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Effort to differentiate essential tremor plus and dystonic tremor using whole exome sequencing: an exploratory study

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Background: The clinical differentiation between essential tremor plus (ETP) and dystonic tremor (DT) is challenging. This study aimed at the genetic diagnosis of ETP and DT.

Methods: Whole exome sequencing was performed on 50 probands (ETP = 25; DT = 25) and analysed to identify variants in known genes linked with dystonia and essential tremor plus phenotypes.

Results: We identified pathogenic/likely pathogenic variants [*THAP1* (n = 1) and *ANO3* (n = 1)] in two patients with DT. In addition, one DT patient had a variant of uncertain significance in *FUS* and four patients had benign variants [*CIZ1* (n = 1), *COL6A3* (n = 1), *GCH1* (n = 1), *TENM4* (n = 1)]. One patient with ETP was detected to have a variant of uncertain significance in *TENM4* and five patients with ETP had benign variants [*COL6A3* (n = 2), *VPS16* (n = 1), *TAF1* (n = 1), *KMT2B* (n = 1)].

Conclusion: Genetic studies may be in an important biomarker in differentiating patients with ET plus from DT which is challenging in a clinical setting.

KEYWORDS

dystonic tremor, essential tremor plus, whole-exome sequencing, genotype, phenotype

Introduction

Tremor is the most common movement disorder and is defined as an involuntary, rhythmic, oscillatory movement of a body part [1]. In the recent classification, tremor syndromes have been classified in two axes [1]. A new terminology essential tremor plus (ET plus) was added for patients with features of essential tremor (ET) and additional neurological signs of uncertain significance such as questionable dystonia, questionable ataxia, and mild cognitive impairment. Dystonic tremor syndromes are tremor syndromes combining tremor

and dystonia as the leading neurological signs. Considering a lack of diagnostic markers regarding the questionable neurological signs, differentiation of ET plus and DT is challenging and adds to diagnostic confusion [2, 3]. There are many genes associated with the dystonic tremor phenotype and tremor may be their only motor manifestation at the onset [4]. Further, patients with ET plus may develop hard signs and may fall into the category of combined tremor syndrome like a dystonic tremor [5–7]. The search for causal genes for ET is still ongoing [8, 9]. Exome studies have reported the association of several genes [*FUS*, *TENM4*, *HTRA2*, *SCN11A*, *NOTCH2NLC*, and *CACNA1*] with ET [5, 8]. But they have been reported in single families only and not in other populations, suggesting that they may be private polymorphisms [5].

Given the potential for a high diagnostic yield from whole exome sequencing, we used this method to screen individuals with ET plus and DT to determine whether there is a genetic overlap in patients with ET plus and DT. To our knowledge, this is the first study to use whole exome sequencing to investigate genetic causes of ET plus.

Materials and methods

Recruitment of patient samples

Patients with ET plus and DT were recruited at GIPMER, New Delhi, (a tertiary care teaching institute) after obtaining approval from the institutional ethics committee (IEC). Written informed consent was obtained from all participating individuals as per the IEC guidelines. A total of 50 consecutive patients (ET plus: 25; DT: 25) were evaluated and their detailed history and clinical information were recorded with the help of a pre-designed form. All patients were examined by two neurologists (SP, CSR). Diagnosis of ET plus and DT was made based on the recent consensus classification. We enrolled patients with ETP having dystonia as a soft sign. A tremor in a body part affected by dystonia was labelled as dystonic tremor (DT). Dystonia was labelled as questionable if there was discordance between the two examiners (S.P., CSR) regarding its presence. If dystonia and tremor were found in different body parts, this was called tremor associated with dystonia (TAWD). For age at disease onset and disease duration, mean ± SD was calculated for each group (ET plus and DT). The severity of tremor was assessed using the Tremor Research Group Essential Tremor Rating Scale (TETRAS). About 5 mL of peripheral blood was collected in EDTA vacutainers from all the subjects recruited in the study.

Genetic analysis

Genomic DNA (gDNA) was isolated from the blood sample using the routine phenol-chloroform method at the Genetics lab (BKT) after obtaining IEC clearance and used for whole exome sequencing.

Whole exome sequencing

Exome library preparations of gDNA were made using SureSelect Human All Exon V5+UTR kit (Agilent Technologies, California, United States); and paired-end sequencing was performed on a NovaSeq 6000 at a commercial facility (MedGenome Labs, Bengaluru, India). Raw data with Phred quality score >Q30 were analysed using bioinformatic protocols previously described [10]. Using a combined variant calling file (VCF) generated for all the samples, both single nucleotide variations and insertions/deletions were called and annotated using KGGSeq [11].

