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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.

Lack of Validation of Variants Associated With Cervical Dystonia Risk: A GWAS Replication Study

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Funding agencies: This study was supported by grants from the Spanish Ministerio de Economía y Competitividad (SAF2007-60700), the Instituto de Salud Carlos III (CP08/00174, PI10/01674, PI13/01461), the Consejería de Economía, Innovación, Ciencia y Empresa de la Junta de Andalucía (CVI-02526, CTS-7685), the Consejería de Salud y Bienestar Social de la Junta de Andalucía (PI-0377/2007, PI-0741/2010, PI-0437-2012, PI-0471/2013), the Sociedad Andaluza de Neurología, the Fundación Alicia Koplowitz, the Fundación Mutua Madrileña and the Jaques and Gloria Gossweiler Foundation. Dr. Pilar Gómez-Garre was supported by the "Miguel Servet" program at the Instituto de Salud Carlos III.

Relevant conflicts of interest/financial disclosures: Nothing to report. Full financial disclosures and author roles may be found in the online version of this article.

Received: 14 May 2014; **Revised:** 12 August 2014; **Accepted:** 1 September 2014

Published online 25 September 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.26044

Abstract

Background: A recent genome-wide association study (GWAS) has identified a putative association, not statistically confirmed, of cervical dystonia within several regions in a British population. Hence, the authors proposed dysfunction of the ion channel NALCN (for sodium leak channel, nonselective) as a plausible cause of cervical dystonia. The objective of our study was to investigate the association of five single nucleotide polymorphisms (SNPs) previously reported with high signals as putative genetic risk factors for cervical dystonia in a British GWAS, including two located in the *NALCN* gene region.

Methods: We performed a case-control association study in a Spanish population. The SNPs selected for

genotyping were two SNPS in the *NALCN* gene (rs61973742 and rs1338041), one SNP in the *OR4X2* gene (rs67863238), one SNP in the *COL4A1* region (rs619152), and one intergenic SNP (rs1249277). Genomic DNA was collected from 252 patients with cervical dystonia, with a mean age of 55.3 ± 14.1 years (mean age at onset, 43.5 ± 15.7 years), and 342 unrelated control subjects with a mean age of 56.3 ± 14.3 years. Genotyping of SNPs was performed using TaqMan assays and SimpleProbe assays.

Results: The SNP rs619152 had to be excluded because of assay failure. No significant differences were found in allele distribution between cases and controls for all analyzed SNPs. Therefore, we found no association with cervical dystonia for the analyzed SNPs in our Spanish population.

Conclusions: We did not find any evidence supporting the association of *NALCN* with cervical dystonia, indicating that this gene is not implicated in the pathogenesis of this disorder in our cervical dystonia population.

Key Words: primary dystonia, cervical dystonia, replication, association study, GWAS

Dystonia is a heterogeneous movement disorder characterized by sustained or intermittent involuntary muscle contractions, frequently causing twisting and repetitive movements, abnormal postures, or both.[1] At present, several loci (DYT1 to DYT25) and some genes have been found to be associated with dystonia, mainly in familial forms. However, most cervical dystonia (CD) cases have a non-Mendelian inheritance pattern. Although symptomatic treatments are available, dystonia's chronicity leads to an impaired quality of life and sustained morbidity. Hence newer treatments are required, but little is known about its underlying molecular pathophysiology, in part because of limited knowledge of the genetic basis of the disorder.

In this context, a recent genome-wide association study (GWAS) has been performed on British resident patients with CD.[2] In this study, 14 single-nucleotide polymorphisms (SNPs) were identified as possibly being associated with CD, but the authors suggested that some of them might be false-positive results. The authors reported a putative association of CD with SNPs in the *NALCN* (for sodium leak channel, nonselective) region. However, this association was not statistically confirmed, because no single SNP passed the genome-wide significance level after GWAS and imputation association analysis. After GWAS analysis, the SNPs with the strongest association with CD were: rs9416795 (located in an intergenic region on chromosome 10) and rs1338041 (located in an intronic region of *NALCN*, coding for sodium leak channel, on chromosome 13). After subsequent imputation, several potential associations were found. The best signals were found clustered around *NALCN*, with the lowest *P* value in rs61973742 and 5 more SNPs in the same gene, including rs1338041, an SNP also found in the nonimputed genotype platform. These two SNPs are not in close linkage with each other. The second cluster was found in rs67863238, in the chromosome 11 *OR4X2* gene region. Remaining imputed SNPs with possible association with CD were rs619152 (located in an intronic region of *COL4A1*, on chromosome 13), also found in the nonimputed genotype platform, and rs1249277 (located in the same intergenic region on chromosome 10 as rs9416795).[2]

Recently, three genes, *CIZ1* (*DYT23*),[3] *ANO-3* (*DYT24*),[4] and *GNAL* (*DYT25*),[5] have been associated with families with CD, the most common form of dystonia.[6] *CIZ1* encodes a zinc finger DNA-binding protein, *ANO-3* encodes a calcium-gated chloride channel, and *GNAL* encodes an olfactory G protein coupled with the expression of dopamine D1 receptor. Therefore, as GWAS authors noted, both *NALCN* (coding an ion channel) and *OR4X2* (coding an olfactory receptor in brain) genes could be plausible candidates for dystonia.

