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RESEARCH ARTICLE

Large-Scale Screening: Phenotypic and Mutational Spectrum in Isolated and Combined Dystonia Genes

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ABSTRACT: Background: Pathogenic variants in several genes have been linked to genetic forms of isolated or combined dystonia. The phenotypic and genetic spectrum and the frequency of pathogenic variants in these genes have not yet been fully elucidated, neither in patients with dystonia nor with other, sometimes

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co-occurring movement disorders such as Parkinson's disease (PD).

Objectives: To screen >2000 patients with dystonia or PD for rare variants in known dystonia-causing genes.

Methods: We screened 1207 dystonia patients from Germany (DysTract consortium), Spain, and South Korea, and 1036 PD patients from Germany for pathogenic variants using a next-generation sequencing gene panel. The impact on DNA methylation of *KMT2B* variants was evaluated by analyzing the gene's characteristic episignature.

Results: We identified 171 carriers (109 with dystonia [9.0%]; 62 with PD [6.0%]) of 131 rare variants (minor allele frequency <0.005). A total of 52 patients (48 dystonia [4.0%]; four PD [0.4%, all with *GCH1* variants]) carried 33 different (likely) pathogenic variants, of which 17 were not previously reported. Pathogenic biallelic variants in *PRKRA* were not found. Episignature analysis of 48 *KMT2B* variants revealed that only two of these should be considered (likely) pathogenic.

Conclusion: This study confirms pathogenic variants in *GCH1*, *GNAL*, *KMT2B*, *SGCE*, *THAP1*, and *TOR1A* as relevant causes in dystonia and expands the mutational spectrum. Of note, likely pathogenic variants only in *GCH1* were also found among PD patients. For DYT-KMT2B, the recently described episignature served as a reliable readout to determine the functional effect of newly identified variants. © 2024 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: dystonia; GCH1; GNAL; KMT2B; SGCE; THAP1; TOR1A; PRKRA; monogenic; primary dystonia

Introduction

Dystonia is a rare movement disorder characterized by abnormal movements and postures that are caused by involuntary sustained or intermittent muscle contractions.[1] The clinical presentation of dystonia is highly heterogeneous, including various ages at onset (AAO), body distribution of symptoms, and associated features.[1] Dystonia can be isolated, combined with another movement disorder such as parkinsonism or myoclonus, or part of a complex neurological or systemic disorder with extracerebral features.[1]

Monogenic forms (ie, because of pathogenic variants in a single gene) can explain a fraction of dystonia patients, often with early disease onset (<20 years) and a non-focal presentation.[2] To date, pathogenic variants in at least 10 genes have been linked to forms of isolated dystonia,[3,4] of which pathogenic variants in *TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *ANO3*, and *PRKRA* have been reported in >25 patients each.[5] Pathogenic variants in four additional genes causing isolated dystonia (ie, *AOPEP*, *EIF2AK2*, *HPCA*, and *VPS16*) have been confirmed more recently and, to date, were only reported in less than 10 families each, except for *VPS16* variants, which have been reported in at least 25 families.[4,5] For *HPCA*, for instance, disease-causing variants have only been reported in a handful of families.[5] Further, many genes have been linked to the diverse group of combined dystonia,[3] of which *GCH1* and *SGCE*[6] play a major role in dopa-responsive dystonia and myoclonus-dystonia, respectively (see also www.mdsgene.org). Of note, pathogenic *GCH1* variants have also been implicated in the pathogenesis of Parkinson's disease (PD),[7] and a recent systematic literature review revealed that ~10% of patients with *GCH1* mutations present with isolated parkinsonism.[8]

A challenge in genetic testing is the interpretation of variants as disease-causing (pathogenic) or not (benign). Different recommendations have been developed for this, for example, the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG)[9] or the pathogenicity scoring applied within the Movement Disorder Society Genetic mutation database (MDSGene).[10] They both use a weighted score combining evidence from recurrence/family studies (segregation or de-novo occurrence), in-silico prediction (eg, the CADD score),[11] variant frequency in public databases such as the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/), and functional studies. However, the latter are often not available, for instance, for *TOR1A*, or labor-intensive such as for *THAP1*[12] and *GNAL*.[13] The situation might be different for *KMT2B*, a large gene with many rare missense variants, the interpretation of which is particularly challenging. Recently, a characteristic so-called "episignature" for KMT2B loss-of-function was described based on aberrant CpG methylation and can be used as a functional readout to evaluate the effect of variants on the protein's function.[14]

The phenotypic and mutational spectrum of dystonia-linked genes is constantly expanding, and newly identified variants require careful evaluation. In this study, we aimed to evaluate the frequency and role of rare variants in seven of the most common isolated and combined dystonia genes (ie, *TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *PRKRA*, *GCH1*, and *SGCE*) by screening more than 2000 patients with dystonia or PD. The overall frequency of rare variants was significantly higher in dystonia patients compared to PD patients, underlining their role as dystonia genes. Further, this large-scale dataset and the functional evaluation of *KMT2B* variants will guide future variant interpretation.

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Materials and Methods

Study Population

We included 1207 patients with dystonia. These patients were recruited in Germany (n = 1014, within the DysTRACT consortium, a large research-based registry of patients with a diagnosis of dystonia, https://www.isms.uni-luebeck.de/en/research/dystract/), in Spain (n = 92), in South Korea (n = 75), or at several other sites (n = 26) (Supplementary Table S1). All patients were examined by movement disorder specialists. Dystonia patients had a median age of 57 years (interquartile range [IQR], 45–68), a median AAO of 36 years (IQR, 21–49), and included 564 males (46.7%) and 643 females (53.3%). Most of the enrolled dystonia patients (726/1207, 60.1%) presented with focal dystonia, 229/1207 (19.0%) had segmental or multifocal, and 125/1207 (10.4%) had generalized dystonia (Supplementary Table S1). Of the patients with focal dystonia, most had cervical dystonia (293/726, 40.4%), upper limb dystonia (80/726, 11.0%), blepharospasm (63/726, 8.7%), or musician's dystonia (225/726, 31.0%). As a disease control group, we included 1036 PD patients from Germany with a median age of 71 years (IQR, 60–78), a median AAO of 61 years (IQR, 52–69.75), of whom 641 were male (61.9%) and 376 were female (36.3%, information missing for 19) (Supplementary Table S1). The study was approved by the local Ethics Committee of the University of Lübeck, Germany, and written informed consent was obtained from all participants before the genetic tests.

