing the presence of WS features were included in the interview.

Cases with a known syndrome (other than WS) or chromosome abnormality were excluded from this study, as were cases with neural tube defects other than spina bifida. Further, cases

were excluded if their mothers had epilepsy or pre-gestational diabetes, or took medications associated with an increased risk of neural tube defects during pregnancy.

Informed consent, and when appropriate, assent, were obtained for all study subjects. This study was approved by the Institutional Review Board of the University of Texas Health Science Center at Houston.

DNA SEQUENCING

DNA was extracted from whole blood using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA) and from saliva using the Oragene DNA Preparation Kit (DNA Genotek, Kanata, Ontario, Canada). Whole genome amplification, using the Qiagen REPLI-g Mini Kit, was performed on 51 samples (1 blood, 50 saliva).

A strategy using VariantSeqr amplicons (NCBI) was used to isolate, by PCR, the exons and promoter regions of *PAX3* and *T*. For *PAX3*, 19 amplicons were isolated. These amplicons covered the 11 *PAX3* exons as well as 1,106 nucleotides upstream and 62 nucleotides downstream of the transcriptional start and stop sites, respectively. The 11 *PAX3* exons code for eight alternative transcripts. Most of the literature has focused on the 10 exons that code for the main transcript (NM_181459.3), which are generally referred to as exons 1–10; in this study, they are referred to as exons 1–4 and 6–11 (Supplemental file 1). For *T*, 13 amplicons, covering nine exons (eight coding exons in two alternative transcripts) as well as 543 nucleotides upstream and 351 nucleotides downstream of the transcriptional start and stop sites, respectively, were isolated.

Sanger sequencing was performed with standard methods (Big Dye Terminator Cycle Sequencing Kits), using an Applied Biosystems (San Francisco, CA) 3730xl automated capillary sequencer. Sequence traces were assembled in Lasergene SeqMan v8.2 (DNASTAR, Madison, WI). Variants were extracted and validated by direct inspection of the chromatographic traces. Primer sequences are provided in Supplementary file 2. Parents were genotyped for novel variants using the Sequenom MassARRAY iPLEX Gold genotyping assay (Sequenom; San Diego, CA) using standard protocols (see URLs).

DATA ANALYSIS

As the majority of cases were reported to be non-Hispanic white, analyses were restricted to this subset of cases unless otherwise specified. Allele frequencies in cases were compared to frequencies in non-Hispanic white populations in the HapMap (Genome Browser release 28, phase 1–3 merge), 1,000 Genomes (Phase 1, version 3.20101123), and/or Exome Sequencing Project (ESP, Exome Variant Server release ESP6500SI) public databases (see URLs), since our family-based study did not include ascertainment of a control sample. Unadjusted odds ratios (ORs) with exact 95% confidence intervals (CI) were estimated using Golden Helix SNP & Variation Suite v7 (Golden Helix, Inc., Bozeman, MT) and STATA version 10 (StatCorp LP, College Station, TX). Odds ratios with a 95% confidence interval that excluded one were considered to be statistically significant. For *PAX3*, analyses were repeated among cases with a feature, or a relative with a feature, of WS.

Known variants that were significantly associated with spina bifida and all novel variants (i.e. not reported in HapMap, 1000 Genomes, ESP, or dbSNP) were assessed for: predicted impact on protein function using Polyphen2; transcription factor binding sites using TESS; micro-RNA binding sites using Targetscan; evolutionary conservation among vertebrates using phastCons, phyloP, and GERP++ scores; and transcript splicing using Human Splicing Finder 2.4 (see URLs).

RESULTS

Ten cases met an exclusion criterion and were not included in this study. The 114 (57 male, 57 female) cases that were sequenced were predominantly non-Hispanic white (91%) and the majority had lumbar level lesions (81%). Among these cases, 6% had a family history positive for neural tube defects, 6% had at least one WS feature and 45% had a relative with at least one WS feature. No case or relative was reported to have WS.

The high proportion of relatives reported to have at least one WS feature (45%) suggests that our telephone interview was not sufficiently specific and similar results were obtained when analyses of *PAX3* variants were conducted using data from all cases and from the subset of cases with a feature of, or a relative with a feature of WS. Consequently, only the results of analyses based on all cases are presented.

PAX3: Sequencing identified 38 variants in non-Hispanic white cases (Supplemental File 3). Of these, 30 were previously reported and the allele frequencies for two were significantly different in cases as compared to at least one reference dataset.

The A allele of rs13022712 in intron 8 was more common in cases as compared to non-Hispanic white controls in all three databases (HapMap: OR=3.36, 95% CI: 1.41–8.05; 1,000 Genomes OR=1.61, 95% CI: 0.97–2.69; ESP: OR: 1.59, 95% CI: 1.00–2.44). However, this difference was only statistically significant for the comparison with the HapMap database (Table 1). The wildtype allele for this variant was predicted to be conserved among vertebrates by phastCons (0.98) and GERP++ (2.81) but not phyloP (0.96).

