

ARTICLE

Identification of candidate genes for familial early-onset essential tremor

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Essential tremor (ET) is one of the most common causes of tremor in humans. Despite its high heritability and prevalence, few susceptibility genes for ET have been identified. To identify ET genes, whole-exome sequencing was performed in 37 early-onset ET families with an autosomal-dominant inheritance pattern. We identified candidate genes for follow-up functional studies in five ET families. In two independent families, we identified variants predicted to affect function in the nitric oxide (NO) synthase 3 gene (*NOS3*) that cosegregated with disease. *NOS3* is highly expressed in the central nervous system (including cerebellum), neurons and endothelial cells, and is one of three enzymes that converts L-arginine to the neurotransmitter NO. In one family, a heterozygous variant, c.46G > A (p.(Gly16Ser)), in *NOS3*, was identified in three affected ET cases and was absent in an unaffected family member; and in a second family, a heterozygous variant, c.164C > T (p.(Pro55Leu)), was identified in three affected ET cases (dizygotic twins and their mother). Both variants result in amino-acid substitutions of highly conserved amino-acid residues that are predicted to be deleterious and damaging by *in silico* analysis. In three independent families, variants predicted to affect function were also identified in other genes, including *KCNS2* (*KV9.2*), *HAPLN4* (*BRAL2*) and *USP46*. These genes are highly expressed in the cerebellum and Purkinje cells, and influence function of the gamma-amino butyric acid (GABA)-ergic system. This is in concordance with recent evidence that the pathophysiological process in ET involves cerebellar dysfunction and possibly cerebellar degeneration with a reduction in Purkinje cells, and a decrease in GABA-ergic tone.

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INTRODUCTION

Essential tremor (ET) is a chronic, progressive neurological disease.¹ Its hallmark motor feature is a 4–12-Hz kinetic tremor that occurs during voluntary movements such as writing, eating and drinking. At disease onset, the tremor not only affects the hands and arms but it may also eventually spread to involve the head (ie, neck), voice, jaw and other body regions.² Given the presence of etiological, clinical, pharmacological response and pathological heterogeneity, there is growing support for the idea that ET may be a family of diseases whose central defining feature is kinetic tremor of the arms, and therefore it might more appropriately be called ‘the essential tremors’.³

Family studies^{4,5} and twin studies^{6,7} provide strong evidence for a genetic contribution to ET, with heritability estimates ranging from 45 to 90% in twin studies.^{6,7} Despite this high heritability, the field of ET genetics has made only limited advances. Previously, we and others reported that ET aggregates in families, with many families containing multiple members with ET.^{8–10} Most studies indicate that at least 30–70% of ET patients have a family history, with the vast majority (>80%) of young onset (≤40 years old) cases reporting ≥1 affected first-degree relative.¹¹ To date, only three published genome-wide linkage scans have been performed, all in North American or Icelandic ET families.^{12–14} These studies provided evidence for genetic loci

harboring ET genes on chromosomes 3q13 (ETM1; OMIM: 190300),¹² 2p22-p25 (ETM2; OMIM: 602134)¹³ and 6p23 (ETM3; OMIM: 611456).¹⁴ Several studies have attempted to replicate linkage to ETM1,^{15–17} ETM2^{15,18,19} and ETM3, without success (no LOD score >2.0), and the genes and causal variants for these loci (ETM1, ETM2 and ETM3) have yet to be identified. Recently, using a linkage and a whole-exome sequencing approach, the *fused in sarcoma/translated in liposarcoma* (*FUS/TLS*) gene (Chr16p11.2) was identified as a candidate gene in a large family with ET from Quebec.²⁰ Subsequent studies,^{21–23} including our own, indicate that variants in *FUS* are an extremely rare or family-specific cause of ET, and without functional studies, the pathogenicity of variants identified so far ((c.868C > T, p.(Q290*)), RefSeq accession number NM_004960.3²⁰ and c.1129C > T p.(R377W), RefSeq accession number NM_004960.3 reported in 1 patient with family history of ET²⁴ is unknown.