Genetic Analysis

We performed a next-generation sequencing-based gene panel analysis including all coding exons of *TOR1A* (NM_000113), *GNAL* (NM_182978), *THAP1* (NM_018105), *KMT2B* (NM_014727), *PRKRA* (NM_003690), *GCH1* (NM_000161), and *SGCE* (NM_003919). Sequencing was carried out between 2016 and 2021 in a total of eight batches containing 51 to 780 samples each at Centogene (Rostock, Germany). Genomic DNA was enzymatically fragmented, and regions of interest were enriched using DNA capture probes (Twist Biosciences, San Francisco, CA; custom design). The final indexed libraries were sequenced on an Illumina (San Diego, CA) platform (NextSeq), with a sequencing quality parameter of 99.5% coverage of the targeted regions and a minimum read depth of 100×. Bioinformatic pipeline for mapping, variant calling, and annotation has been described elsewhere.[15]

ANO3 variants were previously tested in a subset of patients (n = 729) using the same panel, and results were reported elsewhere.[16] For a few patients (n = 7), a genetic diagnosis was already previously found by gene-specific Sanger sequencing (see Table 1). However, these patients were still included in this gene panel study, also to rule out a second genetic cause. Notably, there was no enrichment for patients with a known genetic diagnosis in this study.

Sanger sequencing was performed for validation of rare (minor allele frequency <0.005), presumably protein-changing variants.

For assessing the pathogenicity of detected variants, two different published scoring systems were used: the one used for MDSGene (www.mdsgene.org/methods) and the standards and guidelines from the ACMG,[9] despite its known limitations.[17,18]

Episignature Analysis for *KMT2B* Variants

To assess the functional effect of *KMT2B* variants, the DYT-KMT2B-specific methylation pattern ("episignature") in peripheral blood, comprising 113 specific CpG sites, was analyzed as described,[14] using the Illumina MethylationEPIC BeadChip. The mean of the normalized methylation levels (mean(z)) and the coefficient of variation (CV = standard deviation/ |mean|) were used as quantifiers (Supplementary Methods). For normalization, we used either 17 DYT-SGCE patients or 38 unaffected individuals as controls and performed the calculations (1) using all 113 sites; and (2) using 103 sites that were left after data cleaning following best practices (Supplementary Methods).

Results

Through gene panel analysis and subsequent Sanger sequencing, 171 carriers (109 with dystonia [9.0%], 62 with PD [6.0%]) of 131 different heterozygous, rare, protein-changing variants were detected, of which the majority (n = 111) were not previously reported (not listed in MDSGene after systematic literature research) (Supplementary Table S2). After classification of these variants by using the MDSGene and ACMG scoring systems, 77 variants were considered as (likely) benign (Supplementary Table S2), 33 as (likely) pathogenic (Table 1), and 21 were left as variants of uncertain significance (VUS) (Table 2). The 33 presumably pathogenic variants were detected in 52 patients, of whom 48 had dystonia (48/1207, 4.0%) and four had PD (4/1036, 0.4%).

The additive frequency of rare, presumably pathogenic variants in all seven tested genes was significantly higher in dystonia patients than in PD patients (Fisher's exact test, $P < 0.00001$, respectively). Notably, the frequency of rare, presumably benign variants and VUS did not significantly differ between the two groups (Fisher's exact test, $P = 0.5723$), underlining the relevance of presumably pathogenic variants only. Excluding variants in *GCH1*, which have a known role also in

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TABLE 1 *Overview of carriers of (likely) pathogenic variants in* TOR1A, THAP1, GNAL, KMT2B, GCH1, *and* SGCE

	Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	gnomAD exomes frequency v2.1.1	Novel	Pathoscoring		Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
								ACMG	MDSGene							
Dystonia patients	*TOR1A*	L-3837	c.907_909del	p.Glu303del	22.3	0.000115	No	P	DP	DEU	43	12	m	Negative	Generalized	n.a.
		L-4004								DEU	40	7	m	Negative	Generalized	Neck, limbs, trunk
		L-4591[36]								DEU	35	13	f	Positive	Focal	Hand
		L-7404								DEU	23	10	f	Negative	Generalized	n.a.
		L-11062								KOR	25	9	m	Negative	Generalized	n.a.
		L-11514								DEU	57	25	m	Negative	Generalized	n.a.
		L-11542								DEU	53	11	m	Negative	Generalized	n.a.
		L-11584								ESP	22	10	m	Positive	Focal	Right leg
		L-11627								ESP	24	12	m	Negative	Generalized	n.a.
		L-13343								DEU	45	4	m	Positive	Generalized	n.a.
		L-4286[19]	c.40_45del	p.Ala14_Pro15del	22.3	n.a.	No	LP	PrP	DEU	80	30	f	Positive	Focal	Neck
	THAP1	L-8923	c.16T>C	p.Ser6Pro	29.7	n.a.	No	LP	PrP	DEU	55	18	m	Negative	Segmental	Neck, oromandibular
		L-11557	c.292G>T	p.Glu98*	36.0	n.a.	Yes	P	PrP	ESP	77	10	m	Positive	Generalized	n.a.
		L-11640								ESP	51	15	f	Negative	Segmental	Oromandibular
		L-2257[37]	c.474del	p.Lys158Asnfs*23	26.5	n.a.	No	P	PrP	DEU	78	8	m	Negative	Generalized	n.a.
		L-11577	c.61T>G	p.Lys24Glu	29.8	n.a.	Yes	LP	PoP	ESP	26	16	m	Negative	Generalized	n.a.
		L-13633	c.62C>T	p.Ser21Phe	32.0	n.a.	No	LP	PrP	DEU	29	7	f	Negative	Generalized	n.a.
		L-14814								DEU	37	4	f	Positive	Generalized	n.a.
		L-4155[12]	c.68A>C	p.His23Pro	32.0	n.a.	No	P	PrP	DEU	43	9	m	Positive	Focal	Hand
		L-3841[12]	c.70A>G	p.Lys24Glu	31.0	n.a.	No	LP	PrP	DEU	42	14	f	n.a.	Multifocal	Neck, hand, foot
		L-11501	c.71+2T>C		33.0	n.a.	Yes	LP	PrP	DEU	55	1	f	Negative	Generalized	n.a.
		L-7807								DEU	56	30	f	Negative	Generalized	Orofacial, neck, limbs
	GNAL	L-13315	c.1060_1065del	p.Phe354_Leu355del	21.1	n.a.	Yes	LP	PrP	DEU	62	46	f	Negative	Segmental	Face, shoulder, hand, neck

