Pathogenic variants in *TUBB4A* are not found in primary dystonia

Satya R. Vemula, PhD
Jianfeng Xiao, MD, PhD
Robert W. Bastian, MD
Dragana Momčilović,
MD, PhD
Andrew Blitzer, MD
Mark S. LeDoux, MD,
PhD

Correspondence to
Dr. LeDoux:
mledoux@uthsc.edu

ABSTRACT

Objective: To determine the contribution of *TUBB4A*, recently associated with DYT4 dystonia in a pedigree with “whispering dysphonia” from Norfolk, United Kingdom, to the etiopathogenesis of primary dystonia.

Methods: High-resolution melting and Sanger sequencing were used to inspect the entire coding region of *TUBB4A* in 575 subjects with primary laryngeal, segmental, or generalized dystonia.

Results: No pathogenic variants, including the exon 1 variant (c.4C>G) identified in the DYT4 whispering dysphonia kindred, were found in this study.

Conclusion: The c.4C>G DYT4 mutation appears to be private, and clinical testing for *TUBB4A* mutations is not justified in spasmodic dysphonia or other forms of primary dystonia. Moreover, given its allelic association with leukoencephalopathy hypomyelination with atrophy of basal ganglia and cerebellum and protean clinical manifestations (chorea, ataxia, dysarthria, intellectual disability, dysmorphic facial features, and psychiatric disorders), DYT4 should not be categorized as a primary dystonia. **Neurology® 2014;82:1227-1230**

GLOSSARY

EVS = Exome Variant Server; **H-ABC** = hypomyelination with atrophy of basal ganglia and cerebellum; **HRM** = high-resolution melting; **MREI** = methionine–arginine–glutamic acid–isoleucine; **SNP** = single nucleotide polymorphism.

The advent of exome sequencing has expedited the identification of genes linked to dystonia, a genetically heterogeneous disorder characterized by involuntary and sustained muscle contractions causing twisting and repetitive movements.^{1–5} Recently, exome sequencing was combined with linkage analysis to identify an exon 1 mutation in *TUBB4A* (c.4C>G; p.Arg2Gly) as the cause of DYT4 or “whispering dystonia,” first described by Parker in a family from Heacham in Norfolk, United Kingdom.^{2,3,6,7} The p.Arg2Gly variant is located within the autoregulatory MREI domain (methionine–arginine–glutamic acid–isoleucine) of β -tubulin 4a. Concurrently, an exon 4 mutation in *TUBB4A* (c.745 G>A; p.Asp249Asn) was found to cause leukoencephalopathy hypomyelination with atrophy of basal ganglia and cerebellum (H-ABC).⁸ The phenotypic spectrum of H-ABC includes dystonia, delayed psychomotor development, spasticity, ataxia, dysarthria, short stature, and microcephaly.⁸ MRI in subjects with H-ABC shows cerebellar and striatal atrophy along with diffuse hypomyelination.

METHODS **Standard protocol approvals, registrations, and patient consents.** Human studies were conducted in accordance with the Declaration of Helsinki, with formal approval from the institutional review boards at each participating study site. All genetic and phenotypic analyses and publication of the results were approved by the University of Tennessee Health Science Center Institutional Review Board (01-07346-XP). Written informed consent was obtained from all participating subjects.

Subjects. Our primary dystonia cohort included 575 subjects (table 1), mainly non-Hispanic Caucasians of European descent residing in the United States. All subjects with laryngeal dystonia had spasmodic dysphonia. One subject with segmental dystonia had inspiratory laryngeal dystonia. The diagnosis of laryngeal dystonia was made by a board-certified otorhinolaryngologist or a neurologist with subspecialty expertise in movement disorders. Enrollment of patients with primary dystonia has been described previously.^{4,5} High-quality genomic DNA was extracted from peripheral whole blood.