Despite its remarkably high prevalence, the pathophysiology of ET is still poorly understood and current debate as to whether it is a functional or neurodegenerative disease.^{25,26} There is considerable evidence from clinical, neuroimaging and physiological studies, of cerebellar involvement,^{27,28} and an emerging literature that documents a variety of changes, some of which are degenerative, in the Purkinje

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cell population.^{29,30} There is also a related literature that indicates a possible change in brain gamma-aminobutyric acid (GABA) tone in ET.^{31,32}

MATERIALS AND METHODS

Study participants and clinical diagnosis

To identify genes for ET, we enrolled probands (affected with ET) and relatives in a family study of ET at Columbia University, NY, USA (2011–2014). The study was approved by the Institutional Review Board at Columbia University and written informed consent was obtained from all enrollees. The criteria for enrollment were as follows: (1) the proband had early-onset ET with age at onset ≤ 50 years; (2) the proband had a diagnosis of definite or probable ET; (3) in addition to the proband there were at least two affected relatives in the family; (4) additional affected and unaffected family members were willing to participate in the study; and (5) the families contained more than two affected individuals in different generations. For the genetic analyses, we excluded enrollees who had been diagnosed with Parkinson's disease (PD) or dystonia. The final sample includes 52 families (52 probands (affected with ET)) and 155 relatives. The number of affected individuals enrolled per family ranged from 3 to 7 (mean = 4.1). An in-person evaluation was performed on all participants, during which they completed demographic, medical history and family history questionnaires, and underwent a videotaped neurological examination, from which a total tremor score (range 0–36) was assigned.^{10,33–37} After review of the questionnaires and videotaped examinations, the diagnosis of ET was then reconfirmed by a senior neurologist specializing in movement disorders (EDL) using reliable and valid research criteria.³⁸ All ET diagnoses (possible, probable and definite) required, at a minimum, moderate or greater amplitude kinetic tremor on at least three tasks, and an absence of other etiologies (eg, dystonia, PD and drug-induced tremor). Probable ET required such tremor on at least four tasks and definite ET required this as well as postural tremor of at least moderate amplitude.³⁸ As such, all ET diagnoses also met the requirements outlined in the Consensus Statement on Tremor of the Movement Disorders Society.³⁹ At the time of starting the genetic analyses, 37 families had completed all clinical assessments and evaluations (videotape, diagnosis of ET by EDL and isolated DNA), and were selected for whole-exome sequencing.

An additional 95 unrelated ET cases enrolled in a clinical-epidemiological study at Columbia University²¹ were also screened by Sanger sequencing for KCNS2 coding variants.

Whole-exome sequencing analysis

Genomic DNA was isolated from peripheral blood cells using standard methods. Whole-exome sequencing was performed on the genomic DNA of at least two most distantly related affected (definite, probable or possible ET diagnosis) individuals from each of 37 total families. In some families, >2 affected individuals were sequenced. The pedigrees for 37 families, indicating that family members were exome sequenced, are shown in Supplementary Figure S1. All samples were processed using the Agilent SureSelect XT kit (Agilent Technologies, Santa Clara, CA, USA) for library preparation and

exome captured using the All Exon v5+UTRs library (Agilent Technologies). Paired-end sequencing was performed at $>40\times$ coverage per sample. Obtained libraries were sequenced on the Illumina HiSeq2500 instrument (Illumina, Inc., San Diego, CA, USA). Sequence alignment to the human reference genome (UCSC hg19) was performed using the Burrows–Wheeler Aligner algorithm,⁴⁰ and variant calling was performed using the Genome Analysis Toolkit (Broad Institute, Cambridge, MA, USA).⁴¹ The following criteria were used to generate a list of variants for each family for follow-up genotyping of other family members and cosegregation analysis: (1) on the assumption that disease-causing variants were rare, we excluded all common single-nucleotide polymorphisms (MAF $> 1\%$) in dbSNP v.137; (2) the variant was shared between individuals with ET within a family; and (3) the variant was ranked by the software tool pVAAS (see Web Resources)⁴² (using a dominant model and the maximum number of permutations of 1 000 000) with P -value < 0.05 .