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TABLE 1 *Continued*

Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	gnomAD exomes frequency v2.1.1	Novel	Pathoscoring		Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
							ACMG	MDSGene							
	L-12521	c.1264dup	p.Tyr422Leufs*3	34.0	n.a.	Yes	LP	PrP	DEU	52	16	m	n.a.	Focal	Neck
	L-4486	c.868G>A	p.Gly290Ser	33.0	n.a.	Yes	LP	PoP	DEU	64	37	m	Negative	Focal	Neck
	L-11929	c.1115T>G	p.Ile372Ser	29.6	n.a.	Yes	LP	PrP	DEU	63	40	f	Positive	Generalized	Neck, limbs
	L-7606								DEU	50	32	m	Negative	Segmental	Neck, oromandibular
KMT2B	L-8941[20]	c.3568_3577del	p.Leu1190Serfs*162	42.0	n.a.	No	P	DP	AFG	33	7	f	Negative	Generalized	Lower limbs, trunk, neck
	L-13774	c.3400C>T	p.Gln1134*	33.0	n.a.	Yes	P	PrP	DEU	39	27	f	Negative	Multifocal	n.a.
GCH1	L-3773	c.181G>T	p.Glu61*	38.0	n.a.	No	P	DP	DEU	68	7	f	Positive	Generalized	n.a.
	L-12163	c.229 T>C	p.Ser77Pro	25.0	n.a.	Yes	LP	PoP	DEU	34	15	f	Negative	Generalized	Neck, feet, trunk
	L-858[38]	c.262C>G	p.Arg88Gly	32.0	0.000004	No	LP	PrP	DEU	40	16	f	Positive	Generalized	n.a.
	L-12641	c.283C>T	p.Pro95Ser	31.0	n.a.	No	LP	PrP	DEU	64	2	m	Negative	Multifocal	Neck, lower limbs
	L-11635	c.323G>T	p.Gly108Val	29.2	n.a.	Yes	LP	PoP	ESP	73	18	f	Negative	Generalized	Especially cervical region
	L-14616	c.4G>A[a]	p.Glu2Lys	23.7	n.a.	Yes	LP	PrP	DEU	55	38	m	Negative	Segmental	Face, hand, neck
	L-11143	c.638_641del[b]	p.Lys213fs	14.7	0.000016	Yes	LP	PoP	KOR	17	14	f	Negative	Segmental	n.a.
	L-11944	c.680C>T	p.Thr227Ile	28.7	n.a.	Yes	LP	PrP	DEU	87	6	f	Positive	Generalized	Limbs, neck, trunk
	L-8340								DEU	82	6	m	Positive	Multifocal	Right hand, foot
	L-5967	c.745A>G	p.Arg249Gly	22.7	n.a.	Yes	LP	PoP	DEU	49	38	m	Negative	Segmental	Right hand, arm
	L-14447	c.671A>G[a]	p.Lys224Arg	21.3	0.000386	No	VUS	PoP	DEU	25	8	m	Negative	Generalized	Face, neck, trunk, limbs
SGCE	L-11895	c.109+1G>T		35.0	n.a.	No	LP	PrP	DEU	74	34	f	Negative	Myoclonus-dystonia	n.a.
	L-6808	c.1291_1297dup	p.Gly433fs	33.0	n.a.	Yes	P	PrP	DEU	39	15	m	n.a.	Myoclonus-dystonia	Limbs
	L-2354[39]	c.289C>T	p.Arg97*	36.0	0.0000089	No	P	PrP	DEU	76	2	m	Negative	Myoclonus-dystonia	n.a.

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TABLE 1 *Continued*

	Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	gnomAD exomes frequency v2.1.1	Novel	Pathoscoring		Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
								ACMG	MDSGene							
		L-4151	c.304C>T	p.Arg102*	38.0	n.a.	No	P	PrP	DEU	81	5	m	Positive	Myoclonus-dystonia	n.a.
		L-4168								DEU	69	0.1	f	Positive	Myoclonus-dystonia	n.a.
		L-8162								DEU	51	3	m	Negative	Myoclonus-dystonia	Neck, hand, upper limbs
		L-6589	c.418G>T	p.Glu140*	38.0	n.a.	Yes	LP	PrP	DEU	36	15	m	Negative	Myoclonus-dystonia	Neck, trunk, limbs
		L-12173	c.771_772del	p.Cys258fs	32.0	n.a.	No	P	PrP	DEU	61	4	f	Positive	Myoclonus-dystonia	n.a.
PD patients	*GCH1*	L-11358	c.4G>A[a]	p.Glu2Lys	23.7	n.a.	Yes	LP	PrP	DEU	43	36	m	n.a.	No dystonia	
		L-9896	c.586G>T	p.Ala196Ser	23.4	0.0000398	Yes	LP	PoP	DEU	69	48	m	Negative	No dystonia	
		L-11656	c.671A>G[a]	p.Lys224Arg	21.3	0.000386	No	LP	PoP	DEU	75	70	f	n.a.	No dystonia	
		L-5970								DEU	68	60	m	Negative	No dystonia	

Note: Novel variant means a variant that has not previously been reported in a dystonia patient. For the country of origin, the standard ISO Country code is listed.