Supplemental data
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From the Departments of Neurology and Anatomy & Neurobiology (S.R.V., J.X., M.S.L.), University of Tennessee Health Science Center, Memphis, TN; Bastian Voice Institute (R.W.B.), Downers Grove, IL; Clinic for Child Neurology and Psychiatry (D.M.), Medical Faculty University of Belgrade, Serbia; and New York Center for Voice and Swallowing Disorders (A.B.), NY.

Go to Neurology.org for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

Table 1 Phenotypes and demographics

	No.	Mean age, y	Mean age at onset, y	Male/female
Phenotype				
Spasmodic dysphonia	288	59	46	79/209
Segmental dystonia	210 (65 ^a)	60	46	52/158
Generalized dystonia	77 (14 ^a)	29	15	38/39
Race/ethnicity				
Caucasian	509	56	42	159/350
African American	23	60	48	4/19
Native American	14	54	46	2/12
Asian	13	54	45	2/11
Hispanic	11	57	46	2/9
Jewish	5	60	41	0/5

^a Number with laryngeal dystonia.

Objectives. To determine the contributions of *TUBB4A* to primary dystonia, high-resolution melting (HRM)^{4,5} and Sanger sequencing were used to examine the entire coding region of *TUBB4A* in 575 subjects with primary laryngeal, segmental, or generalized dystonia (table 1). Mutations in *THAPI*, *GNAL*, and exon 5 of *TORIA* were excluded in the entire cohort.^{4,9,10}

Screening for *TUBB4A* variants. Exons 1 and 4 were examined with Sanger sequencing in the forward and reverse directions,^{4,5} whereas exons 2 and 3 were screened with HRM (table e-1 on the *Neurology*[®] Web site at Neurology.org). Compared with Sanger sequencing, HRM is a more rapid and less expensive technology for variant detection.¹¹ With small amplicons (<300 base pairs), the sensitivity of heterozygote scanning is nearly 100%, and false positives are readily excluded with Sanger sequencing.¹¹ However, the value of HRM as a variant screening tool is limited in regions that contain common single nucleotide polymorphisms (SNPs). Sanger sequencing was chosen for exons 1 and 4, and contiguous intronic regions, because these genomic intervals of *TUBB4A* harbor common SNPs (e.g., reference SNP rs390530, rs61741669, and rs2071347) and the variants associated with DYT4 and H-ABC. HRM was performed with the LightCycler 480 Real-Time PCR system and High Resolution Master Mix (Roche, Indianapolis, IN) in accordance with our laboratory protocol.⁵ Using 20 ng of DNA template, 1X HRM Master Mix, 2.5 mM MgCl₂, and 200 nM of each primer in a 10-μL reaction volume, the reactions were performed in either 96- or 384-well plates. Melting curves and difference plots were analyzed by at least 2 investigators blinded to phenotype. For samples with shifted melting curves, PCR products were cleaned using ExoSAP-IT and Sanger sequenced in the forward and reverse directions.

Variant analysis. In silico mutation prediction programs SIFT (<http://sift.jcvi.org/>), MutationTaster (<http://www.mutationtaster.org>), and PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) were used to access the pathogenicity of novel and previously reported missense sequence variants. SIFT is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution at a particular position in a protein will have a phenotypic effect.¹² MutationTaster applies a naive Bayes

classifier to data derived from evolutionary conservation, splice-site changes, loss of protein features, and changes that might affect the amount of messenger RNA to predict disease potential.¹³ PolyPhen-2 predicts pathogenicity by applying a probabilistic classifier to sequence- and structure-based information.¹⁴ The sensitivities and accuracies of SIFT, MutationTaster, and PolyPhen-2 range from 79% to 89% and 84% to 88%, respectively.^{13,15}

