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RESEARCH ARTICLE

A Multi-center Genome-wide Association Study of Cervical Dystonia

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ABSTRACT: Background: Several monogenic causes for isolated dystonia have been identified, but they collectively account for only a small proportion of cases. Two genome-wide association studies have reported a few potential dystonia risk loci; but conclusions have been limited by small sample sizes, partial coverage of genetic variants, or poor reproducibility.
Objective: To identify robust genetic variants and loci in a large multicenter cervical dystonia cohort using a genome-wide approach.
Methods: We performed a genome-wide association study using cervical dystonia samples from the Dystonia Coalition. Logistic and linear regressions, including age, sex, and population structure as covariates, were employed to assess variant- and gene-based genetic associations with disease status and age at onset. We also performed a replication study for an identified genome-wide significant signal.
Results: After quality control, 919 cervical dystonia patients compared with 1491 controls of European ancestry were included in the analyses. We identified one genome-wide significant variant (rs2219975, chromosome 3, upstream of *COL8A1*, *P*-value 3.04×10^{-8}). The association was not replicated in a newly genotyped sample of 473 cervical dystonia cases and 481 controls. Gene-based analysis identified *DENND1A* to be significantly associated with cervical dystonia (*P*-value 1.23×10^{-6}). One low-frequency variant was associated with lower age-at-onset (16.4 ± 2.9 years, *P*-value $= 3.07 \times 10^{-8}$, minor allele frequency $= 0.01$), located within the *GABBR2* gene on chromosome 9 (rs147331823).
Conclusion: The genetic underpinnings of cervical dystonia are complex and likely consist of multiple distinct variants of small effect sizes. Larger sample sizes may be needed to provide sufficient statistical power to address the presumably multi-genic etiology of cervical dystonia. © 2021 International Parkinson and Movement Disorder Society

Key Words: cervical dystonia; genome-wide association study (GWAS); rare disease; movement disorder

The dystonias are a group of disorders characterized by involuntary, repetitive twisting movements, abnormal postures, or other manifestations of excessive muscle contraction.[1,2] Cervical dystonia (CD) is the most common adult-onset dystonia and is characterized by involuntary patterned contractions of cervical musculature resulting in abnormal movements or postural changes of the head and neck. CD is rare, and estimates of incidence and prevalence are limited by a paucity of comprehensive epidemiological studies.[3]

Some forms of dystonia are genetically determined, and several genes have been identified so far.[4–6] However, these genes collectively account for only a small fraction of all isolated dystonias. Thus, additional genetic and/or environmental factors probably play an important role. The identification of genes among affected pedigrees has been complicated by the fact that many appear to be dominantly inherited with partial penetrance. Genome-wide association studies (GWAS) agnostically scan markers across the genome in a large population to identify genetic variations associated with a disease trait. GWAS have successfully identified thousands of markers that are associated with a wide spectrum of diseases,[7] and are the most cost-effective approach to discovering any genetic association with a disease. The power of this method to detect genetic variants depends on study sample size and the underlying genetic architecture. Although variants with large effect sizes can sometimes be detected with sample sizes as small as 100, far more variants with smaller effect sizes can be detected when more than 10,000 cases and controls are examined. Currently, only a few very small GWAS have been published for dystonia. One GWAS involving 212 CD cases from the United Kingdom revealed a genetic variant in *NALCN* gene,[8] but the

association was not replicated in another study involving additional 252 CD patients from Spain.[9] A GWAS of 127 cases with musician's dystonia identified a significant association in a locus of *ARSG* gene, but it has not yet been independently replicated.[10]

These studies demonstrate that GWAS is a promising approach for the discovery of candidate genes and loci related to the dystonias. However, they are limited by small sample sizes and lack of genome-wide gene-based analysis of low-frequency variants. To address these limitations, the present study provides a comprehensive coverage of the genetic susceptibility in idiopathic CD using a large cohort of 919 cases. Additionally, we investigated potential genetic determinants of age-at-onset and tremor among CD cases.

Patients and Methods

Data Sources and Study Population

The dystonia subjects studied in this project are from the Dystonia Coalition, which is an ongoing study to facilitate collaborations and to advance the pace of clinical and translational research for isolated dystonia syndromes (ie, "primary" dystonias).[11–13] The Dystonia Coalition collected DNA samples and detailed clinical information on adult-onset isolated dystonia patients (n = 3119) including several subtypes to identify risk factors and biomarkers to improve prognosis, treatment, and prevention. Because different forms of dystonia may have different genetic substrates, we focused our GWAS on the most common subtype, CD. We included all available CD samples of European ancestry at the time of the study. All cases were evaluated and recruited by individuals with expertise in movement disorders using a standardized protocol, which was videotaped for verification.[11] Acquired forms of CD and CD combined with other movement disorders (eg, parkinsonism) were excluded. We included cases who started with dystonia in the neck region (isolated CD). Cases with minor dystonic symptoms in other body regions were also included, if CD was the main problem (segmental or multifocal dystonias).