Data analysis

The analysis focussed on previously reported dystonia (DYT) genes (n = 20) and hereditary essential tremor (ETM) genes (n = 3) based on their presence in the OMIM database (listed in Supplementary Table S1). The other ET genes were not included as either there was no variant with CADD score >20 identified in HS1BP3 gene (ETM2) or were only loci with no specific causal gene (ETM3) or no single nucleotide variants were reported in ETM6. For variant prioritization, only novel and rare variants with global minor allele frequency (MAF ≤ 0.01) present in public databases including 1000G, dbSNP v141, NHLBI GO ESP, ExAC, DiscovEHR, and gnomAD browser were retained. Furthermore, among them, only protein disturbing variants with CADD score>20 (denotes the top 1% most deleterious substitutions in the human genome) were taken forward.

Data validation

All the novel variants identified in the prioritized dataset were confirmed by PCR-Sanger sequencing. PCR was done using DreamTaq Polymerase (Thermo Fisher Scientific #EP0705) with primers as per the manufacturer's protocol; and later sequencing of the PCR fragments was carried out at the Central Instrumentation Facility, UDSC.

Statistical analysis

Clinical data were analyzed using the "Statistical Package for the Social Sciences (SPSS)" PC-23 version and "Fisher's exact test" was used to compare variables between ET plus and DT groups. For rare-variant burden test in known DYT and ETM genes between 50 cases (25 ET plus + 25 DT) and 100 ethnicity-matched controls with no history of dystonia/tremor, SKAT-O was performed in Efficient and Parallelizable Association Container Toolbox software as previously described [12].

TABLE 1 Demographic and clinical characteristics of patients with essential tremor plus and dystonic tremor.

	Essential tremor plus	Dystonic tremor	*p*-value
Number of patients	25	25	
Gender (M:F)	14:11	14:11	0.488
Family history	21	12	**0.007**
Mean age ± SD (range) years	48.64 ± 15.22 (13–70)	38.28 ± 16.69 (11–66)	**0.0263**
Mean duration of disease ± SD (range) years	8.52 ± 5.94 (3–20)	5.08 ± 4.66 (0.5–18)	**0.0272**
TETRAS A	14.28 ± 6.63 (4–37)	13.48 ± 5.81 (5–31)	0.6521
TETRAS P	10.78 ± 5.35 (1–25)	9.84 ± 3.61 (2–16)	0.4700
TETRAS total	25.06 ± 10.83 (9–55)	23.32 ± 8.13 (11–46.5)	0.5236

p values < 0.05 are bold.

Results

Demographic details

A total of 25 ET plus and 25 DT patients with 28 males and 22 females were recruited in the study. Patients with ET plus compared to those with DT had significantly more positive family history [84.0% (21/25) vs. 48% (12/25); $p = 0.007$], higher mean age [48.64 ± 15.22 vs. 38.28 ± 16.69; $p = 0.026$] and longer duration of disease [8.52 ± 5.94 vs. 5.08 ± 4.66; $p = 0.027$] (Table 1).

Clinical details

For 25 patients with ET plus, questionable dystonia was present in the neck (n = 14), or upper limb (n = 8). Laterocollis (n = 6), was the most common subtype of cervical dystonia present followed by torticpaut (n = 3), laterocaput (n = 2), retrocollis (n = 2), and anterocollis (n = 1). Three patients had questionable dystonia in the neck (retrocollis = 1; laterocollis = 1 and laterocollis + retocollis = 1) and upper limb.

Among 25 patients diagnosed with DT, the body distribution of dystonia was focal in 13, multifocal in 1, segmental in 8, and generalized in three patients. Focal dystonia was present in the upper limb (n = 9 including writer's cramp in 7, eating dystonia in one and non-task specific dystonia in one patient), cranial (n = 2), cervical (n = 1), and trunk (n = 1). DT was present in 19 patients, TAD in five patients, and a combination of DT and TAD was present in only one patient. Detailed clinical information of all the study subjects has been provided in Supplementary Table S2.