The T allele of rs13014735 in intron 9 was also more common in cases as compared to non-Hispanic white controls in the two databases that included this variant (1,000 Genomes, OR=1.56, 95% CI: 0.94–2.60; ESP, OR=1.67, 95% CI: 1.03–2.60). However, this difference was only statistically significant for the comparison with the ESP database (Table 1). The wildtype allele for this variant was consistently predicted to be conserved among vertebrates (phastCons=1, phyloP=2.65, GERP++=5.77) and the variant allele is predicted to create a splice acceptor site that could affect the splicing of exons 10 and 11 in the main transcript (Supplemental file 1).

Seven previously reported, rare variants were also identified in one case each, including three missense variants that are predicted to be highly conserved by at least two of three prediction methods, and are more frequent (although not significantly so) in this case group as compared to the ESP (i.e. OR>19) (Supplemental File 3).

Eight novel variants were also identified in non-Hispanic white cases (Table 2). No case harbored more than one novel variant. A variant in exon 11 (c.*1125C>T) was present in the homozygous state in one case. Genotyping of this variant in the case's parents was unsuccessful. The remaining seven novel variants were each observed in the heterozygous state. For four cases, the observed novel variant was genotyped in both parents, and was found to have been inherited (three maternal, one paternal).

Among the cases of other races/ethnicities, three additional novel variants were identified (Table 2). These variants were each observed in the heterozygous state in a single case. Inheritance could not be determined for these variants because samples were unavailable for one or both parents.

The novel variants identified in *PAX3* included: two variants in the 5' flanking region, of which one (c.-1466T>G) is predicted to be highly conserved (phastCons=1, phyloP=5.46,

GERP++= 5.93); two promoter region variants; two variants in exon 10a; and four variants in exon 11, including one that is predicted to be highly conserved (c.*1125C>T, phastCons=1, phyloP=3.87, GERP++=5.59). Several of these variants are predicted to create or disrupt transcription factor binding, splice acceptor or splice donor sites (Table 2).

T (brachyury): Sequencing identified 28 variants in non-Hispanic white cases (Supplemental File 3). Of these, 27 were previously reported and the allele frequencies for three were significantly different in cases as compared to non-Hispanic whites in 1,000 Genomes (rs3099267: OR=0.53, 95% CI 0.30–0.95; rs3816304: OR=0.56, 95% CI 0.32–0.99; rs35606910: OR=0.56, 95% CI 0.32–0.99) (Table 1). These variants were not observed in HapMap and only one (rs3099267) was observed in the ESP. The allele frequencies for rs3099267 in cases were also significantly different as compared to non-Hispanic Caucasians in the ESP (OR=0.55, 95% CI 0.30–0.91).

Three previously reported rare variants were also identified in one case each, including one missense variant that is predicted to be highly conserved by at least two of three prediction methods (Supplemental File 3).

No novel variants in *T* were identified in the non-Hispanic white cases. Among cases of other races/ethnicities, one novel, promoter region variant was identified (Table 2). As no paternal sample was available for this case, the inheritance status of this variant could not be determined.

DISCUSSION

The spina bifida phenotype observed in mouse *Pax3* mutants and multiple reports of the co-occurrence of spina bifida and WS (Pingault et al., 2010) suggest that variation in *PAX3* may be related to the risk of non-syndromic spina bifida in humans. Although studies that have assessed *PAX3* as a risk factor for non-syndromic neural tube defects in humans have been largely negative (Chatkupt et al., 1995; Hol et al., 1996; Lu et al., 2007; Melvin et al., 2000; Trembath et al., 1999; Volcik et al., 2002), they were limited in size (e.g. Melvin et al. 2002 and Lu et al., 2007 each included less than 80 cases) and/or in scope (e.g. Volcik et al. 2002 considered only three *PAX3* variants and Hol et al. 1996 sequenced only the paired domain regions of this gene) and, therefore, insufficient for establishing or refuting an association.

While our study is also based on a small sample, we took advantage of several large databases that were not available to the previous studies. Using these resources, we identified two common *PAX3* variants that were significantly more common in cases than in at least one reference dataset. We also identified 11 novel *PAX3* variants, including several that are predicted to be highly conserved and/or impact gene function or expression.

We also identified three common *T* variants, for which the minor allele was significantly less common in cases than in public databases, as well as one novel *T* variant. Although the *TIVS7-2* variant, which has been associated with spina bifida in several studies, was not significantly associated with spina bifida in this study, its minor allele was more common in cases as compared to the HapMap data (OR=1.48, 95% CI 1.00–2.21, Supplemental file 3). In addition, one of the associated variants (c.1034+17A>G) is located in intron 7, 62 base pairs from *TIVS7-2*, although it is not in linkage disequilibrium with *TIVS7-2* (r^2=0.04 in 1,000 Genomes, Pilot Phase, CEU).