Genotyping

Follow-up genotyping was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom, San Diego, CA, USA) with Sequenom iPLEX Gold custom assays designed using MassARRAY assay design software version 4.0 (Sequenom). Details of the variants that were genotyped are provided in Supplementary Table S1.

From the cosegregation analysis, we identified a list of annotated 'candidate' variants that were shared exclusively by affected ET patients within a family. Annotation of cosegregating candidate variants was performed at the gene and variant level (Supplementary Materials and Methods).

Availability of data. All phenotype, genotype and exome data will be released and deposited in the database of Genotypes and Phenotypes (dbGaP: <http://www.ncbi.nlm.nih.gov/gap>) of the National Center for Biotechnology Information. The study titled 'Identification of Susceptibility Genes for Essential Tremor' received the dbGaP accession phs000966.v1.p1.

RESULTS

The demographic and clinical characteristics of affected ET patients and unaffected family members that were sequenced are shown in Table 1. The average age at onset of tremor in ET patients was 24.5 years (± 17.2) and duration of tremor was 35.3 years (± 19.5). Ten percent of ET patients reported Ashkenazi Jewish ancestry.

We identified variants predicted to affect function in five ET families (Table 2). Cosegregating variants were considered to be candidates based on the following criteria: (1) the variant occurred at a highly conserved nucleotide or amino-acid residue; (2) amino acid changes were predicted to be damaging to the protein by one or more prediction tools (provean, SIFT and mutation taster); (3) the gene is expressed in the cerebellum (including Purkinje, granule, stellate and basket cells); and (4) the gene function is implicated in the

Table 1 Clinical characteristics of affected ET patients and unaffected family members that were exome sequenced in 37 ET families

Clinical characteristic	ET patients (n = 100)	Unaffected (n = 4)	Total (n = 104)
Male, n (%)	45 (45)	1 (25)	46 (44.2)
Age at tremor onset, mean years (SD)	24.5 (17.2)	NA	NA
Age at interview, mean years (SD)	59.9 (17.7)	44.8 (18.1)	59.3 (17.8)
Ashkenazi Jewish, n (%)	10 (10)	0 (0.0)	10 (9.6)
Duration of tremor, mean years (SD)	35.3 (19.5)	NA	NA
Total tremor score, mean (SD)	18.6 (7.7)	NA	NA
Head tremor on examination, n (%)	29 (29)	0 (0)	NA
Chin tremor on examination, n (%)	9 (9)	0 (0)	NA
Head tremor presence in head and chin, n (%)	10(10)	0 (0)	NA
Tremor absent in chin or head, n (%)	52 (52)	4(100)	NA

Abbreviations: ET, essential tremor; NA, not applicable.

pathophysiological process in ET. In two independent families (families A and B), we identified variants in the nitric oxide (NO) synthase 3 gene (*NOS3*) that cosegregated with disease. In family A, a heterozygous variant, c.46G>A (p.(Gly16Ser)), located in *NOS3*, was present in all three affected ET cases and absent in an unaffected family member. In family B, a heterozygous variant, c.164C>T (p.(Pro55Leu)), was identified in three affected ET cases that included dizygotic twins and their mother. These *NOS3* variants are absent from the 1000 genomes project (TGP) database but are present in the heterozygous state in the exome aggregation consortium (ExAC) data set with an allele frequency of 1.969×10^{-5} and 5.145×10^{-5} . The father was also affected with ET but did not carry the heterozygous variant identified in other family members, and family history information was not available for him as he was adopted, suggesting the possibility of a phenocopy. *NOS3* was ranked 32 (*P*-value 0.0133) in the pVAASST analysis (Supplementary Materials and Methods). Both variants result in amino-acid substitutions of highly conserved amino-acid residues that are predicted to be deleterious and damaging by *in silico* analysis (both mutations by SIFT and mutation taster). The main clinical features of six affected individuals from two families with *NOS3* variants are shown in Table 3.