Abbreviations: ID, identification; cDNA, complementary DNA; CADD, Combined Annotation Dependent Depletion; gnomAD, Genome Aggregation Database; ACMG, American College of Medical Genetics and Genomics; MDSGene, Movement Disorder Society Genetic mutation database; m, male; f, female; n.a., not available; P, pathogenic; DP, definitely pathogenic; LP, likely pathogenic; PoP, possibly pathogenic; PrP, probably pathogenic; VUS, variant of uncertain significance; PD, Parkinson's disease.

[a]Variant found in both PD and dystonia patients.

[b]The nomenclature of this variant is based on NM_001024070.

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TABLE 2 *Overview of carriers of variants of uncertain significance in* TOR1A, THAP1, GNAL, KMT2B, GCH1 *and* SGCE

	Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	gnomAD exomes frequency v2.1.1	Novel	Pathoscoring ACMG	Pathoscoring MDSGene	Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
Dystonia patients	*TOR1A*	L-7938	c.331G>C	p.Val111Leu	22.0	0.000095	Yes	VUS	PoP	DEU	30	21	m	Positive	Segmental	Neck
		L-3584	c.719T>C	p.Leu240Ser	27.1	0.000028	Yes	VUS	PoP	DEU	84	44	f	Negative	Focal	Neck
	GNAL	L-12036	c.74C>A	p.Pro25Gln	14.5	n.a.	Yes	VUS	B	DEU	56	50	m	Negative	Multifocal	Shoulders
		L-8257	c.313A>C	p.Ile105Leu	25.7	n.a.	Yes	VUS	PoP	DEU	53	23	f	Negative	Multifocal	Face, shoulder, neck, limbs
		L-3811	c.580T>G	p.Tyr194Asp	29.5	n.a.	Yes	VUS	PoP	DEU	49	38	f	Negative	Focal	Blepharospasm
	KMT2B	L-12226	c.1550G>A	p.Ser517Asn	22.2	0.000004	Yes	VUS	PoP	DEU	36	20	m	n.a.	Segmental	n.a.
		L-12253	c.4573G>A	p.Gly1525Arg	34.0	0.000065	Yes	VUS	PoP	DEU	47	7	f	n.a.	Segmental	n.a.
		L-12626	c.6475C>G	p.Pro2159Ala	12.5	0.000010	Yes	VUS	B	DEU	58	15	f	n.a.	Focal	Cranial
		L-12035	c.5108T>C	p.Leu1703Pro	24.6	n.a.	Yes	VUS	PoP	DEU	69	63	f	Negative	Focal	Neck
	GCH1	L-11878	c.509 + 3A>G[a]		13.2	0.0000955	Yes	VUS	PoP	DEU	54	38	f	Positive	Segmental	n.a.
	SGCE	L-11614	c.936C>A	p.Asp312Glu	23.3	n.a.	Yes	VUS	PoP	DEU	57	18	m	n.a.	Generalized	n.a.
		L-12406	c.158C>T	p.Ser53Leu	23.4	0.000004	No	VUS	PoP	DEU	60	10	m	Negative	focal	Neck
		L-13044	c.277G>T	p.Gly93Cys	32.0	0.000012	Yes	VUS	PoP	DEU	81	5	m	Positive	Myoclonus-dystonia	n.a.
		L-3624	c.391>G[a]	p.Ile131Val	18.0	0.000182	Yes	VUS	PrP	DEU	20	3	f	n.a.	Generalized	Trunk, neck, limbs, tongue
		L-14626								DEU	41	33	m	n.a.	Generalized	n.a.
PD patients	*TOR1A*	L-11950	c.741C>A	p.Asn247Lys	21.9	n.a.	Yes	VUS	PoP	DEU	59	55	f	n.a.	No dystonia	
	KMT2B	L-11741	c.2822C>G	p.Ser941Cys	23.4	n.a.	Yes	VUS	PoP	DEU	75	65	m	n.a.	No dystonia	
		L-11843	c.2843C>T	p.Thr948Ile	19.8	n.a.	Yes	VUS	PoP	DEU	71	59	f	n.a.	No dystonia	
	GCH1	L-5282	c.202C>T	p.Leu68Phe	26.8	n.a.	Yes	VUS	PoP	DEU	68	59	f	Negative	No dystonia	
		L-7781	c.509+3A>G[a]		13.2	0.0000955	Yes	VUS	PoP	DEU	82	n.a.	m	n.a.	No dystonia	
	SGCE	L-5503	c.1235C>T	p.Pro412Leu	21.4	n.a.	Yes	VUS	PrP	DEU	70	56	f	n.a.	No dystonia	
		L-11375								DEU	74	43	m	Positive	No dystonia	

(Continues)

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PD, the frequency of presumably pathogenic variants was also significantly higher in dystonia patients (Fisher's exact test, $P < 0.00001$, respectively).