Genetic analysis

Whole exome sequencing of 50 samples generated an average of ~28 × 10 [6] reads per sample with an average %Q > 30 ~95.05 (Supplementary Table S2) and ~47.2 average mean coverage per sample. A total of 77,648 variants with MAF≤0.01 were called in the filtered dataset.

Novel and known variants with CADD >20 in DYT and ETM genes

We identified one heterozygous protein-coding rare variant each in two known autosomal dominant isolated dystonia genes, *THAP1* (DYT-*THAP1*) and *CIZ1* (DYT- *CIZ1*), and a novel variant c.979G>A in another autosomal dominant isolated dystonia gene, *ANO3* (DYT- *ANO3*), which was confirmed by Sanger sequencing (Figure 1). All the identified variants had a high CADD score of >25 and among them, the c.416T>G variant in *THAP1* and c.979G>A in *ANO3* were predicted to be pathogenic by pathogenicity prediction tool, MetaRNN[1] (Table 2). Of note, these two variants also had high conservation scores as evaluated by PhyloP100 which is based on multiple alignments of 99 vertebrate genome sequences to the human genome. On the other hand, the c.626G>A variant in *CIZ1* was predicted to be benign with a low conservation score despite having a CADD score of 25.4 (Table 2).

On screening for rare variants with CADD score>20 in known autosomal recessive (AR) isolated dystonia genes in the next step, we found three rare heterozygous variants in *COL6A3* (DYT-*COL6A3*) and one rare heterozygous variant in *VPS16* (DYT-*VPS16*), all predicted to be benign by MetaRNN (Table 2). Of note, all the variants identified in *COL6A3* had a very low conservation score, while variant c.136C>T in *VPS16* had a moderate score. Furthermore, we also identified one rare variant each in known combined dystonia genes, *TAF1* (DYT/PARK-*TAF1*), *GCH1* (DYT/PARK- *GCH1*); and *KMT2B* (DYT-*KMT2B*), all predicted to be benign by MetaRNN (Table 2). Of note, we identified a novel variant c.173C>A in *GCH1*, which was

1 Available at https://varsome.com/

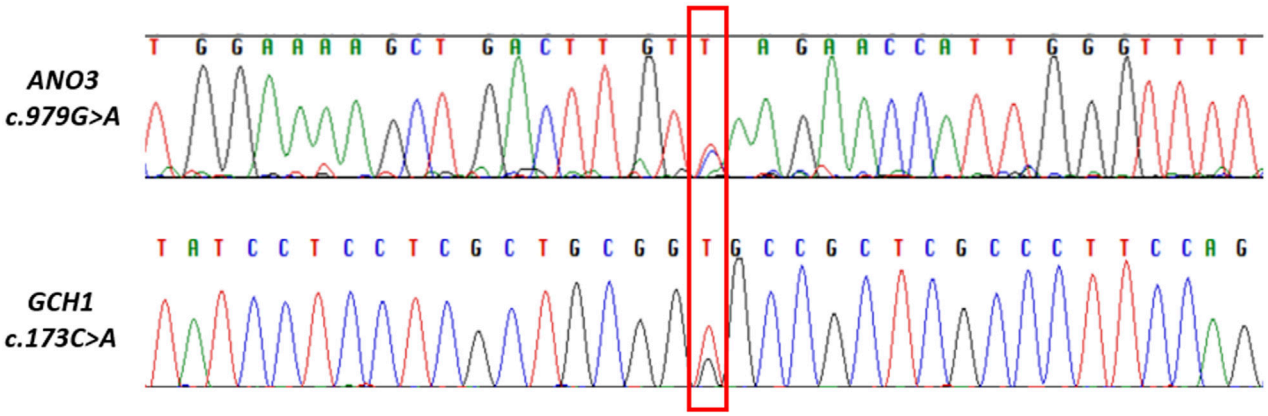

FIGURE 1
Electropherograms of Sanger sequencing showing part of *ANO3* and *GCH1*gene sequences with novel variant (marked in red box), obtained using reverse primers.

confirmed by Sanger sequencing (Figure 1) and which had a high conservation score as compared to the variants identified in *TAF1* and *KMT2B*.

We tried levodopa in this patient and there was good improvement in hand dystonia. The neck dystonia required botulinum toxin injection.

For essential tremor genes, we identified one rare variant in *FUS* (ETM4) and two rare variants in *TENM4* (ETM5) predicted to be variants of unknown significance and benign respectively by MetaRNN; but with a high conservation score (Table 2). Furthermore, SKAT-O analysis performed on rare variants in known DYT and ETM genes did not find any association (p-value≤0.05) between cases and controls possibly due to the limited sample size.