In family C, a heterozygous variant was identified in the gene *KCNS2* (*KV9.2*), c.1137T>A, resulting in a missense amino-acid substitution (p.(Asp379Glu)) and ranked 52 (*P*-value 0.027) in the pVAASST analysis (Supplementary Materials and Methods). The variant is absent from the TGP and ExAC databases. This variant was present in all affected ET patients but was absent in an unaffected family member (Figure 1). *KCNS2* was also identified as a candidate gene in a significant linkage interval (P2 LOD 3.312 and P1 LOD 2.729) on hg19 chr8: g.94960934_101969832 (Supplementary Materials and Methods, and Supplementary Table S2) by nonparametric linkage analysis in 37 ET families. Three *in silico* prediction programs, provean,⁴³ SIFT⁴⁴ and mutation taster,⁴⁵ predict that this amino-acid substitution is intolerable, deleterious and damaging to the

structure and function of the *KCNS2* protein. Furthermore, the asparagine residue at position 379 is highly evolutionarily conserved, suggesting that this residue is essential for channel function (Figure 2). *KCNS2* also shows high homology to *Drosophila Shab*, a K⁺ channel that regulates membrane excitability and synaptic transmission in many central nervous system (CNS) neurons, in addition to the neuromuscular junction, with 42% amino-acid identity and 63% amino-acid similarity. Protein alignment of *Drosophila Shab* with *KCNS2* shows that the asparagine residue (and adjacent amino acids) at position 379 in *KCNS2* is conserved in *Shab* (Figure 2). We also evaluated the effect of the variant on the protein structure (residue range 332–413) using Swiss-Pdb viewer (Deep View version 4.0, see Web Resources; Figure 3). The variant, c.1137T>A (p.(Asp379Glu)), alters the molecular surface, electrostatic potential and creates a strong hydrogen bond that is absent from the wild-type protein (Figure 3). We sequenced the coding regions of the *KCNS2* gene in an additional 95 unrelated ET cases enrolled in a clinical–epidemiological study at Columbia University.²¹ We did not identify novel variants predicted to affect function in *KCNS2*, nor did we identify the *KCNS2* variant, c.1137T>A (p.(Asp379Glu)), identified in family C, in any of the 95 ET cases analyzed.

In family D, we identified a heterozygous variant, c.1048G>C (p.(Gly350Arg)), in hyaluronan and proteoglycan link protein 4 (*HAPLN4*; pVAASST rank 38, *P*-value 0.0167) that was identified in three affected ET cases. DNA was not available for other family members that reside in the United Kingdom and were unwilling to participate. Three *in silico* prediction programs, provean,⁴³ SIFT⁴⁴ and mutation taster,⁴⁵ predict that this amino-acid substitution is intolerable, deleterious and damaging to the structure and function of the *HAPLN* protein. Furthermore, the glycine residue at position 350 is highly evolutionarily conserved, suggesting that this residue is essential for function of the protein. The variant is absent from the TGP database and present in the ExAC database with an allele frequency of 0.00002917.

Table 2 Variants predicted to affect function identified in ET families

Family	Gene	cDNA (Ensembl transcript)	Protein	Function
A	<i>NOS3</i>	c.46G>A (ENST00000297494)	p.Gly16Ser	Enzyme that converts the neurotransmitter NO from L-arginine
B	<i>NOS3</i>	c.164C>T (ENST00000297494)	p.Pro55Leu	Enzyme that converts the neurotransmitter NO from L-arginine
C	<i>KCNS2</i>	c.1137T>A (ENST00000287042)	p.Asp379Glu	K ⁺ channel highly expressed in Purkinje and granular cells
D	<i>HAPLN4</i>	c.1048G>C (ENST00000291481)	p.Gly350Arg	Hyaluronan and proteoglycan link protein 4. Expressed in perineuronal nets in the basal ganglia and cerebellum
E	<i>USP46</i>	c.398C>T (ENST00000441222)	p.Ala133Val	Ubiquitin-specific protease 46 involved in GABA synthesis. Highly expressed in cerebellum

Abbreviations: ET, essential tremor; GABA, gamma-amino butyric acid; *HAPLN4*, hyaluronan and proteoglycan link protein 4; NO, nitric oxide; *NOS3*, nitric oxide synthase 3 gene; *USP46*, ubiquitin-specific protease 4.