Among the dystonia patients, 48 carriers of presumably disease-causing variants were identified in the heterozygous state in *TOR1A* (n = 11, 0.9%), *GCH1* (n = 11, 0.9%), *THAP1* (n = 11, 0.9%), *SGCE* (n = 8, 0.7%), *GNAL* (n = 5, 0.4%), and *KMT2B* (n = 2, 0.2%) (Fig. 1). Of note, no carriers of biallelic pathogenic *PRKRA* variants were found. The dystonia patients with (likely) pathogenic variants had a median AAO of 12 years (IQR, 7–17) and included 26 males (54.2%) and 22 females (45.8%). Compared to the overall sex distribution in the dystonia sample (564/1207 males = 46.7%, 643/1207 females = 53.3%), there was no significant predominance of one sex in the group of carriers of presumably disease-causing variants (Fisher's exact test, $P = 0.3049$). The small number of patients with (likely) disease-causing variants per gene did not allow us to search for statistical differences in sex distribution for each

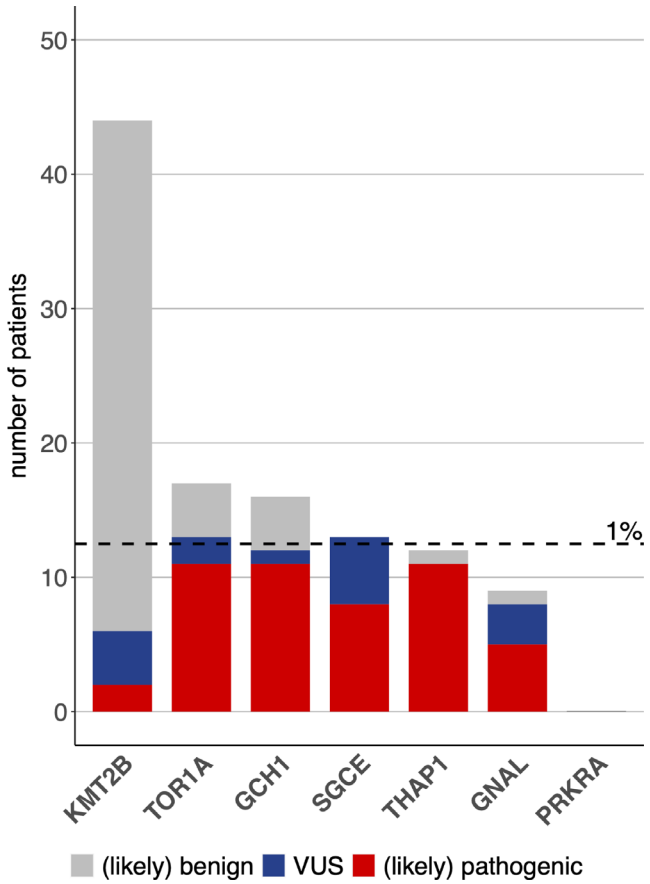

FIG. 1. Prevalence of rare variants in our dystonia sample (n = 1207) according to the predicted pathogenicity. The absolute number of identified carriers of rare (minor allele frequency [MAF] <0.005) variants in *TOR1A*, *SGCE*, *GCH1*, *THAP1*, *GNAL*, *KMT2B*, and *PRKRA* is displayed. Of note, for the four VUSs in *KMT2B*, testing of the episignature was not possible because of lack of DNA. VUS, variant of uncertain significance.

TABLE 2 *Continued*

Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	gnomAD exomes frequency v2.1.1	Novel	Pathoscoring ACMG	Pathoscoring MDSGene	Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
	L-10938	c.1138A>T	p.Ile380Leu	24.2	n.a.	Yes	VUS	PoP	DEU	56	54	f	n.a.	No dystonia	
	L-6057	c.391A>G[a]	p.Ile131Val	18.0	0.000182	Yes	VUS	PrP	DEU	60	60	m	Negative	No dystonia	
	L-8365								DEU	61	n.a.	m	n.a.	No dystonia	
	L-11924	c.502A>C	p.Asn168His	24.9	n.a.	Yes	VUS	PoP	DEU	41	2	m	Positive	No dystonia	

Note: Novel variant means a variant that has not previously been reported in a dystonia patient. For the country of origin, the standard ISO Country code is listed.
Abbreviations: ID, identification; cDNA, complementary DNA; CADD, Combined Annotation Dependent Depletion; gnomAD, Genome Aggregation Database; ACMG, American College of Medical Genetics and Genomics; MDSGene, Movement Disorder Society Genetic mutation database; m, male; f, female; VUS, variant of uncertain significance; PoP, possibly pathogenic; n.a., not available; PrP, probably pathogenic; PD, Parkinson's disease.
[a]Variant found in both PD and dystonia patients.

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genetic subtype. Family history was positive in 16/48 cases (33.3%, four unknown). A total of 23 patients presented with generalized (23/48, 47.9%), 11 with multifocal or segmental (22.9%), six with focal dystonia (12.5%), eight with myoclonus-dystonia (16.7%, all *SGCE*-linked), and for one patient information was missing.

Specifically, 10 patients originating from South Korea, Germany, and Spain that mainly presented with early-onset generalized dystonia carried the known dystonia-causing GAG deletion in *TOR1A*. Additionally, one previously reported patient with adult-onset cervical dystonia carried a 6-bp deletion (c.40_45delGCGCCG, p.Ala14_Pro15del) in *TOR1A*.[19] Pathogenic or likely pathogenic variants in *THAP1* were detected in 11 patients, including three recurrent variants (c.292G>T:p.Glu98*, c.62C>T:p.Ser21Phe, and c.71+2 T>C) that were found in two unrelated patients each and are absent in gnomAD. Seven of 11 DYT-THAP1 patients had generalized dystonia, and 10 had an AAO below 18 years. For *GNAL*, five German patients with four different, not previously described, likely pathogenic variants were identified that all had adolescence to adulthood disease onset and presented with cervical dystonia (focal in 2/5 patients). One *GNAL* variant occurred recurrently (c.1115 T>G, p.Ile372Ser) in our dystonia patients, but is absent from gnomAD controls. After pathogenicity scoring and functional evaluation (episignature) of rare *KMT2B* variants, two variants were classified as pathogenic (see below). For the combined dystonia-parkinsonism gene *GCH1*, 11 dystonia patients were found to carry (likely) pathogenic variants. Ten different, mainly missense variants were identified, of which one, not previously published variant, occurred in two German siblings with dopa-responsive dystonia (c.680C>T, p.Thr227Ile). The identified DYT-GCH1 patients presented with generalized (6/11) or segmental/multifocal (5/11) dystonia that started in childhood in most cases (9/11, information missing for one). In eight patients, the affected body sites at last examination included the neck. Six different variants were detected in *SGCE*, including a truncating variant (c.304C>T, p.Arg102*) in three independent German patients that is absent from control databases. All eight DYT-SGCE patients presented with myoclonus-dystonia.