Hence, to further replicate the British GWAS findings, we investigated the top independent hits found in that study, including variants rs61973742, rs1338041, rs67863238, rs1249277, and rs619152, for their association with CD in our Spanish population.

Methods

Subjects

Our analysis included a total of 252 patients with CD (86 men and 166 women) with a mean age of 55.3 ± 14.1 years (mean age at onset, 43.5 ± 15.7 years), and 342 unrelated healthy control subjects (125 men and 217 women) with a mean age of 56.3 ± 14.3 years. Subjects were recruited at different hospitals of Spain, making up the "multicenter study of genetic factors in primary dystonia consortium." Cervical dystonia was diagnosed by senior neurologists using accepted standard clinical criteria that was agreed on beforehand. The selection of control subjects was clinic-based from outpatient clinics at the same hospitals, and absence of neurological disease was an essential inclusion criterion.

Ethics Statements

The study was approved by the ethics committees from all participating centers and was conducted according to the principles expressed in the Helsinki Declaration. All subjects, both CD patients and controls, signed a written informed consent before blood withdrawal.

Genetic Analysis

DNA Isolation

Genomic DNA was isolated from peripheral blood for each subject according to established protocols

TABLE 1. Association analyses of the four genotyped SNPs with cervical dystonia in the Spanish population

					Minor Allele Frequencies			Allelic Association	
SNP	Chr.	Locus	BP	Alleles	Cases	Controls	$\chi 2$	*P*-value	Odds-ratio
rs1338041	13	*NALCN*	102058862	A>C	0.3975	0.4196	0.6215	0.4305	0.9125
rs61973742	13	*NALCN*	102083273	A>G	0.06618	0.09077	2.478	0.1155	0.7098
rs67863238	11	*OR4X2*	48267856	G>C	0.08541	0.08902	0.05013	0.8228	0.9556
rs1249277	10	intergenic	28720076	G>C	0.1684	0.1584	0.2286	0.6326	1.076

Chr, chromosome; BP, position in the chromosome.

using standard or two automated methods (Maxwell 16 System, Promega Corporation, Madison, WI, USA; MagNA Pure LC, Roche Applied Science, Indianapolis, IN, USA).

Genotyping

Two of the selected SNPs, rs1338041 and rs1249277, were genotyped with TaqMan SNP chemistry (Applied Biosciences Hispania, Alcobendas, Madrid, Spain). Genotyping of the other selected SNPs, rs61973742, rs619152, and rs67863238 was performed with SimpleProbe chemistry (Roche) and melting curve analysis. Asymmetric polymerase chain reaction conditions were used to obtain more copies of the strand complementary to the probe and reduce competitive binding. All genotyping reactions were performed in a LightCycler480 (LC480) instrument (Roche Applied Science, Indianapolis, IN, USA).

Statistical Analysis

All association analyses of the SNPs and CD risk factors in our case-control study were carried out with PLINK software.[7] Hardy-Weinberg equilibrium in the control group and allelic associations between cases and controls were tested by the χ^2-test. Because this is a replication study, no correction was applied to the obtained *P*-values. The results were considered statistically significant when *P*-values were lower than 0.05.

Statistical power calculations were performed using Quanto software (Version 1.2.4; USC, Los Angeles, CA).

Results

We studied five SNPs previously reported with high signals in a British GWAS, including two identified as putative genetic risk factors for CD. All genotyping reactions showed a high genotyping quality, with the exception of the SNP rs619152. Therefore, this SNP was excluded from the analysis. All control genotype frequencies in all of the studied polymorphisms were in Hardy-Weinberg equilibrium. Total genotyping rate in individuals was 96.4%. Of all genotyping, 0.5% was duplicated, resulting in a concordance of 100%. Allele frequencies and association results for all analyzed SNPs are shown in Table 1.