Table 3 Clinical features of ET individuals with *NOS3* variants

Clinical feature	Family A			Family B		
	IV-001	V-002	V-004	IV-001 DZ twin	IV-002 DZ twin	III-003
Age at tremor (years)	14	30	16	12	12	12
Duration of tremor (years)	45	4	21	10	10	31
Total tremor score	33	13	15.5	25	20	26
Tremor presence in head	Yes	Yes	No	No	No	No
Tremor presence in chin	No	No	No	No	No	No
Tremor presence in head and chin	Yes	No	No	No	No	No

Abbreviations: ET, essential tremor; *NOS3*, nitric oxide synthase 3 gene.

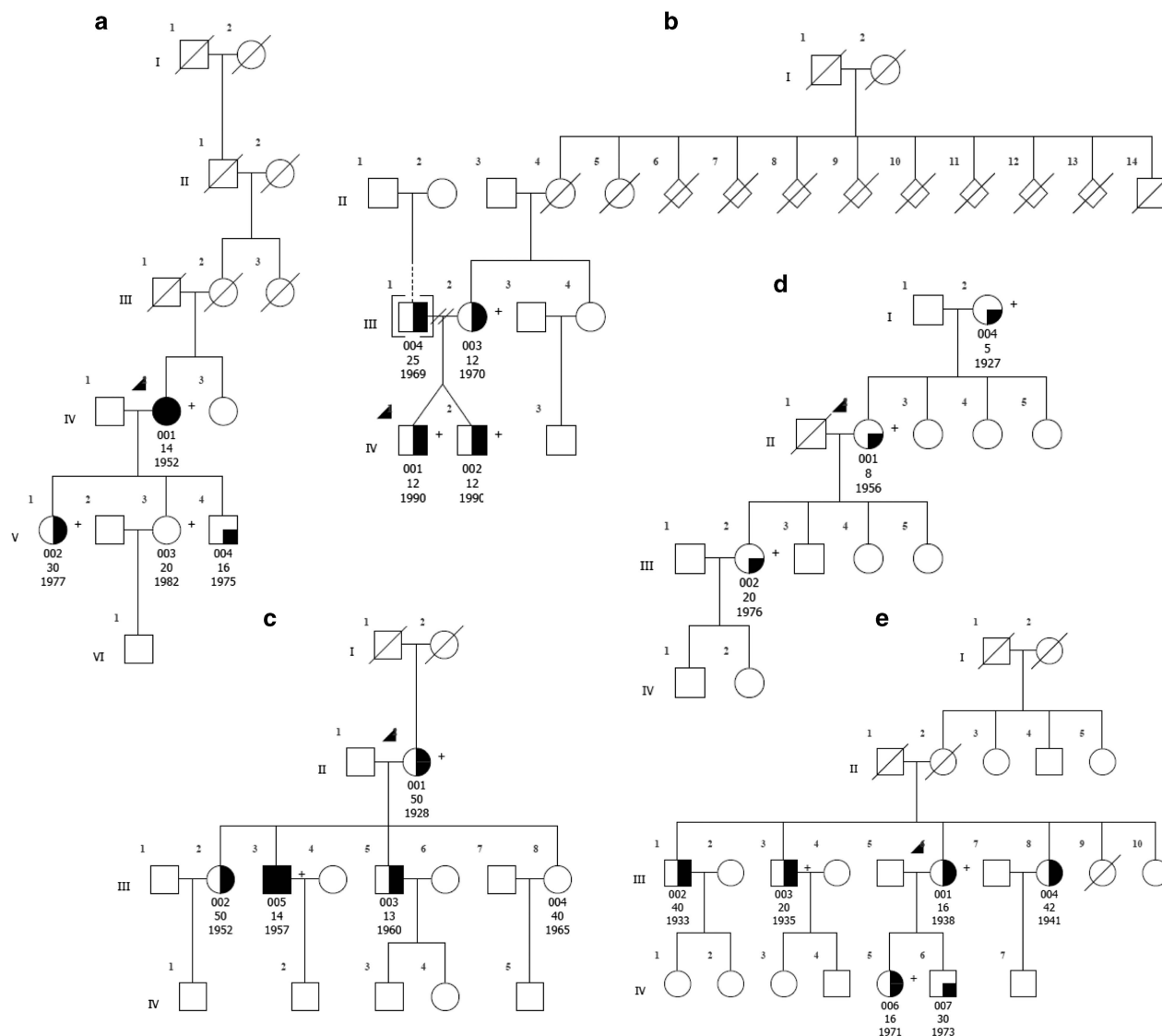


Figure 1 Pedigrees with variants in the genes *NOS3* (families **a** and **b**) *KCNS2* (family **c**), *HAPLN4* (family **d**) and *USP46* (family **e**). Genetic pedigrees for families with likely pathogenic mutations are shown. The generation in each pedigree is indicated by roman numerals. The proband is indicated by an arrowhead. A '+' symbol indicates subjects that were exome sequenced. Below each subject with DNA available for genetic analysis, the subject id (OOX), age at tremor onset and date of birth are indicated. Symbol shading is as follows: definite ET, symbols completely black; probable ET, symbols half vertical black fill; possible ET, symbols with a quadrant in black; and unaffected, clear symbol.

Species	Match	Gene	Amino acid alignment