RESULTS No novel or pathogenic *TUBB4A* variants were discovered in our cohort. However, previously reported nonpathogenic variants in dbSNP (Single Nucleotide Polymorphism Database) and the Exome Variant Server (EVS) of the National Heart, Lung, and Blood Institute were identified (table 2). Among a cohort of 6,503 subjects, EVS reports 7 missense variants in this highly conserved gene (figure e-1). SIFT and MutationTaster suggest that p.Arg2Gly is disease-causing while PolyPhen-2 predicts that this variant is only possibly damaging. Lohmann et al.³ screened 394 unrelated subjects with dystonia and identified a second variant (p.Ala271Thr) in a single subject with segmental craniocervical dystonia. The p.Ala271Thr variant is predicted to be benign by PolyPhen-2 and damaging by MutationTaster and SIFT. Of the 7 variants reported by EVS, 2 are predicted to be pathogenic by MutationTaster and SIFT but benign by PolyPhen-2. The p.Asp249Asn variant associated with H-ABC is predicted to be damaging by all 3 programs.

DISCUSSION Our data indicate that *TUBB4A* variants do not make a significant contribution to the etiopathogenesis of primary dystonia. Careful analysis of published DYT4 and H-ABC phenotypes indicates the presence of significant phenotypic overlap. H-ABC exhibits an earlier onset and more severe phenotype than DYT4. Therefore, DYT4 may be a “form fruste” of H-ABC. Unlike one or more members of the DYT4 pedigree, none of the subjects in our cohort had a “hobby horse” gait, ataxia, dysmorphic facies, dementia, chorea, or severe dysarthria.^{6,7} Moreover, the overt improvement in dystonia and/or gait with alcohol, propranolol, or tetrabenazine that has been reported in subjects with DYT4 is not consistent with clinical findings in the vast majority of individuals with primary dystonia.^{6,7} H-ABC is characterized by defective myelination and atrophy of the striatum and cerebellum.⁸ Although MRI is reportedly normal in DYT4,⁷ technical details of imaging and images have not been provided in publications related to the DYT4 pedigree. It is possible that more severely affected older individuals with the p.Arg2Gly variant do show evidence of hypomyelination and atrophy on MRI.

Although there is overt phenotypic overlap between DYT4 and H-ABC, it is possible that these

Table 2 Frequency and pathogenicity of *TUBB4A* sequence variants

Chromosome position	dbSNP ID	Allele change	MAF, %	Source	GVS function	cDNA change	Protein change	SIFT (score)	PolyPhen-2 (score)	MutationTaster (score)
Database										
19:6495179	rs371914924	G>A	0.008	EVS	Missense	c.1331C>T	p.A444V	D (0)	P (0.81)	D (0.99)
19:6495210	rs374020309	C>T	0.008	EVS	Missense	c.1300G>A	p.E434K	T (0.14)	P (0.83)	D (0.99)
19:6495567	rs373302095	C>T	0.008	EVS	Missense	c.943G>A	p.A315T	T (0.1)	B (0.04)	D (0.99)
19:6495758	rs145794132	C>T	0.008	EVS	Missense	c.752G>A	p.R251H	D (0.01)	B (0.41)	D (0.99)
19:6496137	rs371265470	C>G	0.008	EVS	Missense	c.373G>C	p.E125Q	D (0)	P (0.72)	D (0.99)
19:6501305	rs369521667	G>T	0.008	EVS	Missense	c.270C>A	p.F90L	D (0)	B (0.01)	D (0.99)
19:6501598	rs372230819	G>C	0.008	EVS	Missense	c.94C>G	p.P32A	T (0.09)	B (0.00)	D (0.99)
Published										
19:6495699	Unknown	C>T	NA	3 ^a	Missense	c.811G>A	Ala271Thr	D (0.03)	B (0.20)	D (0.99)
19:6495765	Unknown	C>T	NA	8 ^a	Missense	c.745G>A	Asp249Asn	D (0)	D (1)	D (0.99)
19:6502220	Unknown	G>C	NA	2 ^a	Missense	c.4C>G	Arg2Gly	D (0)	P (0.87)	D (0.98)
Our study										
19:6495122	rs113953942	C>T	1.100	dbSNP	NA	c.*53G>A	NA	NA	NA	NA
19:6495172	rs150446118	A>G	1.123	EVS	NA	c.*3T>C	NA	NA	NA	NA
19:6495223	rs61731566	C>T	1.945	EVS	Synonymous	c.1287G>A	NA	NA	NA	NA
19:6495466	rs111274574	G>A	0.031	EVS	Synonymous	c.1044C>T	NA	NA	NA	NA
19:6495589	rs118102196	G>A	1.707	EVS	Synonymous	c.921C>T	NA	NA	NA	NA
19:6495595	rs149903666	C>T	0.108	EVS	Synonymous	c.915G>A	NA	NA	NA	NA
19:6495736	rs2071347	A>G	30.46	EVS	Synonymous	c.774T>C	NA	NA	NA	NA
19:6496168	rs61741669	G>A	3.883	EVS	Synonymous	c.342C>T	NA	NA	NA	NA
19:6502137	rs390530	C>A	6.137	EVS	NA	c.57+30G>T	NA	NA	NA	NA