A total of 252 patients with CD and 342 unrelated healthy control subjects were included in our study. Taking into account this sample size and assuming a primary dystonia prevalence of 1.52 per 10.000,[8] power analysis indicated that we had a statistical power of 80%, two-sided, to detect statistical differences ($P < 0.01$) in SNPs with minor allele frequencies (MAFs) greater than 0.05 in controls and effect sizes of genetic risk (odds ratio) of 2.2. Indeed, the estimated effect size for each SNP, based on MAFs in our population, ranged from 0.7 to 1.08.

The MAFs did not show any statistical significant difference between cases and controls for any of the studied SNPs. Therefore, we found no association between these SNPs and CD in the Spanish population.

Discussion

Genome-wide association study is a powerful method for the detection of genetic factors in polygenic diseases, but it may produce spurious association findings. Therefore, replication of associations in other ethnic groups is essential to confirm the results. Recently, Mok et al.[2] reported the possible association between a few clusters of SNPs and CD by performing GWAS in 212 British resident CD patients of European descent. The lowest *P*-value was found in the *NALCN* gene region. After imputation, results showed a few clusters of potential significance, with the top hit again in the *NALCN* region. This gene codes for a voltage-independent sodium leak channel. Hence, the authors suggested that dysfunction of a sodium channel could be a good candidate in dystonia.[2]

The current study was conducted to confirm the association of the most informative SNPs from that GWAS (rs61973742, rs1338041, rs67863238, rs1249277, and rs619152) with CD.

Our results showed that the possible association of those SNPs with CD was not replicated in our study. Several factors could lead to this lack of replication. First, allele frequencies reported for control populations vary depending on the ethnic origin (Supplemental Data Table 1), and these differences may

contribute to the controversial results. The SNPs showed a potential effect in the same direction as previously reported, with the exception of rs1338041. For this SNP, we observed a higher MAF in controls than in cases, implying a potential protective minor allele. This result differs from that reported by Mok et al.,[2] who found a detrimental minor allele, which could be attributable to allele frequencies differing between our control population and the British population and also between our population and the 1000 Genomes Project-CEU population. The same situation is observed for the SNP rs61973742; however, in this case the observed potential effect is similar in both studies (Mok et al.[2] and present study).

A second factor could be clinical differences between the patients with CD included in both studies. In this regard, we think that this difference does not apply to our study because both studies only included patients with focal CD and with exclusion of *DYT1*. Finally, in the GWAS, no *locus* was found with a statistically significant association with dystonia. Authors suggested that this fact can be attributable to the small sample size, which underpowered the study to detected loci with a small disease effect. However, our study had over 100% power to replicate similar effect sizes for these SNPs and an 80% power to detect odd ratios of at least 1.8. Therefore, we would expect at least a signal in the right direction if an association of these SNPs with CD were of general validity. Thus, despite the fact that we cannot completely exclude a low effect size for these SNPs, we think that a cause of these discrepant results could be the overestimation of the true effect in the discovery study.

In conclusion, our study did not find any association between the more informative SNPs from the previous British GWAS and CD. ■

Acknowledgments: We thank the patients and healthy individuals who participated in this study. We also thank doctors John Hardy, Kin Mok, and Kailash Bhatia for their help in the SNP selection from their GWAS for our replication study. This research has been conducted using samples from the Andalusian Public Health System Biobank (ISCIII-Red de Biobancos RD09/0076/00085).

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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.

Families With Wilson's Disease in Subsequent Generations: Clinical and Genetic Analysis

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Abstract

Introduction: Wilson's disease is an inherited autosomal recessive disorder of copper metabolism. The prevalence of Wilson's disease in most populations is approximately 1 in 30,000. The risk for offspring is 0.5%. The aim of this study was to establish the frequency of disease among offspring of a cohort of Wilson's disease patients.

Materials and Methods: In February 2014, our registry included 760 cases of diagnosed Wilson's disease. We selected families in which Wilson's disease was diagnosed in the proband's offspring.

Results: Between 1957 and 2014, 1,050 relatives of affected members were screened. Wilson's disease in subsequent generations was observed in nine non-consanguineous families, with 12 affected offspring from nine probands.

Conclusion: We detected a higher (4.08%) than expected (0.5%) frequency of Wilson's disease among proband offspring, which is in accordance with a recent genetic study in the United Kingdom that suggested a higher WD prevalence in the European population. © 2014 International Parkinson and Movement Disorder Society

Key Words: Wilson's disease, family screening, consecutive generations

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Funding agencies: No financial support was received for this work.

Relevant conflicts of interest/financial disclosures: Nothing to report. Author roles may be found in the online version of this article.

Received: 7 May 2014; **Revised:** 18 September 2014; **Accepted:** 28 September 2014

Published online 18 October 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.26057