Genotype-phenotype correlations

Our study has provided some important observations.

Essential tremor plus

Of the six variants identified in this group, none were pathogenic. One patient had a variant of unknown significance in *TENM4* and five had benign variants [*COL6A3* (n = 2), *VPS16* (n = 1), *TAF1* (n = 1), *KMT2B* (n = 1)].

Dystonic tremor

Known or novel variants were observed in a total of seven DT patients but pathogenic/likely pathogenic known [*THAP1* (n = 1) and novel *ANO3* (n = 1)] variants were identified in only two patients.

THAP-1

A 24-year-old male (See Supplementary Video Case S1) presented with a 5-year history of abnormal movement of his body which started from his left hand. Gradually his movements were generalized and mainly involved the neck, upper limb, and trunk. On examination, he had generalized dystonia with more severe involvement of the neck, upper limb, and trunk (see Supplementary Video). He was treated with multiple sessions of injection botulinum toxin with a good response. His father had a similar history of generalized dystonia, but he did not consent to genetic testing.

ANO3

A 38-year-old male (See Supplementary Video Case S2) presented with 8-year history of task-specific focal hand dystonia in the form of writer's cramp. He was working as a marketing executive where he had to write extensively to maintain the company records. His symptoms were insidious in onset and gradually progressive. On examination, he had primarily flexion type of writer's cramp. He was injected with Ona botulinum toxin on multiple occasions with a good response to treatment. His father and uncle had a history of bilateral upper limbs postural tremor but they were not alive.

Discussion

The term ET-Plus was introduced in the last consensus classification [1]. It was defined as a tremor with the characteristics of essential tremor (ET) and additional neurological signs of uncertain significance such as questionable dystonic posturing. However, in the absence of a clear definition of questionable dystonia, there is a high rate of discordance among the experts regarding the diagnosis of ET plus and DT [2, 3]. We conducted this study to know the genetic profile of patients with ET plus and to establish overlap, if any,

TABLE 2 *In silico* tools based pathogenicity prediction of the known or novel variants identified in known Dystonia and Essential Tremor genes in the study.

