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Short communication

Role of *ANO3* mutations in dystonia: A large-scale mutational screening study

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ABSTRACT

Background: The role of *ANO3* variants as a monogenic cause of dystonia is still under debate because of its relatively high frequency also in controls.
Objective: To screen > 1000 patients with movement disorders for rare *ANO3* variants.
Methods: We searched for rare *ANO3* variants in 729 dystonia and 294 Parkinson's disease (PD) patients using a gene panel. Variants were validated by Sanger sequencing. For one variant carrier, family members were available for segregation analysis.
Results: Nine carriers (seven with dystonia [1.0%], two with PD [0.7%]) of seven different rare, protein-changing variants were identified. None of these variants has been previously reported in dystonia patients. Two of the variants in dystonia patients were found recurrently: p.Arg328Cys was detected in two Korean and p.Arg969Gln in two German patients. The frequency of these two variants in our sample seemed to be higher as in ethnically matched samples from the Genome Aggregation Database (GnomAD). Further, we identified a patient with early-onset, generalized dystonia with a de-novo variant in *ANO3* (p.Val561Glu). Of note, she benefitted from deep brain stimulation.
Conclusion: This study confirms the relatively high frequency of rare, protein-changing *ANO3* variants in both dystonia and non-dystonia patients indicating that not all variants contribute to the disease. Thus, disease relevance of novel variants remains difficult to interpret and functional studies are warranted for a better understanding of the role of *ANO3* variants in dystonia. In contrast, de-novo variants in childhood-onset, generalized dystonia seem to represent an as yet underestimated phenotypic expression of changes in *ANO3*.

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1. Introduction

Dystonia is a rare movement disorder characterized by sustained or intermittent muscle contractions causing abnormal, often repetitive movements, postures, or both. Dystonic movements are typically patterned and twisting, and may be tremulous [1]. Apart from this common definition, dystonia is clinically heterogeneous, including various ages at onset, body distributions and possible association with different movement disorders such as myoclonus or parkinsonism. The etiology in most patients remains elusive; however, monogenic causes have been identified in a subset of patients. While the pathogenic role of several causative genes, e.g. *THAP1* and *TOR1A*, is already well-established [2], the role of *ANO3* mutations in dystonia is still under debate because of its relatively high frequency also in controls and the lack of extended families [3].

Mutations in *ANO3* have first been described in families with autosomal dominant tremulous, craniocervical dystonia [4]. Since then, several dystonia patients with variants in *ANO3* have been reported, mostly presenting with segmental or multifocal dystonia, often including cervical dystonia and tremor (www.mdsgene.org) [5]. Recently, two patients with de-novo variants have been described, supporting a pathogenic role of *ANO3* in dystonia [6,7]. *ANO3* codes for the protein *anoctamin* 3 that is expressed in the brain, mainly the putamen. Anoctamins are a family of calcium-gated ion channels and fibroblast cultures of *ANO3* mutation carriers showed abnormalities in calcium signalling [4].

In this study, we aimed to evaluate the role of *ANO3* by screening more than 1000 patients with movement disorders for *ANO3* mutations. While the overall frequency of rare, protein-changing *ANO3* variants did not differ between dystonia and non-dystonia patients, selected variants seem to be more frequent among patients and de-novo mutations may be more often than initially thought which further confirms a role of specific *ANO3* changes in the etiology of dystonia.

2. Methods

We performed a gene panel analysis including all 27 *ANO3* coding exons (NM_031418) and exon-intron boundaries with a mean coverage of 74x in 729 dystonia and 294 PD patients. We included 562 dystonia samples from Germany (Hanover (n = 247, all with musician's dystonia), Berlin (n = 124), Kassel (n = 113), Kiel (n = 58, most with writer's cramp), and Lübeck (n = 20)), 92 samples from Spain (Sevilla), and 75 patients from South Korea (Seoul) (Table 1A). All patients were examined by movement disorder experts. Dystonia patients had a mean age of 55.9 ± 20.3 years, a mean age at onset of 37.6 ± 12.1 years and included 401 (55%) males. Most of the included dystonia patients (478/729, 65.6%) presented with focal dystonia, 53 (7.3%) patients had segmental and 51 (7.0%) generalized dystonia. As a disease control group, we included 294 PD patients from Germany (collected in Berlin, Kiel, and Lübeck) who had a mean age of 65.3 ± 12.6 years (Table 1A).