The median AAO and percentage of patients with generalized dystonia for each genetic subtype are displayed in Figure 2. In both our data and the MDSGene database, the latest median AAO was observed for DYT-GNAL patients (37.0 and 38.0 years, respectively), and DYT-SGCE patients had the earliest disease manifestations (4.5 and 4.0 years, respectively). In MDSGene, at least half of the patients with *TOR1A*, *THAP1*, *KMT2B*, and *GCH1* variants developed generalized dystonia, whereas in our data, percentages were even higher (for *TOR1A*, *THAP1*, and *GCH1*). Of note, comparison of *KMT2B* data is complicated by the extremely small sample size (n = 2). In both data sets, *GNAL* mutation carriers rarely showed generalization (0% and 9.6%, respectively). Altogether, 16 of the here identified, presumably dystonia-causing variants have not previously been reported.

Among the PD patients, four different variants were classified as likely pathogenic, all in *GCH1* (Table 1). The four PD patients had no sign of dystonia (current median age: 68.5 years, IQR, 61.75–70.5). Notably, two variants (c.4G>A:p.Glu2Lys and c.671A>G:p.Lys224Arg) were detected in both dystonia and PD patients in our sample.

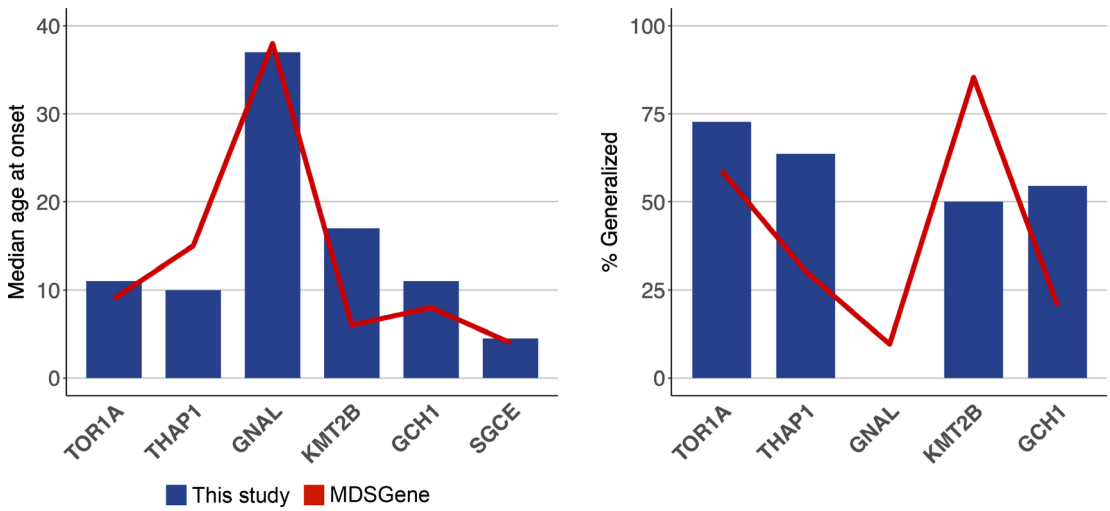

FIG. 2. Comparison of median age at onset (left) and percentage of patients with generalized dystonia (right) between the MDSGene data and our study. Of note, information on the body distribution of dystonia is usually not available for DYT-SGCE patients (only on the presence of myoclonus and dystonia), therefore, *SGCE* is not displayed in the right panel. MDSGene, Movement Disorder Society Genetic mutation database. [Color figure can be viewed at wileyonlinelibrary.com]

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Episignature Analysis for *KMT2B* Variants

To assess the functional effect of rare *KMT2B* variants, the DYT-KMT2B-specific methylation pattern (episignature) in patients' blood was analyzed. Two of the 48 tested variants (c.3400C>T, p.Gln1134* and c.3568_3577delCTGAGTGTGC, p.Leu1190Serfs*162) were shown to result in strong hypermethylation and showed mean(z) and CV values characteristic of loss of KMT2B function (Fig. 3, Supplementary Table S3), which was interpreted as positive functional evidence during pathogenicity scoring. The p.Gln1134* variant (mean(z) = 4.18, CV = 2.06) was found in a 39-year-old German patient with a developmental disorder and dysmorphic features who developed multifocal dystonia at the age of 27 years. Family history was negative, but no family members were available to test if the variant arose de novo. The p.Leu1190Serfs*162 (mean(z) = 3.77, CV = 1.93) variant was found in a previously reported patient[20] with generalized dystonia and mild intellectual disability and occurred de novo. All missense and in-frame indel variants showed values indicative of benign variants. Repeating the calculation with (1) all 113 sites of the published episignature[14] or (2) unaffected individuals as controls instead of DYT-SGCE patients yielded comparable results, rating only the two abovementioned truncating variants as pathogenic (Supplementary Table S3). In total, 26 of 48 tested *KMT2B* variants were reclassified through episignature analysis (mostly from VUS to likely benign). An overview of the results of testing of the episignature has also been added to the MDSGene website at https://www.mdsgene.org.