Control samples without neurological disorders were obtained from the Emory Cardiovascular Biobank study and the Mental Stress-Induced Ischemia, Mechanisms and Prognosis (MIPS). Blood specimens of participants were collected and used to extract genomic DNA. The Dystonia Coalition study and the present GWAS were approved by the Institutional Research Board committee at Emory University. All participants provided written consent. We obtained demographic variables such as age, sex, age-at-onset from the survey and medical records.

Genotypic Measures and Data Processing for the GWAS

Genomic DNA (gDNA) was extracted from blood samples of study participants by a central laboratory at the Coriell Institute for Medical Research (Camden, New Jersey, USA). Each gDNA sample was quantified using the PicoGreen assay, standardized to 50 ng/mL, and processed following the standard Illumina protocol including hybridization, incubation, and scanning. We used Illumina's Multi-Ethnic Genotyping Array (MEGA) platform, which is optimized for genome-wide association studies in multiethnic populations. The MEGA chip directly measures 1.7 million genetic markers, including improved genome-wide coverage of non-European ancestry, exonic content of over 400,000 markers, more than 17,000 variants relevant to clinical and pharmacogenetic studies, and an additional 23,000 variants selected for functional, immunological, oncological, ancestry, forensic, and other common and rare diseases.

Genotypes were measured and called using Illumina's GenomeStudio software. Participants were excluded if they had an overall SNP call rate (ratio of measured SNPs per participant over the total number of SNPs in the data set) < 95% or mismatch between genotypic and phenotypic measurements (eg, sex and ethnicity). Individuals related closer than second-degree cousins were identified. Only one individual from each related pair was kept in the study. In addition, individual SNPs were excluded from the analyses if they had unknown chromosomal location, were missing for more than 5% of the total participant samples, had Hardy–Weinberg Equilibrium (HWE) *P*-value less than 10^{-6} among the controls, or had a minor allele frequency (MAF) less than 0.01 among controls.[14] Base-pair position of each variant was annotated to human genome reference GRCh37/hg19.

Cleaned genotype data of common variants were further pruned using plink software with r^2 less than 0.1, and window size and step of 50 and 5 SNPs, respectively. We used ADMIXTURE[15] analysis with the 1000 Genome phase 3 samples and known continental ancestries to assign genotyped individuals to European (>80% European Ancestry). Principal component analyses were performed using flashPCA software.[16] The dosage data of SNPs in the 1000 Genomes Project phase 3 version 5 reference panel were imputed by the Michigan Imputation Server (https://imputationserver.sph.umich.edu/) using minimac.[17] Following imputation, variant-level quality control was performed using the EasyQC R package (www.R-project.org), and exclusion metrics included: Hardy–Weinberg equilibrium *P*-value $<10^{-6}$, posterior call probability <0.9, imputation quality <0.3, MAF < 0.05, call rate < 97.5%. Variants were also excluded if they deviated >10% from their expected

allele frequency based on reference data from the Genome Aggregation Database (gnomAD).[18] Among 968 genotyped CD cases with phenotypic data, 13, 14, and 22 samples were excluded due to high missing rate, sex-mismatch, and non-European ancestry, respectively. Following variant-level quality control, we obtained 8,053,795 common variants (MAF > 5%) in 919 CD cases and 1491 controls of European ancestry.

Association Analysis

Genome-wide association analysis of CD status was adjusted for age, sex, and genotype-derived principal components in a logistic regression model. Imputed genetic variants were tested for association with CD assuming an additive genetic model. Among 919 CD patients with available phenotypes, genome-wide association analyses of age-at-onset, tremor, and affected area (segmental vs. focal) were adjusted for age, sex, and genotype-derived principal components in linear or logistic regression models. We summarized the GWAS results using FUMA (http://fuma.ctglab.nl/), which is a platform that can be used to annotate, prioritize, visualize, and interpret GWAS results.[19] Genome-wide significant SNPs ($P < 5 \times 10^{-8}$) were grouped into genomic loci if they are dependent on each other at $r^2 > 0.1$ or physically close (distance < 500 kb). Lead SNPs were defined within each locus if they are independent ($r^2 < 0.1$). FUMA uses GWAS summary statistics to compute gene-based *P*-values using the MAGMA tool.[19] The gene-based *P*-value is computed for protein-coding genes by mapping SNPs to genes if SNPs are located within the genes. The Bonferroni correction was used to correct for multiple testing based on the total number of tested protein coding genes. The 1000 Genomes phase 3 was used as a reference panel to calculate LD across SNPs and genes. All other statistical analyses were performed in the R statistical environment version 3.2.5 (http://www.r-project.org/).