<i>H.Sapiens</i>		ENSG00000156486	379 ATVSMTTVGYGDVVP GTTAGK LTA
Mutation			379 ATVSMTTVGYG E VVP GTTAGK LTA
<i>P.troglodytes</i>	All identical	ENSPTRG00000039728	296 ATVSMTTVGYGDVVP G
<i>M. mulatta</i>	All identical	ENSMMUG00000010267	379 ATVSMTTVGYGDVVP GTTAGK L
<i>F.catus</i>	No homologue		
<i>M.musculus</i>	All identical	ENSMUSG00000050963	379 ATVSMTTVGYGDVVP GTTAGK L
<i>G.gallus</i>	No homologue		
<i>T.rubripes</i>	All identical	ENSTRUG00000011051	379 ATVSMTTVGYGDVVP GTTAGK L
<i>D. rerio</i>	No homologue		
<i>D.melanogaster</i>	No homologue		
<i>D. melanogaster (Shab)</i>	42% AA identity	FBgn0262593	644 AGITMTTVGYGDIYP
<i>C.elegans</i>	No homologue		
<i>X.tropicalis</i>	No homologue		

Figure 2 Evolutionary conservation of *KCNS2*. Evolutionary conservation of *KCNS2* at the protein level. The asparagine residue indicated in blue at position 379 in Human *KCNS2* is highly conserved across species including chimpanzee, rhesus macaque, mouse and pufferfish. The non-synonymous substitution to glutamic acid identified in family 68 is indicated in red. *KCNS2* also shows high homology to *Drosophila Shab*, a K^+ with 42% amino-acid identity and 63% amino-acid similarity. Protein alignment of *Drosophila Shab* with *KCNS2* shows that the asparagine residue (and adjacent amino acids) at position 379 in *KCNS2* is conserved in *Shab*.