Discussion

Here, we report on the role and frequency of variants in the isolated and combined dystonia genes *TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *PRKRA*, *SGCE*, and *GCH1* in a large dystonia sample (n = 1207) as well as in a disease control group with PD (n = 1036). In total, 33 different presumably pathogenic variants were identified, one of which was only found in the disease control group (in *GCH1*) and two found in both PD and dystonia patients (in *GCH1*). Additionally, we report 21 rare VUS and 77 variants that were considered (likely) benign after careful evaluation. Among the dystonia patients, pathogenic variants in *TOR1A*, *THAP1*, and *GCH1* were most frequent (0.9% each), followed by *SGCE* (0.7%), *GNAL* (0.4%), and *KMT2B* (0.2%). We did not identify any carrier of a biallelic variant in the dystonia gene *PRKRA*, confirming that this genetic subtype is extremely rare,[5] especially outside of Brazil, where most reported patients originate from and where the prevalence was estimated to be ~5% in isolated dystonia patients.[21]

The frequency of rare, presumably pathogenic variants was significantly higher in dystonia patients compared to PD patients, confirming the overall enrichment of variants in the investigated genes among dystonia patients and underlining their role as dystonia genes. A potential molecular diagnosis was established in 48/1207 (4.0%) dystonia patients. These patients had a median AAO of 12 years (IQR, 7–17), and 48% presented with generalized dystonia. Compared to the median AAO and body distribution in all dystonia patients (36 years (IQR, 21–49), 10% generalized dystonia), this confirms the observation that it is more likely to identify a monogenic cause in patients with an earlier AAO and generalized body distribution of symptoms.[22] An exome sequencing study[2] of smaller size (n = 1100, including 764 dystonia patients) identified diagnostic variants in 19% of included dystonia patients. However, most of these patients had additional neurological symptoms. Among isolated dystonia patients, the diagnostic yield was only 3.9%, comparable to the one in this study that mainly included

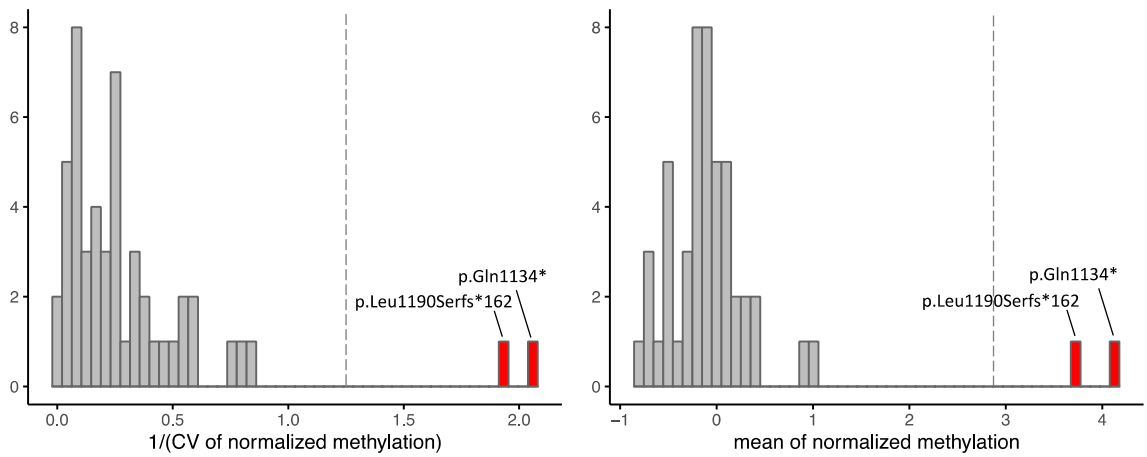

FIG. 3. Histogram of individual mean and coefficient of variation (CV) of the episignature's normalized methylation levels. The dashed vertical lines represent the maximum observed values in 162 non-*KMT2B* samples as described in Mirza-Schreiber et al.[14] Only the two truncating variants show values indicative of a loss of KMT2B function. [Color figure can be viewed at wileyonlinelibrary.com]

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isolated dystonia patients, underscoring that the diagnostic yield largely depends on the patient selection criteria. This is also reflected by the variable outcomes of other, smaller next-generation sequencing studies in dystonia (including 16–189 cases) with overall diagnostic yields of 11.7% to 37.5%.[23]

We identified 11 dystonia patients (0.9%) carrying two different variants in *TOR1A* that were classified as (likely) pathogenic (p.Glu303del, p.Ala14_Pro15del). Although the pathogenic nature of the p.Ala14_Pro15del variant is supported by only one functional study,[19] numerous studies have proven the pathogenicity of the recurrent GAG deletion, mainly characterized by mislocalization of the mutant torsinA from the endoplasmic reticulum to the nuclear envelope and altered nuclear envelope morphology.[24] DYT-TOR1A is the most prevalent monogenic form of isolated dystonia, and, to date, at least 680 dystonia patients (~98% of reported DYT-TOR1A patients) have been described to carry the p.Glu303del variant.[5] The majority had childhood disease onset (~70%) and developed generalized dystonia (~60%). In this study, 10 carriers were identified that mainly (8/10) presented with early-onset generalized dystonia (median AAO, 10.5 years; IQR, 9.25–12), in keeping with previous observations. Nevertheless, the phenotypic spectrum of reported variant carriers is broad, which is also reflected in our study, as two patients only developed focal dystonia affecting one leg or hand, respectively.

We identified 11 dystonia patients (0.9%) carrying eight presumably pathogenic variants in *THAP1*, three of which have not been reported before, including a nonsense mutation (p.Glu98*) that occurred in two unrelated Spanish dystonia patients, but is absent from control databases. Another nonsense mutation (p.Glu97*) was previously described in seven unrelated dystonia patients,[25,26] providing good evidence for the pathogenicity of this variant. The p.Ser21Phe variant was reported in two unrelated dystonia patients[25,27] and was also identified in two of our patients with generalized dystonia. Additionally, we found a novel missense variant at the same amino acid position (p.Ser21Ala) in one patient with generalized dystonia, suggesting that this variant also has a pathological role. Additionally, we report a novel splice site variant (c.71+2T>C), predicted to lead to a splice donor site loss by spliceAI (https://ci-spliceai.com/), that was detected in two independent patients with generalized dystonia. For two of five previously described variants, positive functional evidence was reported (p.His23Pro and p.Lys24Glu)[12] and was taken into account in the pathogenicity scoring. Mutations in *THAP1* are a cause of childhood- or adolescent-onset dystonia with a mixed phenotype,[5] which is reflected in our DYT-THAP1 patients that have a median AAO of 10 years (IQR, 7.5–15.5) and show focal, segmental, and generalized body distributions of symptoms.