Replication Study

We investigated 473 independent CD patients that originated from the Dystonia Coalition (n = 270) or the German Dystonia Registry (DysTract, http://dystract.cio-marburg.de/en/, n = 203) using a similar protocol for enrollment. We also included 481 healthy controls from Northern Germany (Luebeck area). Variant rs2219975 was genotyped using a melting curve analysis on the LightCycler or by Sanger sequencing.

Results

After quality control, the analysis included 919 cases of CD and 1491 controls, all of European ancestry. The PCA plots showed that the genetic ancestry of the study sample was consistent with the HapMap panel, and the genetic background was similar between cases and controls (Fig. S1A,B). Characteristics of these groups are summarized in Table 1. In brief, the CD subjects had a younger average age of 60.7 ± 11.4 years, compared to controls (64.8 ± 11.5 years). More CD participants were females than in the control group (75.7% vs. 48.0%). Among 919 CD subjects, the average age at onset was 45.6 ± 13.5 years.

TABLE 1 *Dystonia patients and controls of European ancestry using for the GWAS stage*

Variables	Cases (N = 919) Mean ± SD or count (%)	Controls (N = 1491) Mean ± SD or count (%)
Age (y)	60.7 ± 11.4	64.8 ± 11.5
Female	696 (75.7%)	716 (48.0%)
Age at onset (y)	45.6 ± 13.5	N/A
Focal	769 (83.7%)	N/A
Segmental	150 (16.3%)	N/A
Tremor	616 (67.0%)	N/A

Abbreviation: GWAS, genome-wide association study.

There was moderate global inflation (inflation factor of 1.07) of over 8 million common variants. Using the 1000 Genome Project-based imputation panel, we identified one genome-wide significant common (MAF > 5%) SNP from a locus on chromosome 3 (Fig. 1 and Table 2). The lead common SNP (rs2219975) of the chromosome 3 locus had a MAF of 0.06 in controls and 0.10 in CD cases. The minor allele C was associated with higher risk of CD (OR of 2.1, *P*-value = 3.04×10^{-8}). Two variants (chr3:99071470 and chr3:99072830, GRCh37/hg19) with lower MAF (~1%) and high LD in the chromosome 3 locus were also associated with CD with *P*-values of 6.74×10^{-9} and 8.49×10^{-9}, and ORs of 46 and 50, respectively (see summary statistics of these two low-frequency variants including minor allele counts in Table S1).

The regional plot (Fig. S2) showed all tested SNPs in the neighboring regions of the lead SNP and additional association signals with CD within these regions. The CD-associated locus is located in intergenic region, and is about 300 kb upstream of *COL8A1*.

Genotyping of the lead SNP rs2219975 in the replication samples found the minor C allele less frequently in cases (overall: 6.4%, DC subgroup: 7.4%; DysTract subgroup: 5.2%) compared to controls (9.0%).

Complementary to single variant association test, gene-based statistical methods can analyze multiple genetic markers simultaneously to determine their joint association with a disease trait. Using the MAGMA gene-based analysis method available in the online tool,