Variant	Transcript change	Protein change	Global MAF rsID	*In silico* predictions			Conservation score phyloP100	Pathogenicity score MetaRNN CADD	Patient ID	Main phenotype	Age	Sex	Body distribution of dystonia	Body distribution of tremor
				CADD	SIFT	PolyPhen								
Autosomal dominant isolated dystonia genes														
THAP1 (DYT6) NC_000008.10: g.42693331A>C	(NM_018105.3): c.416T>G	(NP_060575.1) :p.(Val139Gly)	6.37×10^{-5} rs748328560	**27.7**	**D**	P	**5.19**	**Pathogenic (0.75)**	DYS14	Dystonia (DT7)	11	M	Generalized dystonia	BLUL and LL
CIZ1 (DYT23) NC_000009.11: g.130943041C>T	(NM_001131017.2): c.626G>A	(NP_001124489.1): p.(Gly209Glu)	1.16×10^{-4} rs376517766	**25.4**	**D**	**D**	1.93	Benign (0.23)	DYS12	Dystonia (DT6)	26	M	Focal dystonia (RUL)	BLUL
ANO3 (DYT24) NC_000011.9: g.26552810G>A	(NM_001313726.1): c.979G>A	(NP_001300655.1): p.(Asp327Asn)	Novel	**25.5**	T	P	**8.48**	**Pathogenic (0.84)**	DYS46	Dystonia (DT12)	38	M	Writer's cramp	BLUL
Autosomal recessive isolated dystonia genes														
COL6A3 (DYT27) NC_000002.11: g.238274569G>T	(NM_004369.4): c.5610C>A	(NP_004360.2): p.(Ser1870Arg)	4.48×10^{-3} rs113153193	**23.2**	**D**	P	0.06	Benign (0.01)	DYS45	ETP (ETP11)	47	5	Rt. Torticollis (Q)	BLUL
COL6A3 (DYT27) NC_000002.11: g.238285820G>A	(NM_004369.4): c.2665C>T	(NP_004360.2): p.(Arg889Cys)	1.0×10^{-3} rs201327438	**22.9**	T	P	0.56	Benign (0.30)	DYS85 (DYS41)	Dystonia (DT22)	57	M	Focal cranial	BLUL
COL6A3 (DYT27) NC_000002.11: g.238280816C>T	(NM_004369.4): c.3844G>A	(NP_004360.2): p.(Val1282Met)	1.59×10^{-3} rs535661345	**22.5**	T	B	0.70	Benign (0.02)	DYS66	ETP (ETP17)	62	F	RLC and LUL (Q)	Head &BLUL
VPS16 (DYT30) NC_000020.10: g.2840447C>T	(NM_022575.4): c.136C>T	(NP_072097.2): p.(Pro46Ser)	3.27×10^{-3} rs201176727	**23.9**	T	P	3.30	Benign (0.09)	DYS45	ETP (ETP11)	47	5	Rt. Torticollis (Q)	BLUL
Combined dystonia														
TAF1 (DYT3) NC_000023.10: g.70614053C>T	(NM_001286074.2): c.3364C>T	(NP_001273003.2): p.(Arg1122Trp)	2.0×10^{-4} rs775836470	**25.1**	**D**	B	3.31	Benign (0.19)	DYS79 (DYS64)	ETP (ETP21)	60	F	RC (Q)	Head &BLUL
GCH1 (DYT5a) NC_000014.8: g.55369209G>T	(NM_001024024.2): c.173C>A	(NP_001019195.1): p.(Pro58His)	NA Novel	**21.1**	**D**	B	**5.93**	Benign (0.27)	DYS87 (DYS52)	Dystonia (DT23)	51	M	RC, UL (L>R)	BLUL
KMT2B (DYT28) NC_000019.9: g.36210874C>T	(NM_014727.2): c.625C>T	(NP_055542.1): p.(Arg209Trp)	1.0×10^{-3} rs1002774016	**22.7**	**D**	B	1.59	Benign (0.09)	DYS78 (DYS61)	ETP (ETP20)	69	M	RC (Q)	BLUL
Essential tremor genes														
FUS (ETM4) NC_000016.9: g.31202118C>T	(NM_001170937.1): c.1336C>T	(NP_001164408.1): p.(Pro446Ser)	1.0×10^{-3} rs201533156	**26.5**	**D**	P	**7.40**	Uncertain (0.55)	DYS74 (DYS39)	Dystonia (DT18)	65	F	RC and UL (R>L)	Head &BLUL

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TABLE 2 (*Continued*) *In silico* tools based pathogenicity prediction of the known or novel variants identified in known Dystonia and Essential Tremor genes in the study.

Variant	Transcript change	Protein change	Global MAF rsID	*In silico* predictions			Conservation score phyloP100	Pathogenicity score MetaRNN CADD	Patient ID	Main phenotype	Age	Sex	Body distribution of dystonia	Body distribution of tremor
				CADD	SIFT	PolyPhen								
TENM4 (ETM5) NC_000011.9: g.78440477A>G	(NM_001098816.3): c.3350T>C	(NP_001092286.2): p.(Ile1117Thr)	1.03×10^{-4} rs753518649	**22.5**	T	B	**7.09**	Uncertain (0.60)	DYS81 (DYS67)	ETP (ETP22)	70	M	LLC, RC, LUL (Q)	BLUL
TENM4 (ETM5) NC_000011.9: g.78372634C>T	(NM_001098816.3): c.7411G>A	(NP_001092286.2): p.(Val2471Ile)	1.3×10^{-4} rs1218683396	**24.6**	T	**D**	**5.87**	Benign (0.28)	DYS76 (DYS53)	Dystonia (DT20)	23	M	Writer's cramp	BLUL

Note: SIFT: T–Tolerated; D–Deleterious & PolyPhen: B–Benign; P–Possibly-damaging; D–Probably-damaging.
PhyloP100 scores are based on multiple alignments of 99 vertebrate genome sequences to the human genome. The greater the score, the more conserved the site.
MetaRNN prediction and scores incorporates16 scores (SIFT, Polyphen2_HDIV, Polyphen2_HVAR, MutationAssessor, PROVEAN, VEST4, M-CAP, REVEL, MutPred, MVP, PrimateAI, DEOGEN2, CADD, fathmm-XF, Eigen and GenoCanyon), eight conservation scores (GERP, phyloP100way_vertebrate, phyloP30way_mammalian, phyloP17way_primate, phastCons100way_vertebrate, phastCons30way_mammalian, phastCons17way_primate and SiPhy), and allele frequency information from the 1000 Genomes Project (1000 GP), ExAC, and gnomAD. Larger value means the SNV is more likely to be damaging. Scores range from 0 to 1.
CADD scores >20 denotes the top 1% most deleterious substitutions in the human genome.
Pathogenic mutations are bold.