For *GNAL*, we identified five dystonia patients (0.4%) with four different variants that have not previously been described and are absent from control databases (p.Phe354_Leu355del, p.Ile372Ser, p.Tyr422Leufs*3, p.Gly290Ser). Fitting with previous observations,[5] these include missense as well as nonsense mutations, and patients mainly presented with adult-onset cervical dystonia. One variant occurred recurrently (c.1115 T>G, p.Ile371Ser) in our patients, supporting its role in the development of dystonia. Notably, one of the variant carriers (L-11929) presented with generalized dystonia in combination with chorea, which has not been reported in *GNAL*-related disease before and might expand the phenotypic spectrum. Future functional tests will reveal if the here-identified, novel variants are indeed disease-causing.

Mutations in *KMT2B* as a cause of dystonia were first described in 2016/2017.[28,29] Since then, at least 68 different, mainly truncating mutations have been described.[5] As *KMT2B* is a large gene with 37 exons, we detected many rare variants and demonstrated that functionally evaluating their effect on DNA methylation is a powerful and important tool for interpretation. *KMT2B* encodes the lysine-specific histone methyltransferase 2B, and therefore, links disordered chromatin states to the disease mechanism of dystonia. Because histone methylation is inversely correlated to CpG-methylation, DNA methylation analysis can be used to evaluate the effect of sequence variants. More specifically, loss of KMT2B function was found to result in hypermethylation at 113 specific CpG sites (episignature).[14] After episignature analysis, the majority of rare variants were reclassified as (likely) benign. Only the two truncating variants (p.Gln1134* and p.Leu1190Serfs*162) were shown to result in strong hypermethylation and showed mean(*z*) and CV values characteristic of loss of KMT2B function. Therefore, the frequency of pathogenic *KMT2B* variants in our study (2/1207, 0.2%) is much lower than in a previous study (12/764, 1.6%).[2] This might be because of the fact that the here-investigated patients mainly had isolated, focal dystonia and that DYT-KMT2B is mostly generalized and often accompanied by additional features.[5] In line with this, the two carriers of pathogenic *KMT2B* variants in this study presented with dystonia and a developmental disorder. However, it is also possible that the number of true pathogenic mutations would have been lower in previous studies if functional analysis had been performed. We propose that missense variants in particular should be functionally evaluated to allow correct variant interpretation.

Missense and truncating variants in *GCH1* are frequent causes of dopa-responsive dystonia. Although ~70% of patients with pathogenic *GCH1* variants present with isolated dystonia, only ~10% of carriers have a pure parkinsonism phenotype,[8] in accordance with

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the distribution in our study (11/15 isolated dystonia, 4/15 pure PD). The 11 dystonia patients with a presumably pathogenic *GCH1* variant carried two truncating and eight missense variants. Their median AAO of 11 years is slightly above the reported 8 years, and the prevalent occurrence of generalized or multifocal dystonia fits previous observations.[8] Six of the identified, likely pathogenic variants were not previously reported, including a novel missense variant p.Thr227Ile found in two German siblings with childhood-onset dopa-responsive dystonia. Additionally, four PD patients were found to carry novel likely pathogenic *GCH1* variants, of which two (p.Glu2Lys and p.Lys224Arg) were also detected in a dystonia patient, suggesting that these variants may manifest as either PD or dystonia.

Variants in *SGCE* are mainly linked to childhood-onset dystonia in combination with myoclonus. In line with this, all eight of the here-identified carriers of (likely) pathogenic *SGCE* variants presented with myoclonus-dystonia, and the median AAO was 4.5 years. As reported in the literature, the majority of the here detected variants (5/6) are predicted to have a truncating effect on the protein. This includes the p.Arg102* variant that was found in three independent German patients and is absent from control databases, supporting its role in the pathogenesis of myoclonus-dystonia.

Last, a total of 21 variants were classified as VUS (Table 2) as they are not supported by enough evidence to consider them disease-causing. However, the identification of additional patients or functional testing may reclassify these variants and clarify their role in the development of dystonia.

One limitation of our study design using gene panel sequencing is that it captures only a limited number of genes and cannot be easily adjusted for novel discoveries. Presumably, our study's diagnostic yield would have been higher if newly discovered dystonia genes (eg, *VPS16*) had been included. Notably, a subset of the patients (n = 114) included here were screened for *VPS16* variants by Sanger sequencing, and indeed, one carrier of a clearly pathogenic, truncating variant was found.[30] Other, more recently reported genes for isolated dystonia, such as *EIF2AK2*,[31] *AOPEP*,[32] and *EIF4A2*,[33] or combined dystonia, such as *KCTD17* among many others,[34,35] have not yet been targeted. The best way to screen dystonia patients comprehensively is to perform exome or even genome sequencing. Notably, these methods are much more expensive and are currently underway for a subset of the dystonia patients from DysTract.

For a rare disorder like dystonia, large screening efforts are inevitable to gain a deeper understanding of the underlying genetic architecture as well as the genotype–phenotype relationships; however, this has been rarely done, as sample sizes for this rare disorder are usually very small. We here screened >1200 dystonia patients using targeted gene capture sequencing of seven dystonia genes that enabled us to establish a presumptive molecular diagnosis in 4.0% of patients, most of whom had early-onset generalized dystonia, emphasizing that this patient group should be prioritized for genetic testing. Other patients in the DysTract and Dystonia Coalition sample were shown to be carriers of pathogenic variants in *ANO3* or *VPS16* by other means.[16,30] We were able to confirm previously described pathogenic variants by providing additional patients and also discover novel, presumably dystonia-causing variants in the dystonia genes *TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *GCH1*, and *SGCE*, therefore, expanding their mutational spectrum. In addition, application of the episignature analysis for *KMT2B* variants demonstrated the importance of functional studies for the interpretation of sequence variants and provides meaningful interpretation for almost 50 such variants. Therefore, this large-scale dataset and the functional evaluation of *KMT2B* variants will guide future variant interpretation.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Data

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