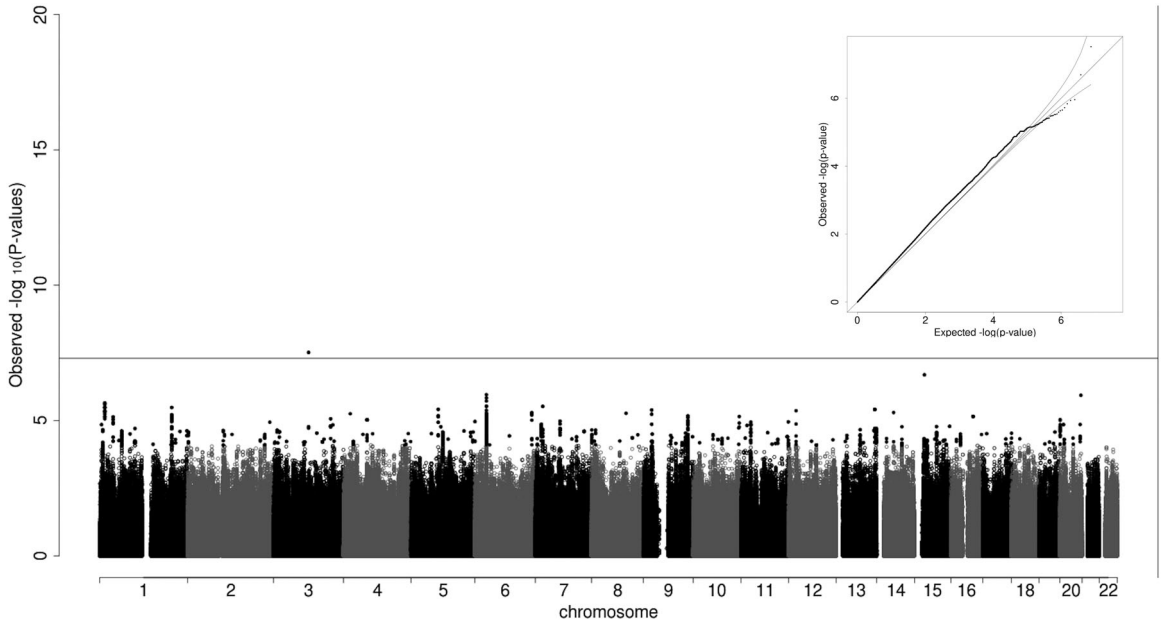

FIG. 1. Summary of the genome-wide association study (GWAS) of cervical dystonia. Manhattan plot showed a significant locus on chromosome 3. The horizontal line represented the genome-wide significant threshold of 5×10^{-8}. The quantile-quantile plot showed overall distribution of *P*-values in the GWAS.

TABLE 2 *Summary of common genetic variants (MAF > 0.05) of genome-wide significant locus associated with cervical dystonia*

SNP ID	rs number	CHR	POS	REF	ALT	AF_Control	AF_CD	OR	T Statistic	*P*-value
3:99070019	rs2219975	3	99,070,019	T	C	0.06	0.10	2.123	5.539	3.04×10^{-8}

Abbreviations: MAF, minor allele frequency; CHR, chromosome number; POS, base-pair position in GRCh37/hg19; REF, reference allele; ALT, alternative (effective) allele; AF, allele frequency of alternative allele; OR, odds ratio.

functional mapping and annotation (FUMA),[19] the *DENND1A* was significantly associated with CD after correction for multiple testing. We also identified another four genes (Table S2; *ATP11A*, *ST7-OT4*, *PPP1R16B*, *SLC39A12*) with nominal *P*-values less than 10^{-4}. None of the previously reported genes potentially related to adult-onset isolated dystonia (ie, *ANO3*, *CIZ1*, *COL6A3*, *GNAL*, *THAP1*, *TOR1A*) were significantly associated with CD in the gene-based analysis. We also examined the genetic associations of individual SNPs with CD in regions harboring six previously reported genes related to dystonias, *ANO3*, *CIZ1*, *COL6A3*, *GNAL*, *THAP1*, and *TOR1A*. None of the SNPs in these regions were significantly associated with CD after multiple testing corrections. Gene expression quantitative trait locus (eQTL) analysis showed that eQTLs from the GWAS of CD were the most enriched for brain tissue as well as specific subregions of the brain (eg, cerebellum, cerebellar hemisphere, spinal cord cervical, hippocampus), although not statistically significant after correcting for multiple testing.

There were no common variants significantly associated with age at onset (ranging 8–77 years) among 919 CD patients adjusted for sex and population structure. Interestingly, one low-frequency SNP on chromosome 9:101222141 (rs147331823, MAF ~0.01) showed strong association with age at onset (*P*-value $= 3.07 \times 10^{-9}$) as shown in Figure S3. The minor allele G was associated with a younger age at onset of 16.4 ± 2.9 years.

The current analyses failed to replicate the CD-associated SNPs in the *NALCN* region on chromosome 13 previously reported.[8] None of these reported SNPs (reported *P*-values range from 9.76×10^{-7} to 2.54×10^{-6}) had nominal *P*-values less than 0.05. We also searched the *NALCN* region including ±200 kb flanking regions. Among all imputed SNPs in the region, none had a *P*-value lower than 10^{-3}.