with DT patients. In this study, only two patients with DT had pathogenic mutations. One patient with ET plus and another with DT had a variant of unknown significance. As for ET genetics, our findings are consistent with the current understanding [5–9]. Although genetic component is likely to play an important role in the pathogenesis of ET with >50% of the affected individuals having a family history, very few disease-causing variants [*DRD3*, *FUS*, *HTRA2*, *NOTCH2NLC*, and *TENM4*] have been identified to date [8]. Of note, all the variants identified in *DRD3* associated with ETM1 have been classified as variants of unknown significance suggesting the variants in this gene to be of minor significance. As for the FUS gene, the pathogenic variant is associated with both Amyotrophic lateral sclerosis type 6 and ETM4 suggesting the pleiotropic nature of this gene. Further, a pathogenic variant in the TENM4 gene is associated only with ETM5 (Supplementary Table S2). Besides their poor replicability in other studies, family members of patients with these variants have manifested phenotypes other than ET, like ataxia, parkinsonism, and autonomic dysfunction [5]. Genetic analyses of ET have been affected by different factors. In the absence of robust criteria many previous studies have included other tremor disorders misdiagnosed as ET, such as DT, spinocerebellar ataxias, and fragile X-associated tremor/ataxia syndrome (FXTAS). Furthermore, in DT, sometimes tremor can be the sole clinical manifestation and dystonia may appear later (e.g., DYT-*ANO3*) [4]. Genetic studies utilising sporadic or familial forms of ET and ET plus recruited following stringent diagnostic criteria are highly warranted to overcome these limitations.

There are some major limitations to this study including a small sample size. Also, many patients had a family history, they could not be tested due to the COVID pandemic at the time of patient recruitment. Further, WES is a powerful tool to identify all potential protein-coding genetic variants associated with a disease phenotype. However, it suffers from some limitations, such as a) low coverage efficiency: Some of the potential disease-causing variants may sometimes be missed owing to low coverage mostly due to poor DNA quality; library preparation, and/or some gene regions with repeat sequences. However, in our study, we obtained an average good quality data of ~28 × 10 [6] reads per sample with an average %Q > 30 ~95.05 (Supplementary Table S1) and ~47.2 average mean coverage per sample; b) copy number variants: Detection of these structural variants in WES data has been challenging due to sophisticated bioinformatics tools which need to be used and sometimes the findings are not validated; and c) regulatory variants: Considering WES focuses on protein-coding variants, variants in non-coding regions of the gene which may have regulatory role on the gene expression are mostly not captured and likely missed in data interpretation. Despite these limitations, to the best of our knowledge, this is the first genetic study with a cohort of ET plus.

Conclusion

In our study, we identified pathogenic/likely pathogenic variants in two patients with DT, however, no pathogenic variants were identified in patients with ET plus. The findings of our study emphasize that genetic studies may be in an important biomarker in differentiating patients with ET plus from DT which may be challenging in a clinical setting.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

The studies involving humans were approved by Institutional ethics committee, Maulana Azad Medical College, New Delhi. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SP: Research project: Conception, organization, execution. Statistical analysis: Design, execution, review, and critique. Manuscript: Writing of the first draft, review, and critique. NY: Research project: Conception, Organization, Execution. Statistical analysis: Design, execution, review, and critique. Manuscript: Review, and critique. SD: Research project: Conception, organization, execution. Statistical analysis: Design, execution, review, and critique. Manuscript: Review, and critique. CR: Research project: Conception, organization, execution. Statistical analysis: Design, execution, review, and critique. Manuscript: Review, and critique. BT: Research project: Conception, organization, execution. Statistical analysis: Design, execution, review, and critique. Manuscript: Review, and critique.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/dyst.2024.13181/full#supplementary-material

SUPPLEMENTARY VIDEO CASE S1
A 24-year-old male has generalized dystonia attributed to a mutation in *THAP-1* gene. His father also has generalized dystonia.

SUPPLEMENTARY VIDEO CASE S2
A 38-year-old male patient has right upper limb task-specific focal hand dystonia in the form of a writer's cramp attributed to a mutation in *ANO3* gene.

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