Sanger sequencing was performed for validation of rare (minor allele frequency [MAF] < 0.005), protein-changing variants in *ANO3* with a CADD score > 15. For one mutation carrier, both parents and a healthy brother were available for segregation analysis. Paternity and maternity were confirmed by analysis of nine polymorphic microsatellite markers.

Pathogenicity of detected variants was assessed by using two different published scoring systems, i.e. the recommendations from the American College of Medical Genetics and Genomics (ACMG) [8] and the one used for MDSGene (www.mdsgene.org/methods) [9].

The study was approved by the Local Ethics Committee of the University of Lübeck, Germany (approval 04–180) and written informed consent was obtained from all participants prior to the genetic tests.

3. Results

Through gene panel analysis, nine carriers of seven different rare, protein-changing variants were identified. Seven of these were dystonia patients (7/729; 1.0%) and two had PD (2/294, 0.7%). The difference in the frequency of *ANO3* variants between these two groups was not significant (p = 1.0, Fisher's exact test). The nine variant carriers had a mean age of 51.4 ± 12.6 years, a mean age at onset of 29.3 ± 15.0 years, and comprised five women and four men. Among the dystonia patients, three presented with focal, three with segmental, and one with generalized dystonia. There was no significant difference between the age at onset of dystonia patients with and without a variant in *ANO3* (p = 0.1, Mann-Whitney-U-Test). The two PD patients had no signs of dystonia (current age: 45 and 78 years, respectively). An overview of the clinical data of variant carriers can be found in Table 1B.

Among the seven variants, six were missense variants and one was a splice site change. Two of the variants were found recurrently: p.Arg328Cys (rs570284825) was detected in two of our 75 Korean patients (2.7%) and has exclusively been reported in East Asian patients in GnomAD, albeit at a lower frequency (14/8624, p = 0.008, Fisher's exact test). Both patients presented with segmental dystonia beginning in early adulthood. The second recurrent variant (p.Arg969Gln, rs777387236) was found in two of our 562 German patients (0.4%) both of whom had focal dystonia. Similar to p.Arg328Cys, the variant was detected more frequently in our dystonia patients than in the ethnically most closely matched population in GnomAD (non-Finnish Europeans: 7/55713, p = 0.0001). Further, a 42-year-old Spanish woman with generalized dystonia since the age of 3 years was found to carry a missense change p.Val561Glu that is absent from public databases. The variant was not found in either her healthy parents or her healthy brother and thus arose de novo (Fig. 1). She first presented with walking difficulties due to lower left leg dystonia. Her symptoms were progressive during childhood when she developed head and hand tremor and axial dystonia. Bilateral GPi (globus pallidus internus) deep brain stimulation resulted in sustained improvement since 2008, allowing her to walk with assistance.

Table 1
Characteristics of included patients and overview of detected rare variants.

A. Overview of all included patients

	Center	Number of patients	Age (y)	Age at onset (y)	Sex (m/f)	Type of dystonia (focal/segmental/generalized/ n.a.)
Dystonia	Germany (Hanover, Berlin, Kassel, Kiel, Lübeck)	562	58.7 ± 21.0	41.8 ± 13.0	314/248	418/32/27/85
	Spain (Sevilla)	92	46.3 ± 18.3	21.4 ± 7.8	42/50	31/20/20/21
	South Korea (Seoul)	75	45.8 ± 12.7	NA	45/30	29/1/4/41
	Total	729	55.9 ± 20.3	37.6 ± 12.1	401/328	478/53/51/147
Parkinson's Disease (PD)	Germany (Berlin, Kiel, Lübeck)	294	65.3 ± 12.6	NA	156/96 (42 NA)	not applicable