Discussion

The results from this study are derived from the largest GWAS for any type of dystonia to date. CD is considered a rare disorder, and this study was made possible only by pooling samples through a multicenter collaborative effort. However, the sample size of this

study (919 cases and 1491 controls) is considerably smaller than other GWAS for more common disorders such as Parkinson's disease and the finding of a genome-wide significant SNP on chromosome 3 could not be confirmed in a small replication sample. There might be several reasons for the lack of replication: (1) rs2219975 has a rather low frequency (~7%) compared to other genetic risk variants, and thus, larger sample sizes are needed to detect and replicate an association; (2) The frequency of this variant highly depends on genetic ancestry (eg, 0.5481 in Africans and 0.07303 in non-Finnish Europeans, https://gnomad.broadinstitute.org/variant/3-99070019-T-C?dataset=gnomad_r2_1). Small differences in the origin of cases and controls may lead to false positive associations in a GWAS; (3) The individual effect size of genetic risk variants might be low given the known etiological heterogeneity of CD. The sample size was sufficient for genome-wide discovery of CD-related genes with large effects (eg, at alpha level of 5×10^{-8}, OR 1.5 and 1.68 for MAF of 0.2 and 0.1, respectively), but insufficient for genetic associations with small effects or very low-risk allele frequency.

In the present GWAS of CD, we could not confirm a previously reported association of CD in *NALCN* from an independent GWAS. We also were unable to replicate the association of dystonia with the *ARSG* gene, although the latter may reflect the different type of dystonia studied (eg, task specific dystonia).[10]

The locus on chromosome 3 is located upstream of *COL8A1*. The *COL8A1* gene encodes one of the two alpha chains of type VIII collagen. Defects in *COL8A1* are associated with corneal dystrophy and age-related macular degeneration. It has not been associated with any type of dystonia, but it is interesting to note that dystonia has been reported to be potentially caused by biallelic mutations in *COL6A3*, another member of the collagen family.[20]

Gene-based analysis identified *DENND1A* on chromosome 9 to be significantly associated with CD. *DENND1A* encodes DENN domain containing protein 1A, a member of the connecdenn family. It serves as a guanine nucleotide exchange factor that is expressed in brain and plays a role in vesicle function. It has not yet been associated with dystonia, although pathogenic variants in different guanine nucleotide exchange factors encoded by *GNAL* and *GNAO1* have been linked to adult onset focal and segmental dystonias and a childhood-onset movement disorder that combines chorea and dystonia,[4] respectively. *SLC39A12* (solute carrier family 39 member 12) encodes a zinc transporter and belongs to a subfamily of proteins that share structural characteristics of zinc transporters. Mutations in *SLC39A14*, another member of the same ZIP transporter family, have been linked to hypermanganesemia associated with infantile-onset dystonia and parkinsonism-dystonia.[21,22]

Although we did not identify any genome-wide significant common variant associated with age at onset for CD, a low-frequency variant (MAF ~1%) was associated with a decrease in age at onset (16.4 ± 2.9 years, P-value $= 3.07 \times 10^{-8}$), located within the *GABBR2* gene on chromosome 9. This gene encodes a subunit of GABA-B receptors, which play an important role in neuronal excitability. This gene has not been previously associated with dystonia, although GABA receptor activators such as baclofen and benzodiazepines can be effective in treating some types of dystonia. Because the strong association was based on nine carriers of the minor allele, future association study is warranted to validate the finding.

In conclusion, we have conducted the largest GWAS of any type of dystonia so far assembled, and attempted to address heterogeneity by relying on a standardized exam and by focusing on a single dystonia subtype, CD. Despite these measures, GWAS did not reveal any robust locus, indicating a presumably rather small impact of an individual common variant at the population level. Future genetic studies of dystonias require the whole scientific community to join effort to reveal the complex genetic architecture of CD and other dystonias.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study. GWAS data of cervical dystonia are shared via dbGaP Study Accession: phs001803.v1.p1.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

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Author Roles

Y.V.S. and H.A.J. were involved in the conception, organization, and execution of the research project; R.B., R.R., I.M., S.R., K.B., K.H., J.J., N.J.P., E.R., M.V., B.B., M.S.L., A.E., P.A., S.P., S.A.F., W.G.O., R.S., S.C., S.N., A.B., A.P., A.B., S.H.F., M.K., U.K., M.N., T.B., S.L., M.B., S.Z., A.M., M.G., J.V., T.O., A.A.K., F.B., A.C., G.K., A.F., S.F., L.S., J.D.B., V.V., A.A.Q., C.K., J.P., K.L., and H.A.J. contributed to evaluation of cases, and data collection and biospecimen collection; Y.V.S., C.L., I.R.K., and Q.H. were responsible for the design and execution of statistical analysis; The first draft was written by Y.V.S. and H.A.J., and developed further by C.K., K.L., M.S.L., and W.G.O. All coauthors contributed to critical review and editing of the manuscript.

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