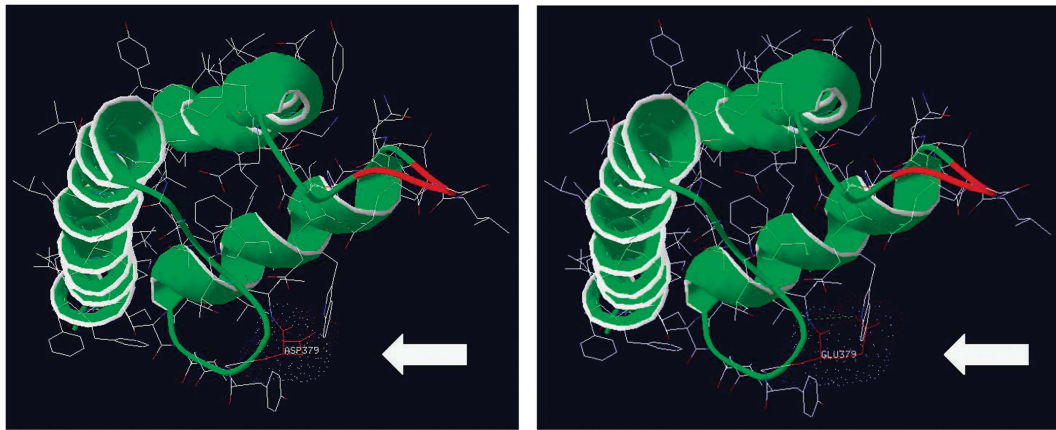


Figure 3 Model 3D protein structure of *KCNS2*. A model 3D structure of *KCNS2* was generated using Swiss-Pdb viewer (Deep View version 4.0). The modeled residue range includes amino acids 332–413, which includes the *KCNS2* variant c.1137T>A (p.(Asp379Glu)), identified in family C. The amino acid 379 is indicated. The variant alters the molecular surface, electrostatic interactions (indicated by colored dots) and creates a strong hydrogen bond (green dotted line).

In family E, we identified a heterozygous variant, c.398C>T (p.(Ala133Val)) in ubiquitin-specific protease 46 (*USP46*), which cosegregated with ET in the family (pVAAST rank 5, *P*-value 0.00228). The variant was present in five ET cases. DNA was not available for testing from other family members. Although only one *in silico* prediction program (mutation taster) predicts that the amino-acid substitution in *USP46* is disease causing, the alanine residue at position 133 is highly conserved in several species (chimpanzee, macaque, cat, mouse, chicken, pufferfish and zebrafish). The variant is absent from the TGP genomes and ExAC databases.

DISCUSSION

Among the 37 early-onset ET families analyzed, we identified four promising candidate genes in five families. Functional studies will be needed to validate our findings and determine their mechanism of action (dominant gain-of-function or dominant negative) and role in ET.

In two families (A and B), we identified variants predicted to affect function in *NOS3*. *NOS3*, one of three enzymes that converts L-arginine into the neurotransmitter NO, is the major NO synthase (NOS) isoform expressed in endothelial cells and has an important role in vasculature homeostasis.⁴⁶ *NOS3* is also highly expressed in the CNS (including the cerebellum), neurons and endothelial cells.⁴⁶ In the brain, NO mediates neuronal survival, synaptic plasticity, vascular smooth muscle relaxation and endothelial cell permeability. The cerebellum expresses high levels of NOS in granule, stellate and basket cells.⁴⁷ The NOS pathway has been implicated in the pathogenesis of Alzheimer's disease (AD), PD, and cerebrovascular disease and stroke, and previous studies have also demonstrated aberrant expression of the *NOS3* gene in neurons, glial and endothelial cells in AD brains.^{46–48} The Glu/Glu genotype at p.(Asp298Glu) (rs1799983; ClinVar pathogenic allele) has been reported as a risk factor for AD in several case–control studies.

Recent findings from several studies, including our own, indicate that the pathophysiological process in ET involves the cerebellum. There is also some discussion as to whether this is associated with cerebellar degeneration as well as a decrease in GABA-ergic activity in deep cerebellar neurons, disinhibition in output to the deep cerebellar neurons and an increase in rhythmic activity of the thalamocortical circuit.^{25,26} Significantly, in the current study, we identified variants in three genes in independent ET families, which include *KCNS2* (*KV9.2*)

(family C), *HAPLN4* (*BRAL2*) (family D) and *USP46* (family E), each of which is highly expressed in Purkinje cells.