B. Overview of carriers of *ANO3* variants

	Dystonia patients								PD patients		
	L-5698	L-11097	L-11116	L-2706	L-5261	L-11567	L-11560	mean	L-11353	L-7808	mean
cDNA change	c.433-2A > G	c.982C > T	c.982C > T	c.2906G > A	c.2906G > A	c.2894T > G	c.1682T > A	n.a.	c.787A > G	c.2834G > A	n.a.
Protein change	Splice site change	p.Arg328Cys	p.Arg328Cys	p.Arg969Gln	p.Arg969Gln	p.Leu965Trp	p.Val561Glu	n.a.	p.Met263Val	p.Arg945Gln	n.a.
CADD score	24.7	25	25	32	32	27.5	33	28.5 ± 3.8	15.13	28.1	21.6 ± 9.2
GnomAD frequency	NA	EAS 0.0008	EAS 0.0008	EUR 0.00006	EUR 0.00006	NA	NA	n.a.	EUR 0.000008	EUR 0.000009	n.a.
Pathoscoring											
ACMG	VUS[a]	VUS[b]	VUS[b]	VUS[b]	VUS[b]	VUS[a]	LP[c]	n.a.	VUS[d]	VUS[b]	n.a.
MDSGene	PoP	PoP	PoP	PrP	PrP	PoP	PrP		PoP	PoP	
Origin	Germany	South Korea	South Korea	Germany	Germany	Spain	Spain	n.a.	Germany	Germany	n.a.
Age (y)	43	39	44	61	65	46	42	48.6 ± 10.1	45	78	61.5 ± 23.3
Age at onset	25	28	36	15	45	21	3	24.7 ± 13.7	35	56	45.5 ± 14.9
Sex	female	female	male	male	male	female	female	n.a.	female	male	n.a.
Family history	neg	unknown	unknown	neg	neg	neg	neg	n.a.	unknown	neg	n.a.
Dystonia type	focal	segmental	segmental	focal	focal	segmental	generalized	n.a.	no dystonia (PD patient)	no dystonia (PD patient)	n.a.
Affected region	Hand (writer's cramp)	right arm	cervical, right shoulder	Mouth (musician's dystonia)	Hand (writer's cramp)	jaw, both hands	cervical, head tremor, arms and hands, axial, limbs	n.a.	no dystonia	no dystonia	n.a.
Other	mirror symptoms	dystonic tremor	partially responsive to levodopa			static under treatment: biperiden, levodopa/ carbidopa	progressive, good response to DBS	n.a.	PD	PD	n.a.

EAS: east Asian; EUR: non-Finnish European; VUS: variant of unknown significance; LP: likely pathogenic; PoP: possibly pathogenic; PrP: probably pathogenic; n.a.: not applicable; NA: not available.

[a] Fulfilled ACMG criteria: PM2, PP3.

[b] Fulfilled ACMG criteria: PP3.

[c] Fulfilled ACMG criteria: PS2, PM2, PP3.

[d] Fulfilled ACMG criteria: none.

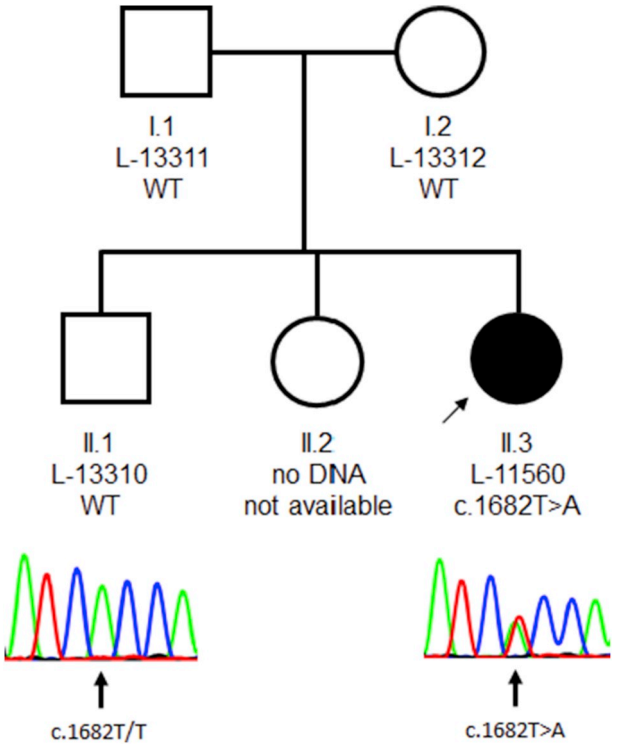

Fig. 1. *Detection of the de-novo variant c.1682T > A (p.Val561Glu) in ANO3.* Pedigree of the patient (L-11560) and electropherogram from Sanger sequencing showing the heterozygous de-novo change at position c.1682 of the *ANO3* gene (on the reverse strand).