Abbreviations: B = benign; cDNA = complementary DNA; D = damaging; dbSNP = Single Nucleotide Polymorphism Database; EVS = Exome Variant Server; GVS = Genome Variant Server; MAF = minor allele frequency; NA = not applicable; P = possibly damaging; T = tolerated.
^aReference number.

neurogenetic disorders are more discrete at the molecular level. In this regard, there have been numerous studies linking tubulin, a major component of microtubules, to a variety of neurologic conditions ranging from congenital fibrosis of extraocular muscles to lissencephaly.^{16–19} Mutations within the N-terminal MREI domain of β -tubulin 4a, such as p.Arg2Gly, disrupt autoregulation of *TUBB4A* transcript levels.²⁰ In contrast, the p.Asp249Asn mutation is located within the T7 loop of β -tubulin 4a, which interacts with the guanosine triphosphate nucleotide bound to the N-site of α -tubulin.²¹ As such, Asn249 β -tubulin 4a could alter microtubule polymerization.

Taken together, these results suggest that (1) p.Arg2Gly is a private mutation, (2) DYT4 should not be classified as a primary dystonia, (3) DYT4 may be a form fruste of H-ABC, and (4) genetic testing for *TUBB4A* mutations should be limited to pedigrees with H-ABC phenotypes.

AUTHOR CONTRIBUTIONS

M.S.L. designed the study. S.R.V., J.X., and M.S.L. performed experiments and analyzed data. R.W.B., D.M., A.B., and M.S.L. examined patients and collected blood specimens. S.R.V. and M.S.L. wrote the manuscript with input from all authors.

STUDY FUNDING

Supported by grants to M.S.L. from the Dystonia Medical Research Foundation, Tyler's Hope for a Dystonia Cure, NIH (R01 NS069936 and R01 NS082296), and the NIH U54 Dystonia Coalition (1U54NS065701) Pilot Projects Program. The Dystonia Coalition is part of the NIH Rare Diseases Clinical Research Network. Funding and/or programmatic support for this project has been provided by NS067501 from the NIH Office of Rare Diseases Research and the National Institute of Neurological Disorders and Stroke. The views expressed in written materials or publications do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention by trade names, commercial practices, or organizations imply endorsement by the US Government.

DISCLOSURE

S. Vemula, J. Xiao, R. Bastian, D. Momčilović, and A. Blitzer report no disclosures relevant to the manuscript. M. LeDoux serves on the speakers bureaus for Lundbeck, UCB Pharma, and Teva Neuroscience; serves on the Xenazine Advisory Board for Lundbeck and Azilect Advisory Board for Teva; receives research support from the NIH, Dystonia Medical Research Foundation, Tyler's Hope for a Dystonia Cure, Prana, and CHDI; and receives royalty payments for *Animal Models of Movement Disorders* (Elsevier). Go to Neurology.org for full disclosures.

Received October 4, 2013. Accepted in final form December 29, 2013.

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