KCNS2 (*KV9.2*), a K⁺ channel α -subunit that is highly and selectively expressed in the brain, modulates the activity of the *KV2.1* and *KV2.2* channels.⁴⁹ A similar localization of expression of *KCNS2* (*KV9.2*) and *KV2.1* and *KV2.2* has been observed in the Purkinje and granular cells in the cerebellum.⁴⁹ *KCNS2* also shows high homology to *Drosophila Shab*, a K⁺ channel that is widely expressed in the CNS and peripheral nervous systems, and that interacts with the *Drosophila Shaker* channel in the regulation of synaptic transmission.⁵⁰ Indeed, these channels regulate membrane excitability and synaptic transmission in many central neurons and also in the neuromuscular junction.⁵⁰ *Drosophila Shaker* mutants display leg shaking under anesthesia,⁵¹ abnormal spike bursting in motor circuits⁵² and greatly enhanced neurotransmission at neuromuscular junctions.⁵³ A tremor phenotype has been described in patients with variants in related potassium channel family members (eg, *KCNA1*, *KV1.1*).⁵⁴ Further, in other K⁺ channels mouse models (*KV3.1* and *KV3.3* K⁺ channel), mice display severe motor deficits, including tremor, myoclonus and ataxic gait.^{55,56}

In family D, the gene, brain link 2 (*BRAL2*; also known as *HAPLN4*) was identified as a candidate. It is of special interest that *BRAL2* is highly expressed throughout the GABA-ergic neurons of the cerebellum, including Purkinje cells and basket neurons, both of which have been shown in some studies to be abnormal in ET^{57,58} in postmortem studies of humans with ET. Furthermore, the Purkinje cells, and their entire GABA-ergic output, are directed at the neurons in the deep cerebellar nuclei. Therefore, a reduction in Purkinje cell output or a reduction in the receptivity of deep cerebellar nuclei to GABA could result in tremor.⁵⁹ Of particular interest is that mice deficient for *BRAL2* have attenuated perineuronal nets, altered localization of brevicin and show a slight decrease in the number of synapses in deep cerebellar nuclei neurons. Interestingly, in a mouse model of a related link protein family member, *BRAL1*, CNS axonal nerve conduction is markedly decreased without changes in the clustering or transition of ion channels at the nodes or in the tissue morphology around the nodes of Ranvier. *BRAL1* mice did however changes in the extracellular space diffusion parameters, suggesting a reduction in the diffusion hindrances in the white matter of mutant mice. Behavioral studies or other phenotypic observations such as tremor have not been described for the *BRAL2*-deficient mice, but

further characterization of these mice in light of our findings is warranted.⁶⁰

USP46 was identified as a candidate gene in family E. *USP46* belongs to a family of deubiquitinating enzymes that regulate diverse cellular functions by cleaving ubiquitin from specific protein substrates. They have been implicated in a wide variety of biological processes and disease processes including cancer and neurodegenerative disease.^{61–64} *Usp46* is strongly expressed in different brain regions including the cerebellum. In a *Usp46* mouse mutant model,⁶⁵ defects in the GABA-ergic system were identified with a marked reduction in *GAD67* expression. Because *GAD67* catalyzes the decarboxylation of glutamate to GABA, *Usp46* has been proposed to be involved in GABA synthesis. Thus, it is likely that *Usp46* extensively affects the GABA-ergic system and controls a broad range of behavioral phenotypes.^{65,66}

One of the strengths of the study is the use of diagnostic criteria that are both reliable and valid, and that were designed specifically for genetic studies of ET, the Washington Heights Inwood Genetic Study of ET. All diagnoses were assigned by a senior movement disorder neurologist based on a detailed, videotaped neurological examination that contained a rigorous assessment of tremor and tremor phenomenology. All ET diagnoses required tremors during a minimum of three separate tasks, with diagnoses of probable and definite requiring tremor during four tasks. Another strength is our focus on families with an early age at ET onset, with a mean age at onset of 24.5 years (± 17.2), which may have enriched the sample for disease susceptibility variants.

Although we have identified a number of interesting candidates, future functional studies are needed to evaluate the pathogenicity of the variants identified in these candidate genes. However, gene and variant level annotation suggest that the variants are predicted to be deleterious and damaging, and functional annotation suggests that many of the candidate genes are likely to have a role in ET pathogenesis. Follow-up studies in cellular and animal models will be needed to determine the role of these genes in ET.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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