Pathogenicity scoring using the criteria from MDSGene classified the de-novo (p.Val561Glu) and one of the recurrent variants (p.Arg969Gln) as “probably pathogenic” whereas using the criteria from ACMG resulted in classification of all variants as “variants of uncertain significance” but p.Val561Glu which was scored as “likely pathogenic” (MDSGene, Table 1B).

4. Discussion

Here, we describe seven different rare, heterozygous *ANO3* variants including six missense changes and a splice site variant that have not previously been reported in dystonia patients. These variants were found in 1.0% of dystonia and 0.7% of PD patients which confirms the relatively high frequency of rare, protein-changing *ANO3* variants in both dystonia and non-dystonia individuals. Similar frequencies of rare, protein-changing *ANO3* variants have also been reported in German controls (0.5%) [3]. Thus, there is no overall enrichment of *ANO3* variants among dystonia patients. Therefore, interpretation of novel variants remains difficult and pathogenicity of a given *ANO3* variant needs to be supported by additional data such as recurrence or segregation within families according to the criteria from the American College of Medical Genetic and Genomics (ACMG) [8].

To date, 36 dystonia patients (50% male) with 19 different variants have been described in the scientific literature (www.mdsgene.org) [5,6,10–12]. Most patients had segmental or multifocal dystonia with cervical dystonia being the main symptom accompanied by head or limb tremor in most patients. There are two peaks of age at onset, one in childhood (0–9 years) and one in adulthood (40–49 years; for detailed information see www.mdsgene.org). Most of the mutation carriers identified in this study fit these characteristics. Three of the dystonia patients presented with multifocal or segmental dystonia (42.8%), two had focal cervical dystonia (28.6%) and two had dystonic tremor (28.6%). However, it is important to note that probably not all of the detected variants in dystonia patients contribute to the disease since a considerable number of variants can also be found in both PD patients and healthy controls. Specifically, we do not consider the two variants detected in the PD patients as causative for their disease as they are neither recurrent nor segregate with the disease. However, studies that are even more extensive are warranted to elucidate further, whether there is a role of *ANO3* variants in PD.

Deep brain stimulation of GPi seems to be a useful treatment in, at least, a subset of patients with ANO3 dystonia as we showed in our Spanish patient with the de-novo variant and as has previously been described in both other carriers of a de-novo *ANO3* variant with childhood-onset, generalized dystonia [6,7]. However, these three successfully operated patients are still a small group and further cases are needed to evaluate the spectrum of DBS response in *ANO3* mutation carriers, especially in those with late onset.

To assess pathogenicity of the detected variants, we aimed to investigate segregation but family history in all our cases was either negative or unknown. Thus, affected family members were not available. However, both parents and an unaffected sibling of a severely affected patient with early onset could be included and the mutation was demonstrated to be de novo which strongly supports pathogenicity. Similarly, the two reported patients in the literature with de-novo variants likewise had early onset (ages 9 and 3 years) of generalized dystonia with upper limb postural tremor and myoclonic-like jerks. The initial symptoms were either writer's cramp [7] or leg dystonia [6]. In addition, pathogenicity of the missense changes p.Arg328Cys and p.Arg969Gln might be supported by their recurrence and enrichment compared to ethnically matched individuals from GnomAD. However, the recurrence in just two patients each may also be a chance finding. Either additional cases or functional read-outs are needed to draw a definite conclusion. Of note, the dystonia patients in our sample presented mostly with focal dystonia (65.6%) and included a relatively high portion of patients with musician's dystonia (33.9%), while segmental distribution of dystonia is commonest in *ANO3* variant carriers. Thus, samples with a broader spectrum of dystonia distributions may include even higher numbers of mutation carriers.

In summary, we further underline the importance of de-novo mutations in *ANO3* in childhood-onset, generalized dystonia that seem to represent a yet underestimated phenotypic expression of changes in *ANO3*. However, disease relevance of novel, presumably inherited, missense changes remains difficult to interpret. Thus, functional studies are warranted for a better understanding of the role of *ANO3* variants in dystonia. This might include measurement of calcium signalling according to the role of Anoctamins and also shed light on the mechanism (s) of the variable expressivity and presumably reduced penetrance of pathogenic variants in ANO3.

Financial disclosure/Conflict of interest

The authors do not have any conflict of interest.

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