As with all studies, this study had limitations including a small sample size, reliance on interview data for clinical information, including WS features, and lack of parental DNA samples for some cases. Nonetheless, this study provides a more comprehensive evaluation of the relationship between spina bifida and variation in *PAX3* and *T* than has previously

been reported. For both genes, we provide evidence that common variants are associated with the risk of spina bifida. Although rare and novel variants in both genes were also identified in cases, determination of the impact of such variants on the risk of spina bifida will require studies in larger samples and, ultimately, evidence of the functional impact of any variants that appear to be disease related.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Significantly associated *PAX3* and *T* known variants in non-Hispanic white cases with spina bifida, 2006–2010

rs number	Alleles[a, b]	Location[c]/ Position	Conservation Scores[d]	Cases (N=104)		1,000 Genomes[e] Phase 1 (N=381)		HapMap[f] (N=113)		Exome Sequencing Project[g] (N=8,600)	
				Allele 1	Allele 2	OR[h]	95% CI[i]	OR[h]	95% CI[i]	OR[h]	95% CI[i]
PAX3											
rs13022712	c.1173 +26G>A	Intron 8; 223084833	0.98, 0.96, 2.81	169	23	1.61	0.97–2.69	3.36	1.41–8.05	1.59	1.00–2.44
rs13014735	c.*211+195C>T	Intron 9; 223066237	1, 2.65, 5.77	187	25	1.56	0.94–2.60			1.67	1.03–2.60
T											
rs3099267	c.1034+17A>G	Intron 7; 166574308	0, −0.40, −1.78	186	14	0.53	0.30–0.95			0.55	0.30–0.91
rs3816304[j]	c.*339A>G	Exon 8; 166571464	0, −0.11, 0.53	193	15	0.56	0.32–0.99				
rs35606910[j]	c.*273C>T	Exon 8; 166571530	0, −1.60, −6.33	193	15	0.56	0.32–0.99				

[a] Genomic Build 37.2; Group term GRCh37.2

[b] Positions and alleles are listed in reverse orientation (i.e., increasing position number from 3' to 5' on the coding strand)

[c] *PAX3* exon/intron numbering based on 11 total exons present in 8 alternative transcripts; *T* exon/intron numbering based on 8 total coding exons in 2 alternative transcripts

[d] Vertebrate evolutionary conservation score from phastCons (range: 0–1) phyloP (range: −12–6), and GERP++ (range: −12–6), respectively, from the conservation track of the UCSC Genome Browser

[e] 1000 Genomes phase 1 release version 3.20101123

[f] HapMap Genome Browser release 28 (phase 1, 2 and 3 merge)

[g] Exome Variant Server release ESP6500SI

[h] Odds ratio for allelic association test; only reported for variants with significant associations

[i] Exact 95% confidence interval

[j] rs3816304 and rs35606910 are 66 base pairs apart in the 3' non-coding region of exon 8 and are in tight linkage disequilibrium (r^2=1.0).

Table 2

Novel *PAX3* and *T* variants in cases with spina bifida, 2006–2010

Gene/Subgroup	Position	Alleles[a, b]	Location[c]	Predicted impact (Conservation Scores[d])	Cases (N)	Inheritance
PAX3						
Non-Hispanic whites						
	223164750	c.-1466T>G	5' flank	Creates E2F-p107 TFBS[e] (1, 5.46, 5.93)	1	Maternal
	223164053	c.-719A>G	5' flank	- (0.02, 0.44, 0.53)	1	Unknown
	223163960	c.-629G>T	Promoter	Disrupts WT1-KTS TFBS[e] (0, −0.01, 0.28)	1	Maternal
	223162055	c.86 -123T>G	Intron 1	Creates splice donor site (0, 0.07, −0.64)	1	Maternal
	223066035	c.*211+400C>T	Exon 10a	Non-coding exonic region (0.02, 0.42, 1.49)	1	Unknown
	223066039	c.*211+396delA	Exon 10a	Non-coding exonic region (0.01, 0.49, 0.58)	1	Paternal
	223065753	c.*374_375insGT[f]	Exon 11	Creates splice acceptor site[f] (0.01, −0.28, −1.37)	1	Unknown
	223065002	c.*1125C>T[f]	Exon 11	Non-coding exonic region (1, 3.87, 5.59)	1[g]	Unknown
Other race/ ethnicities						
	223163940	c.-606G>C	Promoter	- (0, −0.19, −1.16)	1	Unknown
	223065775	c.*352G>A[f]	Exon 11	Non-coding exonic region (0, 0.61, 2.57)	1	Unknown
	223065659	c.*468C>A[f]	Exon 11	Non-coding exonic region (0, −0.15, −2.82)	1	Unknown
T						
Other race/ ethnicities						
	166582137	c.-499G>T	Promoter	- (0, 0.82, 1.44)	1	Unknown

[a] Genomic Build 37.2; Group term GRCh37.2

[b] Positions and alleles are listed in reverse orientation (i.e., increasing position number from 3' to 5' on the coding strand)

[c] *PAX3* exon/intron numbering based on 11 total exons present in 8 alternative transcripts; *T* exon/intron numbering based on 8 total coding exons in 2 alternative transcripts

[d] Vertebrate evolutionary conservation score from phastCons (range: 0–1), phyloP (range: −12–6), and GERP++ (range: −12–6), respectively, from the conservation track of the UCSC Genome Browser

[e] Transcription factor binding site

[f] Position relative to the stop codon in alternative transcript NM_181458.3

[g] Homozygous variants; all other novel variants were in the heterozygous state

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